ESR Studies on the Antioxidant Activity of Troglitazone

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To elucidate the differences in the antioxidant potency of troglitazone and α-tocopherol in the oxidation of low density lipoprotein (LDL), the structure of the phenoxyl radical of troglitazone and the antioxidant activity of troglitazone in homogeneous solution and liposomal membranes were investigated. The antioxidant activity of troglitazone in homogeneous solution based on the *cis*-parinaric acid fluorescence method was not substantially different from that of α-tocopherol. The ESR study using a spin label technique showed that troglitazone more effectively scavenged the radicals generated in the water phase outside of the liposomal membranes than those generated from the interior of the membranes than did α-tocopherol. These results suggest that the difference in the antioxidant potency of troglitazone and α-tocopherol in the oxidation of LDL is partly due to the mobility and the position of these compounds in the liposomal membrane.

Key words ESR; antioxidant activity; troglitazone; spin label

It has been reported that the antioxidant activity of troglitazone (**1**), which possesses the same chroman moiety as α-tocopherol (**2**) (Fig. 1), is stronger than that of the latter in the oxidation of low density lipoprotein (LDL).[1] These substances are believed to exert antioxidant activity through the reaction of their hydroxyl group with the radical species, leading to the scavenging of the radical species and the simultaneous formation of their phenoxyl radicals. The strength of the antioxidant activity depends on the reactivity with the radical species and the stability of the phenoxyl radical formed.

However, the antioxidant activity of α-tocopherol is substantially less in the liposomal membrane than in homogeneous solution.[2] It is now generally accepted based on a number of studies that α-tocopherol is incorporated into the membranes and is located in such a way that its hydroxyl group is at or near the water–membrane interface, and that the hydrophobic phytyl side chain is embedded inside the membrane.[3,4] This reduces its mobility, both lateral and vertical. In fact, it was reported that the rate of scavenging of radicals by α-tocopherol decreased as the radicals went deeper into the membrane interior, implying that the restricted vertical mobility of α-tocopherol and/or radicals reduces the antioxidant activity of α-tocopherol in the membrane.[5] In considering the antioxidant activity of a drug in a heterogeneous environment such as biological membranes or lipoproteins, the following factors should be taken into consideration: (1) reactivity with the radical, (2) location of the drug, (3) concentration in a microscopic field, and (4) mobility.

In the present study, the structures of the phenoxyl radical of troglitazone and α-tocopherol and their antioxidant activities in homogeneous solution and liposomal membranes were investigated, and the most probable location of troglitazone within the lipid bilayer is proposed and compared with the localization of α-tocopherol.

Experimental

Materials Troglitazone was synthesized and purified in the Medicinal Chemistry Research Laboratories of Sankyo Co., Ltd. (Tokyo, Japan). 14:0 PC (L-α-phosphatidylcholine dimyristoyl), 18:2 PC (L-α-phosphatidylcholine dilinoleoyl), AAPH (2,2′-azobis(2-amidinopropane) dihydrochloride) and AMVN (2,2′-azobis(2,4-dimethylvaleronitrile) were purchased from Wako Pure Chemicals Co. (Osaka, Japan). *cis*-Parinaric acid was purchased from Molecular Probes (Junction City, OR, U.S.A.). 5NS (5-doxyl-stearic acid), 7NS (7-doxyl-stearic acid), 12NS (12-doxyl-stearic acid), and 16NS (16-doxyl-stearic acid) were purchased from Aldrich Co. (Milwaukee, WI, U.S.A.). All other reagents were commercially available and of guaranteed grade.

Formation of Phenoxyl Radicals The phenoxyl radicals of troglitazone and α-tocopherol were produced by chemical oxidation of the respective compounds. A 10^{-4} M solution of the compound in tetrahydrofuran (THF) was reacted with a small amount of lead dioxide or metal sodium at room temperature, and the ESR spectrum was measured. The chemical structure of each radical was confirmed by a simulation of the ESR spectrum.

