PHARMACOLOGY AND TOXICOLOGY

Effect of pH and Glutathione on Lipid Peroxidation in Tocopherol-Enriched Liposomes

L. F. Dmitriev, M. V. Ivanova, and A. V. Lebedev

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Tocopherol-enriched liver microsomes are more resistant to lipid peroxidation at an alkaline pH of 8.5 in comparison with microsomes incubated at pH 7.5. An alkaline pH provides conditions for two-electron oxidation of tocopherol, which causes the lipid molecule to revert to the initial state (O_2/H exchange). A possible mechanism of inhibition of lipid peroxidation within the physiological range of pH with participation of a glutathione-dependent enzyme is discussed.

Key Words: *liver microsomes; tocopherol; glutathione*

Vitamin E (α-tocopherol, TP) is known to be a potent chain-break antioxidant. Published data suggest that the antioxidative activity of TP in biological membranes is predominantly responsible for its biological effect, an important role being played by the sulfur-containing compounds glutathione and cysteine [1]. Although glutathione (GSH) is frequently considered to be a potential inhibitor of lipid peroxidation (LPO) [8,9,12,13], the mechanism of its inhibiting action remains poorly understood. The present study examines the GSH-dependent enzymatic mechanism of LPO inhibition by TP in liver microsomes.

MATERIALS AND METHODS

Microsomes were isolated from rat liver routinely (centrifugation at 105,000 g). Incorporation of TP into microsomes was performed as follows: 5 μl of a TP solution in ethanol (concentration range from 1 to 2 mM) were added to the microsomal suspension (containing approximately 20 mg protein) and the mixture was vortexed for 60 or 90 sec depending on the concentration of TP being incorporated. TP was extracted from microsomes as described previously [4]. The concentration of TP was determined fluorimetrically [10]. The microsomes were incubated at 30°C; the content of malonic dialdehyde (MDA) was measured by the reaction with 2-thiobarbituric acid [2]. Superoxide was determined using nitroblue tetrazolium as the acceptor.

Cardiological Research Center, Moscow (Presented by E. I. Chazov, Member of the Russian Academy of Medical Sciences)

RESULTS

We determined the formation of MDA and utilization of oxygen in TP-enriched microsomes in order to elucidate whether the antioxidative effect of GSH (its synergism with TP) [9] is a result of direct action or a result of the work of an enzyme system. The efficacy of TP may be properly evaluated *in vitro* by measuring the duration of the induction period which usually precedes MDA formation.

The formation of MDA was studied in native TP-enriched microsomes with an initial TP concentration of 0.40±0.08 nmol/mg protein. The microsomes were incubated for 30 min in the presence of NADPH at pH 7.5 and 8.5 (Fig. 1). As is seen from the figure, the microsomes incubated at the alkaline pH were more resistant to peroxidation

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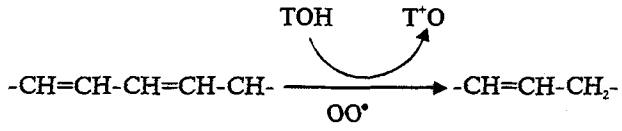
than those incubated at pH 7.5, while NADPH-cytochrome C reductase activity remained unchanged, meaning that the rate of LPO initiation was constant. Thus, TP acting as an antioxidant in microsomes is more effective at pH 8.5 than at pH 7.5, which is in conformity with the data obtained on liposomes [5].

In parallel with MDA we measured superoxide at the same pH values (the life-time of O_2^- is known to increase as the pH rises, $O_2^- + H^+ \leftrightarrow HO_2^\cdot$). There are at least two possible paths of superoxide formation in TP-containing microsomes: transfer of an electron from flavoprotein and two-electron oxidation of TP ($LO_2^\cdot + TOH \rightarrow LH + T^+O + O_2^-$) [5].

In light of this, we studied superoxide formation in microsomes at neutral and alkaline pH values by measuring the O_2^--dependent reduction of nitroblue tetrazolium (Fig. 2). The rate of superoxide formation in the xanthine-xanthine oxidase system was found to rise threefold as the pH increased from 7.5 to 8.5, while in the microsomes we observed a more than 3-fold increase. This difference in the rate of superoxide generation implies that superoxide appears not only due to oxygen reduction by flavoprotein (in which case a pH shift to an alkaline range should cause a 3-fold increase of O_2^-). There may be another pathway of O_2^- generation at pH 8.5. We assume that superoxide is formed due to breakage of a C-O bond in the fatty acid molecule ($LO_2^\cdot$) attacked by TP.



