Antioxidant effect of diethyldithiocarbamate on microsomal lipid peroxidation assessed by low-level chemiluminescence and alkane production

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Different thiol-containing compounds, such as diethyldithiocarbamate (DDC), glutathione, penicillamine, and dithioerythritol have been chosen to study their effect on ascorbate/Fe-ADP-induced lipid peroxidation, detected by low-level chemiluminescence and alkane production. In the concentration range used, these thiols exerted a temporary protection against lipid peroxidation by lengthening the induction period; after overcoming this induction period, no substantial inhibition of either chemiluminescence or alkane production was observed. DDC was effective in protecting against lipid peroxidation in the nanomolar range, whereas the group of other thiol-containing molecules operated in the millimolar range.

Diethyldithiocarbamate *Lipid peroxidation* *Chemiluminescence* *Alkane formation* *Free radical*

1. INTRODUCTION

Low-M_r thiols have been shown to play a significant role in protecting membranes against oxidative damage. The molecular mechanism generally implies free radical interactions, which, within the frame of lipid peroxidation, may occur by either intercepting the formation of O_2 free radicals at the initiation stage, or reacting with them at a later stage, mainly the propagation phase, or, in a secondary fashion, by acting as polar donors in the recovery of vitamin E after its reaction with free radicals.

Although free radical–thiol interactions do formally occur in model systems (see [1–4]), their physiological relevance for some thiols such as glutathione remains to be determined. However, glutathione [5–7] and other thiols [5] such as diethyldithiocarbamate (DDC), cysteamine, mercaptoethanol, etc. were found to protect microsomal fractions against Fe-induced lipid peroxidation, presumably through a microsomally associated factor [5–7], which is dependent on vitamin E [8]. DDC has often been reported to inhibit completely the xenobiotic-induced accumulation of malondialdehyde as an indicator of lipid peroxidation [9].

Although some reports emphasize the 'inhibitory' effect of some low-M_r thiols on lipid peroxidation, it appears that they exert only a temporary protection, which in some cases is concentration dependent, up to the moment in which they are exhausted. We have assessed the effect of some thiols on ascorbate/Fe-ADP-induced lipid peroxidation of microsomal fractions by two sensitive techniques which reflect oxidative reactions, low-level chemiluminescence and alkane production.

2. MATERIALS AND METHODS

2.1. *Biological materials and chemicals*

Rat liver microsomes were prepared as in [6]. DDC, D-(−)-penicillamine, and dithioerythritol

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were obtained from Sigma (Munich), cyanidanol from Zyma GmbH (Munich), and GSH from Boehringer (Mannheim).

2.2. *Assays*

Low-level chemiluminescence [10] and alkane (ethane and *n*-pentane) production [11] were measured as previously described. Chemiluminescence was taken as an index of lipid peroxidation based on the correlations ($r = 0.99$) described between light emission and malondialdehyde accumulation [12,13]; measurement of low-level chemiluminescence allows continuous monitoring of peroxidation reactions [10,13].

2.3. *Experimental conditions*

Peroxidation of microsomal membranes was carried out by incubating the microsomal fractions (0.16–2.0 mg protein/ml) in 0.1 M potassium phosphate buffer (pH 7.4), containing 2 mM ADP/16 μM $FeSO_4$; reactions were started upon addition of 0.5 mM ascorbate to the cuvette containing the microsomal suspension through thin polyethylene tubing (for low-level chemiluminescence experiments). Alkane formation measurements were carried out in 43 ml flasks which were capped with a PVC septum impermeable to alkanes. Thereafter, the reaction was started as described above. Head-space gas samples (5 ml) were withdrawn at different time points with gas-tight Hamilton syringes and rapidly replaced by the same volume of O_2; values were corrected for alkane dilution [11]. Both assays were performed at 37°C under constant oxygenation and stirring (chemiluminescence assays) or agitation (alkane formation measurements).