Antioxidant Activity in Homogeneous Solution For the assay in homogeneous solution, the *cis*-parinaric acid fluorescence-based procedure developed by Tsuchiya *et al.* was used.[6] The assay was performed using a lipophilic azo initiator, AMVN, and *cis*-parinaric acid in hexane. AMVN-induced peroxyl radicals oxidize *cis*-parinaric acid,

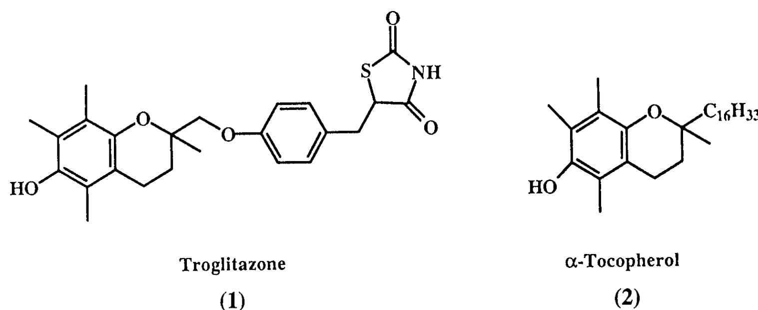

Fig. 1. Structures of Antioxidants

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which is monitored by the decay of their characteristic fluorescence (excitation at 304 nm and emission at 421 nm). The reaction mixture (3 ml) consists of 100 mM AMVN and 30 μM *cis*-parinaric acid in hexane. Methanol solutions of the antioxidants (troglitazone and α-tocopherol) were added to the reaction mixture and the fluorescence was measured.

Antioxidant Activity in Heterogeneous Solution For the assay in heterogeneous solution, the spin label technique developed by Takahashi *et al.* was used.[5] Liposomes with a bilayer membrane were prepared by the conventional method. Chloroform solution of PC in a test tube was evaporated to dryness so that a thin layer of the lipid formed on the inner surface of the test tube. An appropriate amount of 0.1 M NaCl aqueous solution was added, and the film was slowly peeled off by shaking to obtain white, milky, multilamellar liposome suspensions. These were sonicated to obtain unilamellar liposomal membranes and subjected to oxidation. Radical initiator, spin probe and test drug were previously added to PC solution if necessary, and the liposomes were prepared in the same manner. To initiate the radical chain reaction, a water-soluble AAPH and a lipid-soluble AMVN were used. In the experiment using AAPH, an appropriate amount of AAPH was added to the liposomal suspensions and incubated at 37 °C. In the experiment using AMVN, the suspension of liposomes containing AMVN was incubated at 50 °C. An aliquot of the reaction mixture was collected at regular intervals, transferred into a flat quartz cell, and subjected to ESR measurement.

The ESR apparatus used was an X-Band Spectrometer (JEOL R1X). Measurements were performed under the following conditions: modulating magnetic field: 0.1 mT, microwave output: 8 mW, time constant: 0.3 s, and sweeping rate: 10 mT/4 min. The concentration of spin probes in the liposomal membranes was measured from their ESR signals using a standard calibration curve.