CH=CH-+O_2- (pH=8.5)

The effect of GSH and ascorbate on LPO in TP-enriched microsomes is demonstrated in Fig. 3. As is seen from the figure, in TP-enriched microsomes GSH exhibits a marked protective effect. The intensity of oxygen utilization and MDA formation in these experiments depended on the content both of TP and of GSH and ascorbate. For instance, the duration of the induction period increased from 6 to 12 min (0.2 mM GSH) and from 6 to 16 min (0.5 mM ascorbate). It should be noted that in microsomes incubated with and without GSH the O_2/MDA molar ratio remained constant and, consequently, inhibition of LPO occurred at the stage of LOOH formation.

When microsomes were incubated with GSH, again, as in the experiment with pH 8.5, we determined O_2^- generation assuming it not to be restricted by oxygen reduction by flavoprotein. The

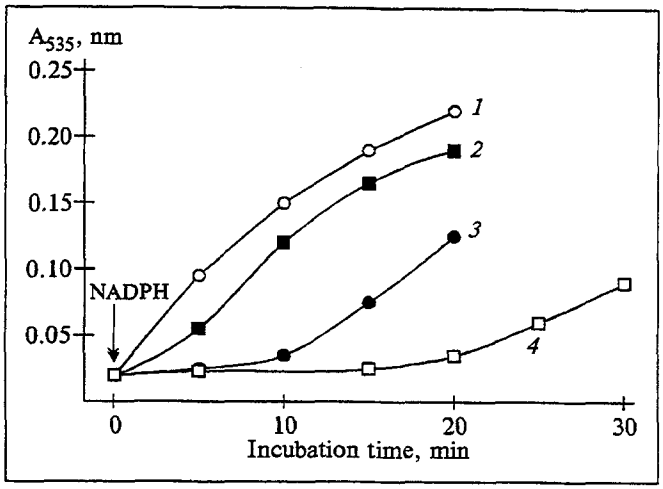


Fig. 1. Effect of pH on MDA formation kinetics in control (*1*, *2*) and TP-enriched (*3*, *4*) microsomes. Incubation conditions: 0.1 mM Tris-buffer, pH 7.5 (*1*, *3*) or 8.5 (*2*, *4*), 0.5 mM ADP, 1 mg/ml protein.

expected increase in superoxide generation was not detected in TP-enriched microsomes in the presence of the substrate of GSH-dependent enzyme. This is quite understandable when the interaction between GSH and superoxide is taken into account [1] (the second-order constant in this case is 6.7×10^5 $M^{-1}sec^{-1}$). Thus, GSH plays a dual role: it inhibits the formation of LOOH hydroperoxides and removes superoxide.

There are three possible explanations of the effect of GSH: 1) GSH quenches lipid radicals and/or superoxide; 2) GSH reduces TP radicals; 3) GSH is a substrate in the enzymatic reaction. The role of GSH as a trap for lipid radicals (L· and $LO_2^\cdot$) can presumably be excluded, since no protective effect of GSH has been found in liposomes [6]. As for the GSH-superoxide interaction, it ob-

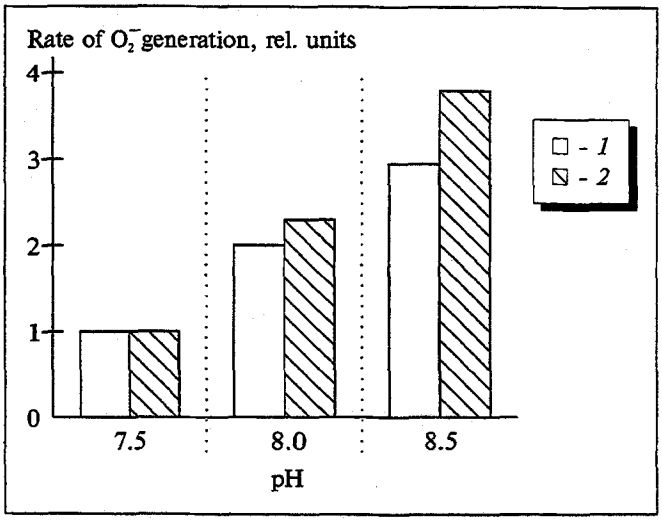


Fig. 2. Effect of pH on rate of superoxide generation in two different systems. *1*) xanthine-xanthine oxidase-containing solution, *2*) liver microsomes.

