Inhibition of Lipid Peroxidation in the Erythrocyte Membrane by Quaternary Morpholinium Salts with Antioxidant Function

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The antioxidant activity of five new compounds from a group of quaternary ammonium salts with antioxidant function has been investigated. The effect of the compounds on the degree of lipid oxidation in the erythrocyte membrane subjected to UV radiation was studied. It was found that all the salts used decreased oxidation of the lipids in the erythrocyte membrane. The antioxidant activity of the ammonium salts studied increased with their alkyl chain length. Three compounds with the longest alkyl chains were the most active antioxidants, and their antioxidant properties were comparable to those of a flavonoid extracted from hawthorn. The antioxidant effectiveness of the ten-carbon alkyl chain compound was comparable with that of the known antioxidant 3,5-di-t-butyl-4-hydroxytoluene (BHT). The least effective antioxidant studied proved to be the eight-carbon alkyl chain compound. The effect of these compounds on fluidity of the erythrocyte membrane has been studied, and for all an increase of fluidity of the membrane was observed. The changes in erythrocyte ghosts fluidity depended both on concentration and type of compound. A fluorimetric study also indicated that rigidity of the erythrocyte membrane increased with degree of its oxidation, but with antioxidants present membrane rigidity increased less.

Introduction

The negative effects of peroxidation on living organisms, that causes damage to cell components by reactive forms of oxygen, are commonly known (Bartosz, 1995; Halliwell and Gutteridge, 1989). The degraded components of a membrane alter its properties. Membrane lipids, above all unsaturated fatty acids, are most prone to oxidation for structural reasons. Toxic for the living organism are also the products of peroxidation that contribute to increased illnesses of people and animals. Thus it seems justified to conduct investigations in search of more and more effective antioxidants that could protect cells and their membranes against oxidation, mainly by blocking free radical reactions. Investigations published in recent years on this topic are concerned both with natural flavonoids and new synthetic antioxidants (Ariga and Hamano, 1990; Kilinc and Rouhani, 1992; Rios *et al.*, 1992; Gabrielska *et al.*, 1995; Chen *et al.*, 1996; Rasetti *et al.*, 1996/97; Witek *et al.*, 1997; Vaya *et al.*, 1997; Gabrielska *et al.*, 1997; Karten *et al.*, 1997).

The objective of the present investigations was to determine antioxidant activity of five new compounds from a group of quaternary ammonium salts with a hindered phenol substituent as an antioxidant function. The compounds are alkoxymethochlorides of morpholine-ethyl 3,5-di-t-butyl-4-hydroxydihydrocinnamate. The effect of the salts on the degree of lipid oxidation in the erythrocyte membrane subjected to UV radiation was studied. The results obtained allowed for a correlation between antioxidant activity of the compounds studied and their hydrophobicity, which may be helpful when synthesizing new more potent antioxidants.

Materials and Methods

Reagents

Quaternary ammonium salts were used with antioxidant function, which are a new group of anti-

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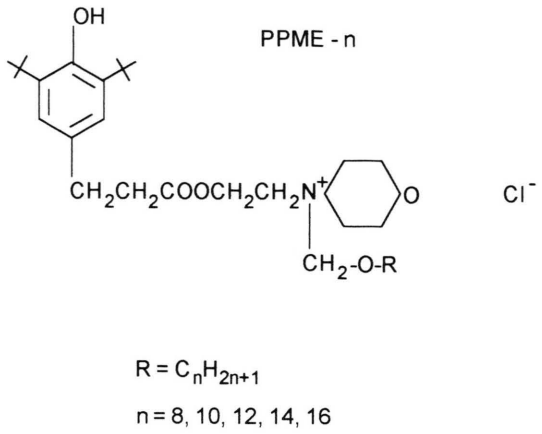

Fig. 1. Structure of the compounds studied.