Results and Discussion

Phenoxyl Radicals of Troglitazone and α-Tocopherol After oxidation of troglitazone in THF with lead dioxide or metal sodium, a rather stable radical exhibiting an ESR spectrum composed of 7 broad peaks was obtained. This spectrum changed with time into one showing hyperfine structures (Fig. 2a). According to the simulation of the spectrum, the hyperfine splitting constants (hfs, gauss) of the protons at the 5-, 7-, and 4-positions were determined as $a_H^{5\text{-}CH_3}=5.25$, $a_H^{7\text{-}CH_3}=4.78$, and $a_H^{4\text{-}CH_2}=1.33$, respectively. These results indicate that the spectrum thus obtained was due to the phenoxyl radical. The spectrum of the phenoxyl radical obtained by the oxidation of α-tocopherol is also shown in Fig. 2b. The hfs were $a_H^{5\text{-}CH_3}=5.98$, $a_H^{7\text{-}CH_3}=4.57$, and $a_H^{4\text{-}CH_2}=1.47$. According to Burton *et al.*, the excellent antioxidant properties of α-tocopherol reside in the fused heterocyclic ring.[7] In this ring, the p-type lone pair on oxygen is kept almost perpendicular to the aromatic plane. This p-type lone pair overlaps with the singly occupied molecular orbital of the radical which stabilizes the phenoxyl radical formed upon abstraction of the phenolic hydrogen. The degree of overlap depends on the overlap parameter θ (θ represents the Ar–O–C dihedral angle). The antioxidant activity increases as θ decreases. With decreasing θ, the spin density in the aromatic ring and the strength of the O–H bond decrease.[8] Troglitazone has a very similar θ to that of α-tocopherol (16.8° for troglitazone[9] and 17° for α-tocopherol). Supporting evidence for θ with regard to the spin density of the phenoxyl radical of troglitazone is provided by the ESR spectrum. Mukai *et al.* reported that the antioxidant activities of α-tocopherol derivatives depend on the sum of the π-spin density at the two ortho carbon atoms at the 5- and 7-positions of the chromanol ring.[10] The spin density is defined by $a_i^H=Q\rho_i$, where a_i is the hfs and ρ_i is the spin density of i atom, respectively, and $Q=27$(gauss).[11] The antioxidant activity increases as the sum of the π-spin density at the two ortho carbon atoms decreases. The phenoxyl radical of troglitazone has a very similar sum of π-spin density to that of α-tocopherol (0.37 for the former and 0.39 for the latter). These results suggest that troglitazone and α-tocopherol have similar antioxidant activity in homogeneous solution.

Antioxidant Activity of Troglitazone in Homogeneous Solution In hexane, *cis*-parinaric acid has maxima at 304 and 421 nm in its fluorescence excitation and emission spectra. *cis*-Parinaric acid with its four conjugated double bonds is extremely sensitive to peroxyl radical-induced oxidation. The fluorescence decay of *cis*-parinaric acid in hexane due to its peroxidative destruction by AMVN-

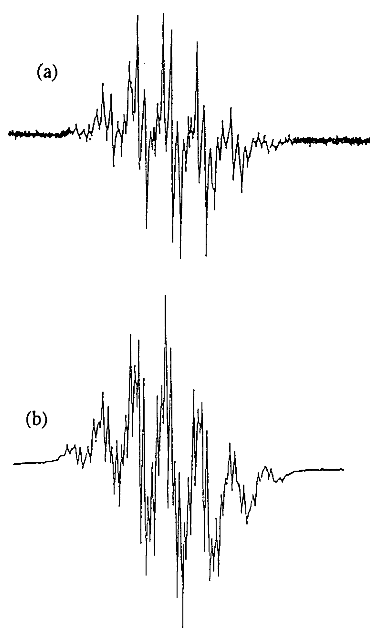

Fig. 2. ESR Spectra of Phenoxyl Radicals of Troglitazone (a) and α-Tocopherol (b) Produced by the Reaction of Troglitazone and α-Tocopherol with Lead Dioxide

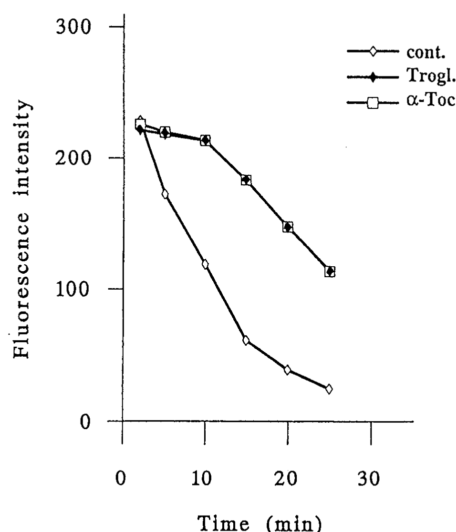

Fig. 3. Comparison of Antioxidant Activity of Troglitazone and α-Tocopherol in a *cis*-Parinaric Acid Fluorescence-Based Assay