3. RESULTS AND DISCUSSION

Fig.1 illustrates the experimental model we have chosen to study the effect of different thiol-containing compounds on low-level chemiluminescence. The intensity of ascorbate/ADP-Fe-induced chemiluminescence (fig.1) and alkane formation (fig.2) of rat liver microsomes starts to rise after overcoming a lag phase or induction period of about 6–10 min (τ_0); the presence of this lag phase has been reported in similar peroxidizing systems [7], except that malondialdehyde accumulation was used as a parameter to assess lipid peroxidation. This induction period is probably related to the occurrence of internal membrane antioxidants, such as vitamin E (0.16 μg vitamin E/mg protein or 0.70 μg vitamin E/mg PUFA [14]), and its duration might be related to the content and free radi-

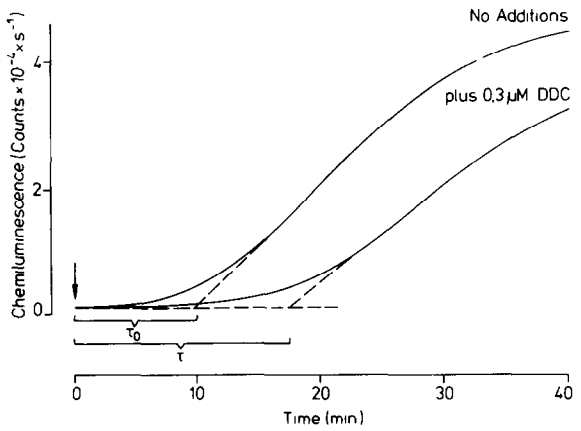

Fig.1. Effect of DDC on ascorbate/ADP-Fe-induced chemiluminescence of microsomal fractions. Assay conditions as described in section 2. Microsomal concentration 0.34 mg protein/ml. Time course of the chemiluminescence reaction in the absence and presence of DDC. τ_0 and τ, induction periods in the absence and presence of DDC, respectively.

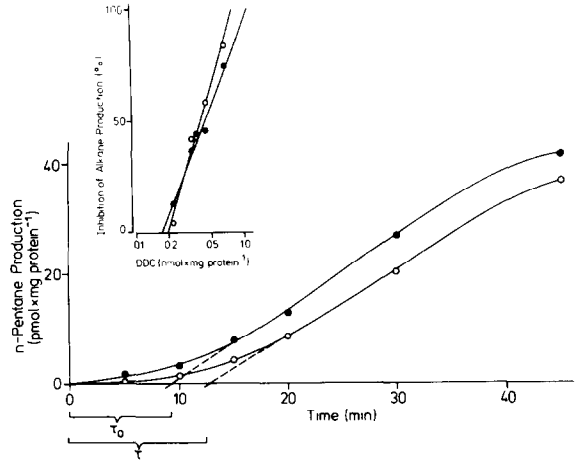

Fig.2. Effect of DDC on ascorbate/ADP-Fe-induced alkane production of microsomal fraction. Assay conditions as in fig.1. Microsomal concentration 0.91 mg protein/ml. Time course of *n*-pentane production in the absence (●) and presence (○) of 0.33 nmol DDC/mg protein. Inset: dependence of the inhibition of ethane and *n*-pentane production of DDC concentration.

cal quenching capacity of vitamin E. Brain homogenates, with a low vitamin E:PUFA ratio, do not show this lag phase of O_2-induced chemiluminescence [15], whereas liver homogenates [15] and isolated hepatocytes [13] have a 20–25 min induction period.

In the presence of DDC, the duration of the induction period (τ) is lengthened but the maximal chemiluminescence intensity (fig.1) and alkane formation (fig.2), once the lag phase is overcome, is similar to that obtained in the absence of DDC. The chromatographic pattern of the alkanes is not changed in the presence of the thiol.

The $\tau:\tau_0$ ratio indicates the relative increase in duration of the lag phase in the presence of DDC and reflects the resistance of microsomes to lipid peroxidation. The $\tau:\tau_0$ ratio is exponentially related to the concentration of DDC relative to microsomal protein (fig.3). It is noteworthy that nanomolar concentrations of DDC are effective in giving temporary protection against ascorbate/ADP-Fe-induced lipid peroxidation. As shown in fig.3 this effect might involve a specific binding of DDC to microsomal components, since it is dependent on the concentration of microsomes in the assay system. DDC did not largely inhibit lipid peroxidation but rather exerted a temporary protection by lengthening the induction period. This can be easily assessed by monitoring continuously the oxidative reactions with the photon-counting technique; sampling at a certain given time, as done for malondialdehyde determination, could lead to the observation of low malondialdehyde values which just fall within the lag phase period and could be mistakenly attributed to an inhibition of lipid peroxidation [9]. The inset in fig.2 shows the relationship between inhibition of alkane formation (ethane and *n*-pentane) and DDC concentration taken after 20 min incubation. In the case where DDC concentration produces a τ value longer than the incubation time, a small or negligible amount of alkanes would be detected. A DDC concentration with a τ value shorter than the incubation period would imply 'no effect' exerted by the thiol.