oxidants (Fig. 1). They were analytical grade, and synthesized in our laboratory. The compounds PPME-n were obtained as white crystalline products by quaternization of 2-morpholineethyl 2-(2',6'-di-*tert*-butyl-4'-hydroxyphenyl)-propionates with n-alkoxymethyl chlorides in diethyl ether. The yields and melting points were as follow (*n*, yield%, m.p.^{0}C): **8**, 45, 174–176; **10**, 42, 178–181; **12**, 48, 182–184; **14**, 40, 179–182; **16**, 46, 176–178). The structure and purity of the compounds were confirmed by ^{1}H-NMR spectra (Bruker Avance DRX_{300} instrument, in deuteriochloroform, TMS as internal standard). The spectrum of the compound PPME-12 (given as an example) had the following signals (δ ppm): **0.883** (3H, t, CH_3-alkyl); **1.261** (20H, s, $(CH_2)_{10}$); **1.428** (18H, s, **t-Bu**); **1.558–1.638** (2H, m, -OCH_2-alkyl); **2.538–2.634** (6H, m, -CH_2-N-$(CH_2)_2$-); **2.811–2.899** (4H, m, aryl-CH_2-CH_2-); **3.286** (2H, s, N-CH_2-O-); **3.705–3.736** (4H, m, -$(CH_2)_2$-O-); **4.167–4.204** (2H, t, COO-CH_2); **5.069** (1H, s, -O**H**); **6.988** (2H, s, aryl).

The flavonoid compound was prepared by ethyl acetate extraction and chloroform precipitation from hawthorn (Yoshibe *et al.*, 1992). The composition and degree of polymerization were determined by HPLC before and after thiolysis (Shibata *et al.*, 1994).

TMA-DPH [(1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene-*p*-toluenesulfonate] was from Molecular Probes Inc. (Eugene, OR).

TBA – thiobarbituric acid was obtained from Chemical Company (St. Louis, Missouri, USA).

TCA – trichloroacetic acid was obtained from Fluka Chemie AG (Buchs, Switzerland).

BHT – 3,5-di-*t*-butyl-4-hydroxytoluene.

Oxidation studies

Lipids contained in the erythrocyte membrane were oxidized. Erythrocyte membranes were prepared, according to the Dodge *et al.* (1963) method, from fresh heparinized pig blood. Erythrocyte ghosts were suspended in a phosphate solution of pH 7.4 yielding a protein concentration of *ca.* 1 mg/ml. Besides the suspension containing erythrocyte ghosts only, suspensions containing proper amounts of the antioxidant compounds studied were prepared and lipid peroxidation in the erythrocyte membrane was induced by UV radiation (bactericidal lamp intensity was 3.5 mW/cm^2).

The end-product of lipid peroxidation is, among others, malonic dialdehyde, and the degree of lipid peroxidation was determined on the basis of malonic dialdehyde released in the samples, using its colour reaction with thiobarbituric acid (Bartosz, 1995; Stock and Dormandy, 1971).

During exposure of the ghost mixture, 1 ml samples were taken and 1 ml of trichloroacetic acid (TCA; 15% TCA in 0.25 M HCl) and 1 ml of thiobarbituric acid (TBA; 0.37% TBA in 0.25 M HCl) were added. The samples taken were stoppered with a ball and heated at 100 °C for 15 min, and then cooled fast and centrifuged, the absorption of supernatant was measured at 535 nm.

Fluorimetric studies

Fluorimetric studies were performed on erythrocyte ghosts. Erythrocyte membranes was prepared according to the Dodge *et al.* (1963) method from fresh heparinized pig blood. Erythrocyte membranes subjected to the action of the antioxidants studied or erythrocyte ghosts oxidized in the absence or presence of antioxidant compounds were labelled with the fluorescent probe TMA-DPH at 1 μM concentration. Protein concentration in the samples was of *ca.* 100 μg/ml. The measurements were performed with SFM 25 spectrofluorimeter (KONTRON). On the basis of fluorescence intensity measurements the polarization coefficient was calculated according to the formula (Lakowicz, 1983; Campbell and Dwek, 1984; Lentz 1988):

$$p = \frac{I_{II} - GI_{\perp}}{I_{II} + GI_{\perp}} , \quad (1)$$

where I_{II} – intensity of fluorescence emitted in direction parallel to the polarization plane of the exciting light, $I_{\perp}$ – intensity of fluorescence emitted in direction perpendicular to the polarization plane of the exciting light, G – diffraction constant, dependent on wavelength.