The reaction system contained *cis*-parinaric acid (30 μM) and AMVN (100 mM) in hexane. The peroxidation of *cis*-parinaric acid was affected by the addition of 60 μM antioxidants (excitation at 304 nm and emission at 421 nm with 5 nm slits, at 40 °C).

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derived peroxyl radicals was linear in time and could be continuously measured in this system. Antioxidant-dependent peroxyl radical scavenging prevented *cis*-parinaric acid fluorescence decay, thus providing an index for the antioxidant efficiency. Figure 3 shows the effects of AMVN and antioxidants on the fluorescence of *cis*-parinaric acid in hexane. The addition of AMVN induced peroxidation of *cis*-parinaric acid, resulting in its fluorescence decay (Fig. 3; control).

In this system, troglitazone and α-tocopherol produced a delay in *cis*-parinaric acid fluorescence decay indicative of their efficient scavenging of peroxyl radicals. There was no substantial difference in the radical scavenging efficiency of the two antioxidants. A low concentration of absolute methanol as a solvent for them had little effect on this reaction. These results also indicated that there was no substantial difference in the antioxidant activity between troglitazone and α-tocopherol in homogeneous solution.

Antioxidant Activity of Troglitazone in Heterogeneous Solution The above data suggested that troglitazone and α-tocopherol have similar *in vitro* antioxidant activity. However, it was reported that the former shows stronger antioxidant activity in the oxidation of LDL than the latter.[1] To elucidate the discrepancy in the antioxidant activity of these compounds, it is important to consider the location of troglitazone in the liposomal membrane. The intra-membrane locations of troglitazone and α-tocopherol, which may be responsible for the difference in their antioxidant potencies, were compared using the spin label technique. The locations were investigated by comparing the rate of consumption of the spin probes, 5NS, 7NS, 12NS, and 16NS, which possess a nitroxyl group at the 5, 7, 12, and 16 positions of stearic acid, respectively. This was conducted in 18:2 PC and 14:0 PC liposomes in the absence and presence of the antioxidants, and the consumption of the spin probe was induced by the reaction with the peroxyl radicals derived from AAPH or AMVN.

Figure 4 shows the rate of consumption of 5NS and 16NS incorporated into 18:2 PC liposomes during oxidation initiated with AMVN in the absence and presence of the antioxidants. The spin probe incorporated into the liposomal membrane suppressed the AMVN-induced oxidation and was consumed as the oxidation proceeded. When the probe and the antioxidant were incorporated simultaneously into the membrane, the antioxidant suppressed consumption of the probe.

In this system, although there was no substantial difference between the effects of the compounds, both troglitazone and α-tocopherol reduced the rate of consumption of 5NS more efficiently than that of 16NS. The spin probes were consumed even when they were incorporated into saturated 14:0 PC liposomal membranes which do not undergo oxidation as shown in Fig. 5.

The efficiencies of troglitazone and α-tocopherol in suppressing consumption of the spin probe were estimated from Eq. 1, where R_0 and R_t represent the rates of consumption of the probe in the absence and presence of the compounds, respectively.[5]

$$P=(R_0-R_t)/R_0 \qquad (1)$$

Figure 6 shows the efficiencies of the compounds in suppressing the AMVN-induced consumption of different NS incorporated into 18:2 PC and 14:0 PC liposomal membranes. These efficiencies were lower in 14:0 PC liposomes than in 18:2 PC liposomes, and no distinction efficiency in compound was detected for all NSs, although the efficiencies tended to decrease the deeper the nitroxide group was in the interior of 14:0 PC.

