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SINGLET OXYGEN PRODUCTION ASSOCIATED WITH HYDROPEROXIDE INDUCED LIPID PEROXIDATION IN LIVER MICROSOMES

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SUMMARY : Ter-butyl hydroperoxide (TBH) induced microsomal lipid peroxidation has been measured by oxygen consumption and malonaldehyde (MDA) formation. It has been found that the singlet oxygen ($^1O_2$) trap 2,5 diphenylfuran depressed both oxygen consumption and MDA formation. In contrast, histidine, another $^1O_2$ trap does not effect neither oxygen consumption, nor MDA production. On the other hand, β-carotene, a $^1O_2$ quencher strongly depresses oxygen consumption but slightly affects MDA production. Such results are consistent with the generation of $^1O_2$ as transient by product of peroxidative microsomal lipid decomposition.

INTRODUCTION : Previous studies on liver microsomal lipid peroxidation have indicated that singlet oxygen ($^1O_2$) formation may occur during the peroxidative process (1, 2). Moreover, in this field of investigations, it has been suggested in a recent study (3) that $^1O_2$ is formed during the metal-catalyzed decomposition of linoleic acid hydroperoxide. These assumptions were based on two pieces of evidence : (i) chemiluminescence emitted during lipid peroxidation and related to be $2^1\Delta g \rightarrow 2^3\Sigma\bar{g}$ transition is inhibited by $^1O_2$ traps. (ii) Cis-1,2-dibenzoylethylen, a product of the oxygenation of 2,5 diphenylfuran by $^1O_2$ was isolated from the reaction mixtures. However, it has been further demonstrated that furan analogues used as $^1O_2$ traps were not specific for $^1O_2$ (4, 5) and very recently (6) it has been suggested that a $^1O_2$-like factor, (a moiety having properties of $^1O_2$ in the presence of $^1O_2$ traps) was generated during lipid peroxidation. In this occurence, the generation of $^1O_2$ during lipid peroxidation is highly questionable and require additional informations. We have therefore investigated this question once more and results reported in this paper are in agreement with the generation of $^1O_2$ as transient by-product of the peroxidative decomposition of microsomal lipid.

MATERIALS AND METHODS

CHEMICALS : The substances used in these studies were obtained from the following sources : Terbutyl hydroperoxide from Koch-Light laboratories, NADPH from Boehringer, 2,5-diphenylfuran from Eastman Organic chemicals, Histidine and βcarotene from Sigma Chemical Co.

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PREPARATIONS OF MICROSOMES : Liver microsomes were prepared from Sprague-Dawley male rats weighting 180-200 g. Animals were sacrified, livers were removed and washed with cold 0.15 M NaCl. Washed livers were immediately minced and homogenization was carried out in a 0.04 M Tris-HCl buffer (pH 7.50) containing 0.34 M sucrose. The homogenate was then centrifuged at 600 g for ten minutes to remove large debris and unbroken cells. The supernatant was then centrifuged at 10,000 g x 30 minutes. This step was repeated once. The 10,000 g supernatant was centrifuged at 105,000 g x 30 minutes and the microsomal pellet was resuspended in Krebs-phosphate buffer (pH 7.40) free of calcium (KRP) and recentrifuged at 105,000 g x 30 minutes. Final pellet was resuspended in KRP. Protein concentration of the microsomal suspension was determined according to Böhlen et al (7) and adjusted at 10 mg/ml with KRP. Average of enzymic activities implicated in the oxidative metabolism were : superoxide dismutase activity measured as previously described (8) = 0. Catalase activity measured at 37°C (pH 7.40) according to Halbach (9) : 5.40 nmoles H_2O_2 decomposed/min/mg microsomal protein. NADPH oxidase-like activity measured as previously described (10): 7.80 nmoles O_2 consumed/min/mg microsomal protein.

OXYGEN CONSUMPTION : Oxygen consumption was carried out in a cylindrical glass chamber (vol 1 ml, T.37°C) equiped with a Clark electrode recorded to a Gilson Oxygraph.

MALONALDEHYDE PRODUCTION : Malonaldehyde generation resulting of the peroxidative decomposition of fatty acids was determined by the thiobarbituric assay essentially as described in (11).