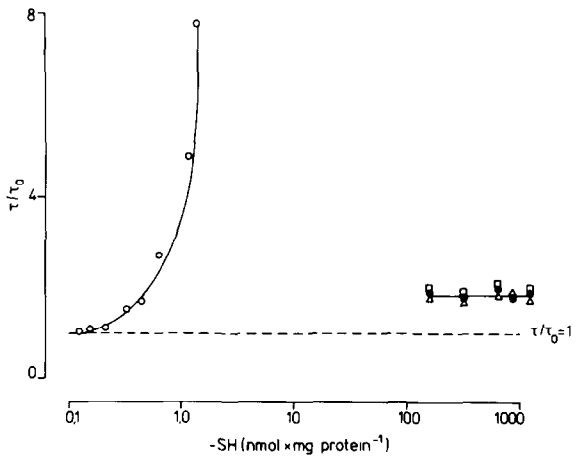

Fig.3. Effect of different SH-containing molecules on τ/τ_0 ratio as estimated by low-level chemiluminescence. Assay conditions as in fig.1. Microsomal protein concentration: 0.16–1.2 mg protein/ml. 0.3 μM DDC (○). 0.5 mM GSH (●), or penicillamine (△), or dithioerythritol (□). (+)-Cyanidanol-3 exerted a half-maximal inhibitory effect at a concentration of 2–10 μM, (not shown).

A second group of thiols, including glutathione, D-(−)-penicillamine, and dithioerythritol, was tested with the same experimental model of fig.1. All of them, and at variance with DDC, were effective in the millimolar concentration range in lengthening the induction period, and their effect was not dependent on microsomal protein concentration in the assay. This is summarized for the different thiols in fig.3. At a concentration of 0.5 mM this second group of thiols exerts a lengthening of the lag phase expressed by a $\tau:\tau_0$ ratio of 1.8–2.1; in order to produce a similar $\tau:\tau_0$ ratio, 0.53 nmol DDC/mg protein is required. It is clear that there is a different mechanism between both groups. In addition, cyanidanol, a well-known free radical scavenger that does not contain thiol groups [16], displays a comparable effect to that of DDC in terms of concentration requirements; however, our previous results with 4-hydroxynonenal-enhanced chemiluminescence of isolated hepatocytes [17] and with the model system reported here show that cyanidanol does not exert a temporary protection by lengthening the induction period, but rather inhibits irreversibly the development of chemiluminescence. Half-maximal inhibition is obtained with about 2–10 μM cyanidanol (not shown), in agreement with a previous report on isolated hepatocytes [17].

DDC has been shown to be involved in (a) inhibition of microsomal monooxygenase activity [18]; this effect was used to explain the antihepatotoxic properties of DDC against xenobiotic-induced

liver injury [9]; (b) inactivation of superoxide dismutase, probably through the formation of a copper-chelating product devoid of dismutating activity [19,20], at variance with the $O_2^{\cdot-}$-dismutating activity exhibited by Cu–penicillamine complexes [20]; (c) as an antidote for acute cadmium intoxication [21]; (d) as an inhibitor of ethanol metabolism and some aldehyde metabolizing enzymes (see [1]). A common mechanism for these diverse effects is difficult to envisage at the present stage. Of the possibilities listed above, only the inhibition of microsomal monooxygenase appears to apply relative to this study. In our model system (non-enzymatically induced peroxidation monitored by chemiluminescence), however, (i) no NADPH is present, which is a requirement to observe the inhibition of the hydroxylating activity of cytochrome *P*-450, and (ii) the concentration of DDC required to observe such an inhibition is 1000-fold higher than that used here [18].

It seems, therefore, that the effect of DDC here observed at nanomolar concentrations relies on a free radical quenching capacity of the compound rather than on an influence on microsomal metabolic processes. The higher efficiency of DDC with regard to the other group of different thiols might rely on the hydrophobicity of the former against the hydrophilic nature of the latter. This could suggest a membrane-related effect of DDC, thus protecting the target more effectively against O_2 radicals formed during the ascorbate/ADP-Fe-induced chemiluminescence.

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