The results suggest that both compounds can scavenge the phospholipid peroxyl radicals that are closer to the surface of the membrane more efficiently than the peroxyl radicals derived from AMVN, probably because lipophilic AMVN resides largely in the more fluid, central region of the PC membranes, while 18:2 PC produces phospholipid peroxyl radicals at the 9- and 13-positions which tend to move to the surface during their generation.[5] It is difficult to determine the difference in the location of the antioxidants from these results, although they do indicate that both are located at or near the surface of the mem-

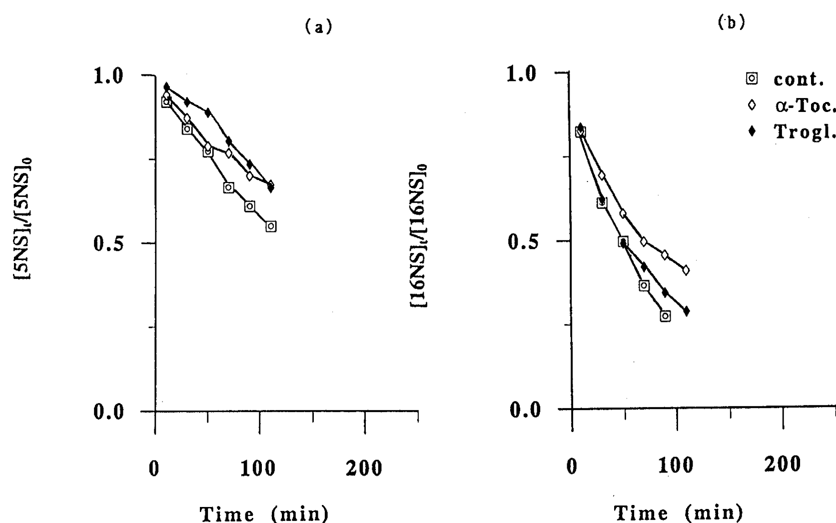

Fig. 4. Consumption of 5NS (a) and 16NS (b) Incorporated into 18:2 PC Liposomes during Oxidation Initiated with AMVN

Oxidation reaction carried out at 50 °C in the absence and presence of α-tocopherol or troglitazone. Concentrations were [5NS] = [16NS] = 23 μM, [18:2 PC] = 11.6 mM, [AMVN] = 3.5 mM, [α-Toc.] = [Trogl.] = 33 μM.

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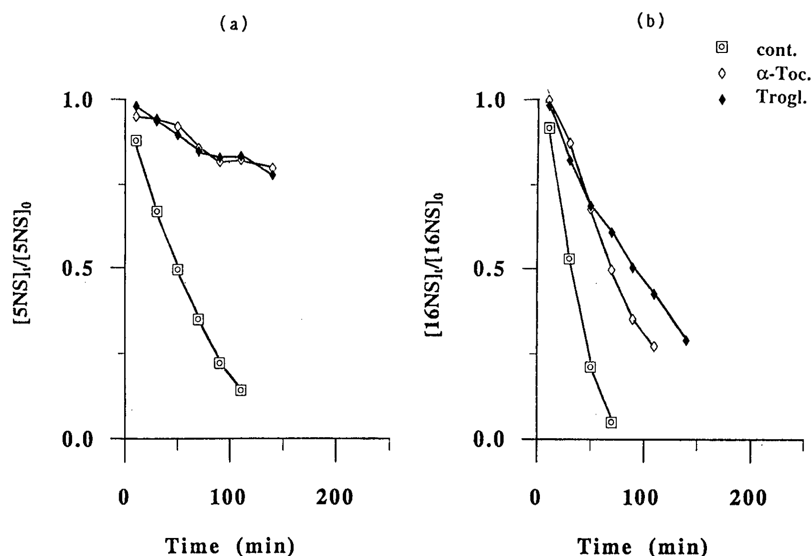

Fig. 5. Consumption of 5NS (a) and 16NS (b) Incorporated into 14:0 PC Liposomes during Oxidation Initiated with AMVN

Oxidation reaction carried out at 50 °C in the absence and presence of α-tocopherol or troglitazone. Concentrations were [5NS] = [16NS] = 23 μM, [14 : 0 PC] = 11.6 mM, [AMVN] = 3.5 mM, [α-Toc.] = [Trogl.] = 33 μM.