Results and Discussion

As was already metioned, two kinds of experiments were performed, and the results on lipid oxidation are presented in Fig. 2, part A. This figure shows the effect of one of the compounds (PPME-16) at three selected concentrations on the extent of lipid oxidation in the erythrocyte membrane (expressed in terms of absorption) as dependent on UV irradiation time. It is seen that the degree of erythrocyte lipid oxidation increases with irradiation time. This increase is, however, small in the presence of PPME-16 and the effect depends markedly on concentration of the compound. As seen, the PPME-16 salt applied at 10 μM concentration effectively protects lipids against oxidation, which is evident from the low absorption after a 120 min irradiation, compared with lipid oxidation in the control sample. Part B of Fig. 2 shows the relation between the degree of oxidation and time in the presence of all the compounds studied at the same concentration equal to 10 μM. The figure contains also the well-known lipid antioxidant BHT and one natural, plant antioxidant – flavonoid, extracted from hawthorn fruit. These two standards have been included in order to compare the antioxidant activity of the ammonium salts studied with the activity of these known antioxidants. Using the relations between degree of oxidation and time of irradiation, made for each of the compounds at various concentrations (1 μM – 100 μM), a relation has been obtained between percentage of oxidation inhibition and antioxidant concentration after a 120-min UV irradiation.

Percentage of the inhibition of oxidation was calculated for each compound relative to control as follows: % inhibition = $(1 - A_{535}/A'_{535}) \times 100$, where A_{535} is absorption in the control sample and A'_{535} is absorption in samples containing a compound studied after 120 min irradiation. Then concentrations of the compounds at which 50% peroxidation occurred were read. Values of these concentrations represented with a bar in Fig. 3 are

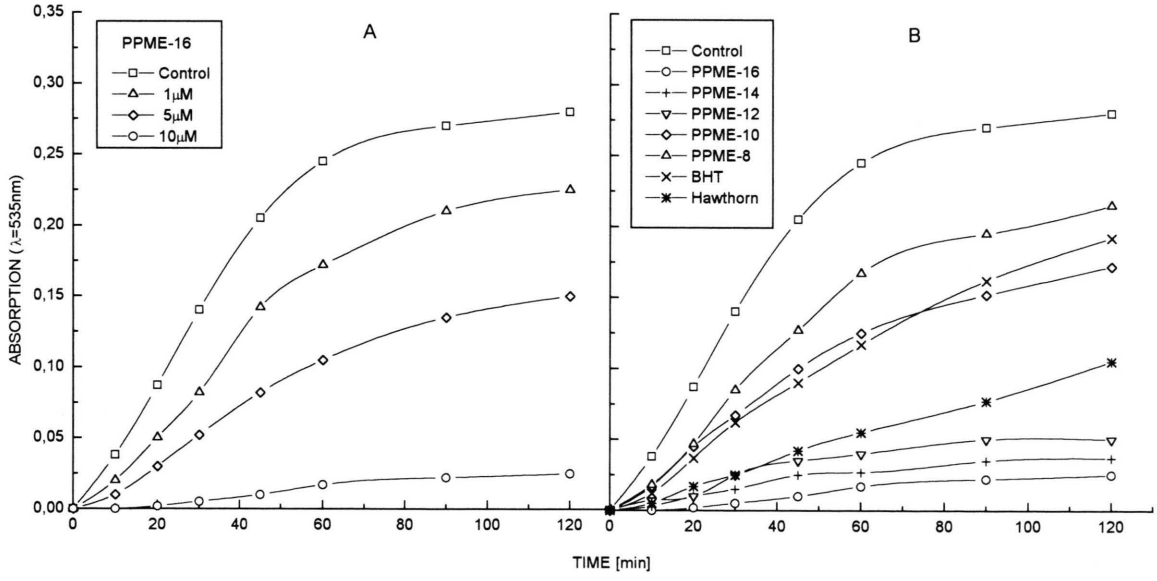

Fig. 2. Relation between absorption of light and time of lipid oxidation in the erythrocyte membrane for three concentrations of hexadecyloxymethylmorpholinium chloride – PPME-16 (A). Relation between absorption of light and time of lipid oxidation in the erythrocyte membrane for various compounds applied with a 10 μM concentration (B).