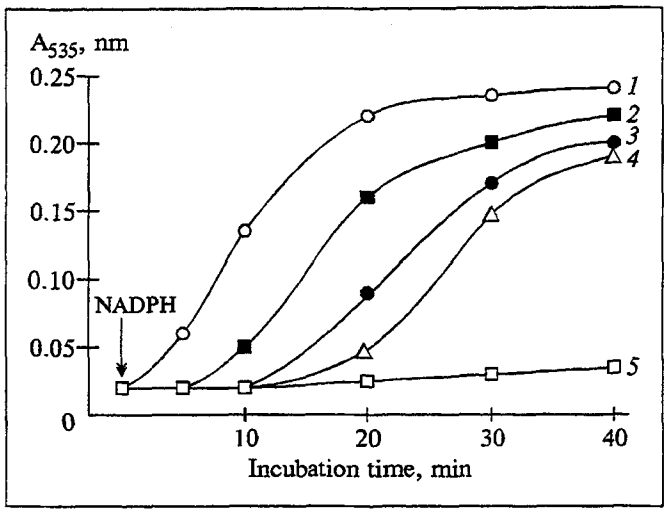
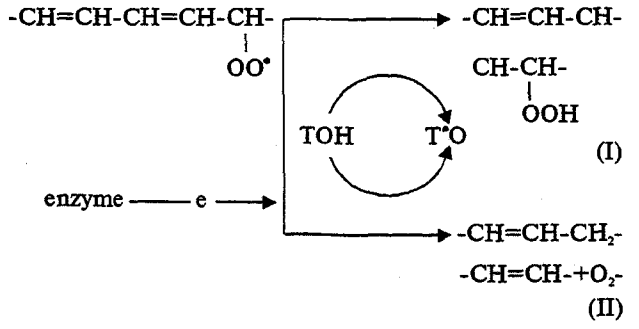


Fig. 3. Kinetics of MDA formation in control (*1*) and TP-enriched (*2–5*) microsomes: *2*) control, *3*) with GSH, *4*) with ascorbate, *5*) with GSH and ascorbate. Incubation conditions: 0.1 mM Tris–buffer, pH 7.5; 0.2 mM ADP, 0.2 mM GSH, 0.5 mM ascorbate, 1 mg/ml protein.

viously cannot be completely responsible for the effect of GSH on LPO, especially for GSH and TP synergism [9].

If GSH, like ascorbate, were to reduce TP radical, the effects of ascorbate and GSH would be additive and the induction period in the presence of these two compounds would last 20-25 min. However, as is seen from Fig. 3, the induction period is much longer. It is known that GSH does not control the state of TP in liposomes [6]. Thus, the third version most reasonably explains the protective effect of GSH.

Although we have no direct evidence for a new enzymatic LPO-controlling mechanism involving TP and GSH (the substrate of the putative enzyme), such a mechanism seems highly likely to exist. Most probably, GSH participates in the reduction of TP radicals by GSH (TO·) reductase or of peroxide radicals by GSH (LO_2·) reductase. There are thus two possible mechanisms of interaction between TP and peroxide radicals: first, the well-known chain-break reaction, accompanied by hydroperoxide formation [11] and, second, superoxide radical reduction (involving a GSH-dependent enzyme) followed by hydrogen acceptance from TP, which prevents the formation of LOOH.



According to this scheme, which includes mechanisms I and II, interaction between TP and peroxide radicals $[LO_2]^-$ results not just in a simple chain break, but in a cyclic transformation of the lipid molecule, i.e., reversion to the initial state, and in the release of superoxide. In contrast to mechanism I, enzymatic mechanism II implies the possibility of complete inhibition of LPO within physiological pH values, which is especially important in view of the fact that TP and ascorbate act as H atom donors [3,7].

LPO in biological membranes is known to be governed by several factors. Based on our findings we suggest that, along with the well-known antioxidant enzymes (superoxide dismutase and glutathione peroxidase), there is an additional enzyme coupled with TP. TP is most effective when paired with GSH-dependent enzyme. The low activity of this enzyme is responsible for the low efficacy of TP. We conclude that the formation of lipid peroxides depends on three main factors: GSH-dependent enzyme, TP, and ascorbate. This assumption should certainly be experimentally verified, specifically by studying the TP utilization kinetics during the incubation of microsomes with GSH.

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