RESULTS AND DISCUSSION

It is established that microsomal lipids peroxidation occurs through a free radical chain reaction involving the classical sequence of initiation and propagation. Among the most likely chain initiating process are those involving hydroperoxides and metal ions according to the following sequence :

$$M^{n+} + ROOH \longrightarrow M^{n+1} + OH^- + RO^\cdot \quad (1)$$

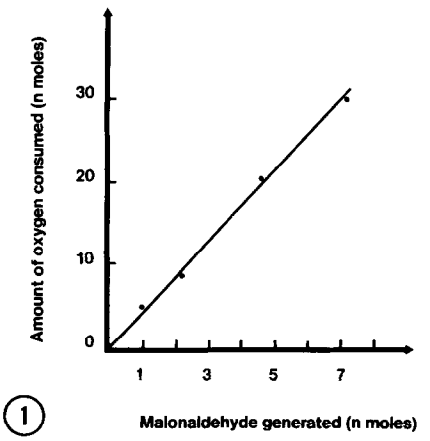
RO· radical further react with unsaturated fatty acids and contributes to the initiation of the propagation reactions :

$$RO^\cdot + AH \longrightarrow R\text{-}OH + A^- \quad (2)$$

$$A^\cdot + O_2 \longrightarrow A\text{-}O_2^\cdot \quad (3)$$

$$A\text{-}O_2^\cdot + AH \longrightarrow AOOH + A^\cdot \quad (4)$$

where AH is a polyinsaturated fatty acid. Moreover, the organic hydroperoxide AOOH is unstable and yields by intramolecular cyclization and decomposition new organic free radicals and malonaldehyde (MDA)(for general review, see : 12). Accordingly, addition of Terbutyl hydroperoxide (TBH) to a microsomal suspension resulted in a rapid peroxidation of lipids which is evident by the appearance of oxygen consumption and MDA production. Fig. 1 indicates the relationship between O_2 uptake and MDA production. It was observed that O_2 uptake is fairly correlated with MDA generation and in this occurence, measurement of oxygen consumption provides a convenient assay for the evaluation of the peroxidative reaction. Fig. 2 indicates that TBH-induced



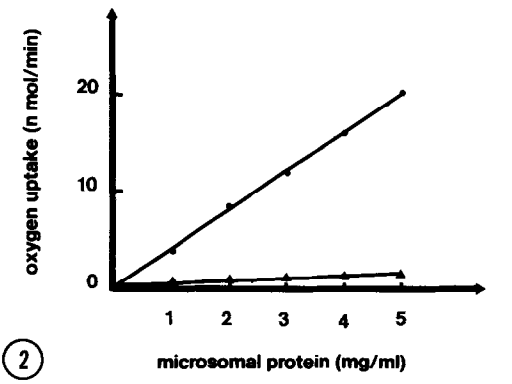


Figure 1

Relationship between oxygen consumption and malonaldehyde production associated with TBH induced microsomal lipid peroxidation. Oxygen consumption and MDA production were measured as indicated in methods. Incubation was conducted at 37°C in the oxygraph glass chamber. Assay medium was composed of Krebs phosphate buffer (pH 7.40) and 2 mg eq microsomes/ml. Peroxidative process was induced by the addition of 10 mM TBH and oxygen uptake was monitored. During the time course of the reaction, 0.5 ml of the microsomal suspension was removed from the oxygraph chamber and acidified with cold trichloracetic acid (5% acid, w/v, final concentration) and centrifuged. MDA content of the supernatant was determined whereas the corresponding oxygen consumption was measured. Such procedure was repeated at various times of incubation.

Figure 2

Relationship between oxygen uptake associated with lipid peroxidation and concentration of microsomes. Experiments were carried out as described in fig. 1. Values reported in the figure are estimated from the linear portion of the curves of oxygen uptake. ●——● intact microsomes, ▲——▲ heat denaturated microsomes.

lipid peroxidation as measured by oxygen uptake is linear with respect of microsome concentration indicating that the amount of TBH present in the assay medium is not limiting factor of the peroxidative reaction. Moreover, the reaction requires the integrity of microsomes since in the presence of heat denaturated microsomes only a slight O_2 uptake occurs.

So, we assume that the experimental conditions used are convenient for the determination of the autocatalytic sequence of peroxidation of lipids as described above in reactions 2, 3 and 4. In order to investigate the possible involvement of $^1O_2$ in the lipid peroxidation process either as active intermediate or as by-product of the reaction we have determine the effects of compounds known to be either $^1O_2$ traps such as 2,5-diphenyl furan (DPF) and histidine or $^1O_2$ quencher such a β-carotene on both O_2

uptake and MDA formation associated with TBH induced lipid peroxidation. It is known that DPF reacts with $^1O_2$ to form cis-dibenzoylethylen through the formation of an endoperoxide. This is an oxygenation reaction resulting in the disappearance of $^1O_2$ from the medium. As well, histidine and $^1O_2$ react to give products of cleavage of imidazole ring resulting in the consumption of $^1O_2$. Moreover, the reaction rates of DPF and histidine with $^1O_2$ have been estimated to be 1.4×10^8 M^{-1} sec^{-1} and 5×10^7 M^{-1} sec^{-1} respectively (13) which indicate a quite similar reactivity of the two $^1O_2$ traps.