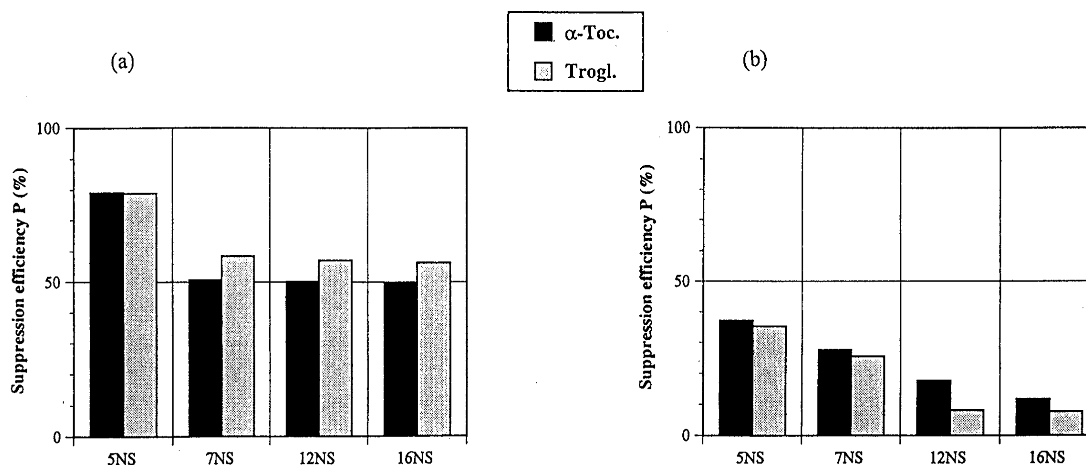

Fig. 6. Suppression Efficiency, P (%), by Troglitazone and α-Tocopherol in the Consumption of NS Induced by AMVN at 50 °C in 18 : 2 PC (a) and 14 : 0 PC (b) Liposomal Membranes

Concentrations were [5NS] = [16NS] = 23 μM, [18 : 2 PC] = [14 : 0 PC] = 11.6 mM, [AMVN] = 3.5 mM, [α-Toc.] = [Trogl.] = 33 μM.

branes and scavenge the phospholipid peroxyl radicals that are closer to that surface.

Figure 7 shows the suppression efficiencies of the compounds for the AAPH-induced consumption of different NS incorporated into 18:2 PC and 14 : 0 PC liposomal membranes. In 18 : 2 PC liposomes, troglitazone suppressed the consumption of 5NS more efficiently than α-tocopherol (Fig. 7a); however, the difference in their efficiency in consuming 16NS was small.

If there was no substantial difference in the reactivities of the two antioxidants toward peroxyl radicals, the one located nearer the surface of the membrane would suppress the consumption of all NS induced by the peroxyl radicals generated in the water phase outside the membrane more efficiently than those located in the inner part although the efficiency of both compounds may lessen the deeper the nitroxyl group is in the membrane interior. The reactivities of troglitazone and α-tocopherol toward peroxyl radicals in a homogeneous system were almost the same. Therefore, it was concluded that troglitazone is located closer to the surface of the membrane and can scavenge the aqueous radicals that are closer to the surface more efficiently, but neither compound can scavenge all of the radicals since most of 16NS was also consumed.

In 14 : 0 PC liposomes, the compound suppression efficiencies clearly depended on NS and decreased as the nitroxide group went deeper into the interior of the membrane (Fig. 7b). However, the efficiency of troglitazone was higher than that of α-tocopherol for all NSs. In 18 : 2 PC liposomes, the difference in the suppression efficiencies was less the deeper the nitroxyl group was in the interior of the membrane. This may be due to the difference in fluidity of the liposomal membranes; these membranes are more fluid and softer when they contain more unsaturated phospholipids, and the fluidity near the surface is not affected by the type of acyl chains of the PC.[12] In more fluid liposomal membranes such as 18 : 2 PC, the difference in the intra-membrane locations of the compounds may not affect the efficiencies in suppressing

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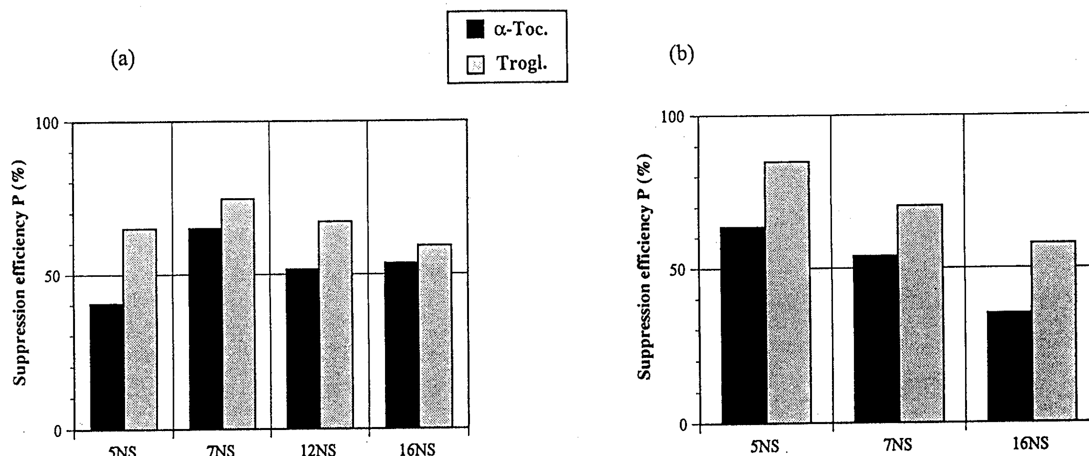

Fig. 7. Suppression Efficiency, P (%), by Troglitazone and α-Tocopherol in the Consumption of NS Induced by AAPH at 37 °C in 18:2 PC (a) and 14:0 PC (b) Liposomal Membranes

Concentrations were [5NS] = [16NS] = 23 μM, [18:2 PC] = [14:0 PC] = 11.6 mM, [AAPH] = 180 mM, [α-Toc.] = [Trogl.] = 33 μM.

16NS.

α-Tocopherol possesses a phytyl side chain which is embedded in the membrane interior and acts to decrease the mobility of α-tocopherol as an anchor, and also decreases its antioxidant activity within the membranes.[4] Troglitazone does not possess such groups which could anchor in the inner part of the membranes, although it possesses a hydrophilic thiazolidine dione group. It therefore should move freely within the membrane, predominantly located at or near the membrane surface. This may be partly due to the difference in the suppression efficiencies of the two compounds in liposomal membranes.

Conclusions

On the basis of the results obtained here, it is proposed that, in comparison with α-tocopherol, troglitazone may function as an antioxidant in the oxidation of PC-liposomal membranes near the membrane surface by preventing the attack of radicals generated outside the membranes. As the progression of oxidation of LDL is likely to occur from the aqueous interface towards the interior, and troglitazone resides nearer the surface of the liposomal membrane than does α-tocopherol, it seems reasonable to assume that troglitazone is the stronger of the two in the oxidation of LDL. Further investigation, particularly in comparing the mobility of the two compounds in the membranes, is clearly needed to elucidate the difference in their antioxidant potency.

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