Curves reported in fig. 3 indicate the effects of varied concentrations of DPF and histidine on O_2 uptake and MDA formation. It was observed that DPF strongly inhibits both O_2 uptake and MDA production whereas in contrast histidine added at identical concentration slightly affects these reactions. This is a surprising result since DPF and histidine react similarely with $^1O_2$ as indicated above. However, this discrepancy may be related to the possible reaction of DPF with other compounds than $^1O_2$ and especially with the peroxy radicals involved in the initiation process of the lipid peroxidation as recently reported (6). The lack of effect of histidine suggests in this occurence that $^1O_2$ was not involved in the development of the peroxidative reaction. If $^1O_2$ is generated as by-product of the peroxidative reaction it should react with electron-rich biological molecules of the neighbourhing before the reaction $^1O_2 \rightarrow {}^3O_2$ may occur. Addition in the medium of an $^1O_2$ quencher such as β-carotene which is known to react with $^1O_2$ to form $^3O_2$ at a diffusion controlled rate should result in the release of molecular oxygen under the triplet state as follows :

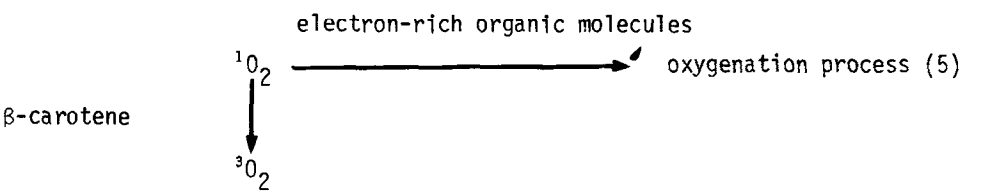
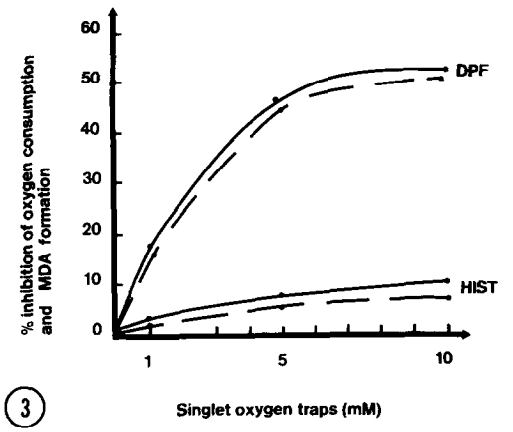


Fig. 4 indicates the effect of varied concentration of β-carotene on O_2 uptake and MDA production during the microsomal lipid peroxidation. Whereas MDA was slightly suppressed, oxygen consumption was strongly inhibited by addition of β-carotene. The curve of inhibition of O_2 uptake (fig.4 curve A) exhibits a typical kinetic of a competitive reaction according to reaction 5. Moreover, identical experiment performed in the presence of 10 mM histidine (fig. 4 curve 13) indicates a competitive effect between β-carotene and histidine. In this occurence, it is clear that the inhibition of oxygen



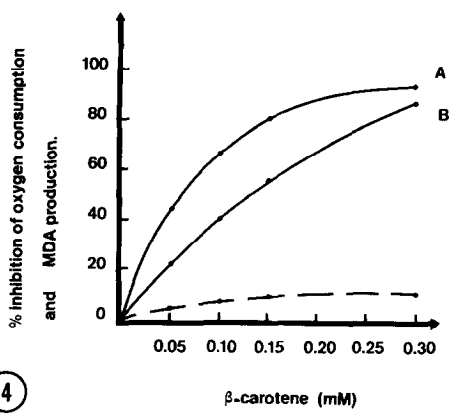


Figure 3

Effects of $^1O_2$ traps on oxygen uptake and MDA production associated with lipid peroxidation. Experiments were carried out as in fig. 1. Solid lines indicate oxygen uptake and dotted lines MDA production. DPF was dissolved in acetone and histidine hydrochloride in water.

Figure 4

Effet of β-carotene on oxygen consumption and MDA production associated with lipid peroxidation. Experiments were carried out as in fig. 1. β-carotene was dissolved in methanol and its concentration was estimated at 450 nm using a molar extinction of 13.800 M^{-1} cm^{-1}. Solid lines indicate oxygen uptake curve A : standard medium, curve B : + 10 mM histidine. Dotted line : MDA production.

uptake induced by β-carotene is an apparent inhibition due to the release in the medium of oxygen under ground state ($^3O_2$) from $^1O_2$. On the other hand the slight effect of β-carotene on MDA production indicates that it does not interfere with the peroxidative process.

From these results we concluded that during microsomal lipid peroxidation induced by TBH, $^1O_2$ is effectively generated as transient by-product but apparently does not participate to the propagation sequence of the peroxidation reaction.

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