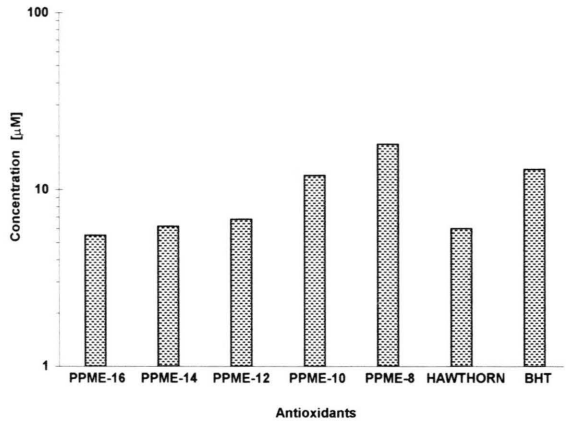

Fig. 3. Compounds' concentrations that cause 50% inhibition of oxidation of erythrocyte membrane lipids.

taken as a measure of antioxidant activity of the compounds towards erythrocyte lipids. It is seen that compound PPME-16 is the most potent antioxidant, since it gives 50% inhibition of lipid oxidation at lowest (compared with other compounds) concentration of 5.5 μM. The results obtained allowed also to conclude that the antioxidant activity of the compounds decreases with decreasing length of their alkyl chain, the PPME-8 compound being thus the least active. The activity of hawthorn flavonoid is high and comparable with that of PPME-14, while activity of BHT is worse than that of PPME-10 compound. The relation between antioxidant activities of the respective compounds is the following: PPME-16 > Hawthorn > PPME-14 > PPME-12 > PPME-10 > BHT > PPME-8.

Also the effect of selected antioxidant compounds on fluidity of the erythrocyte membrane has been investigated. The experiments were performed for two concentrations of the respective compounds (5.0 μM and 10 μM), using the fluorescence probe TMA-DPH. The results of the measurements are given in Table I as fluorescence polarization coefficients P as a function of antioxidant concentration. For all the salts studied a decrease in the value of polarization coefficient was observed for the fluorescence probe used in erythrocyte membranes treated with antioxidants, indicating an increase in fluidity of the membrane. The increase in erythrocyte membrane fluidity depends both on concentration and type of compound. Practically, for all the compounds an

Table I. Polarization coefficient of the erythrocyte membrane vs. concentration of the antioxidant.

Compounds	Polarization coefficient P		
	Control	Concentration c [μM]	
	0	5.0	10.0
PPME-8	0.380	0.370	0.361
PPME-10	0.369	0.360	0.356
PPME-12	0.375	0.365	0.350
PPME-14	0.370	0.353	0.329
PPME-16	0.366	0.351	0.321

increase in membrane fluidity (decrease in polarization coefficient) with increasing salt concentration is observed. The largest changes in erythrocyte ghosts fluidity are induced by compounds with 16 and 14 carbon atoms in their alkyl chain (PPME-16, PPME-14).

Fluidity of the erythrocyte membrane has also been investigated as dependent on the time of exposure to UV radiation, in the presence and absence of the salts used. The results indicate (Table II) that the polarization coefficient of the erythrocyte membrane increases with increasing degree of its oxidation. The increase in polarization coefficient is a measure of increase in membrane rigidity. In the presence of an antioxidant, membrane rigidity increases less with increasing irradiation time. The smallest increase in ghosts rigidity is observed for the three compounds of longest alkyl chains (PPME-16, PPME-14 and PPME-12). The effect of the two remaining compounds (PPME-10, PPME-8) is slight.

It is known that antioxidant activity of a compound depends mainly on the number and localization of the hindered hydroxyl groups (Chen *et al.*, 1996). Our investigations have shown that hydrophobicity has a strong influence on both its antioxidant properties, and, on fluidity of the erythrocyte membrane. Compound PPME-16 with the longest alkyl chain exhibits about a 3 times higher antioxidant activity towards lipids in the erythrocyte membrane than – PPME-8, the least hydrophobic of the five compounds. Generally, antioxidant activity of the studied ammonium salts increases with length of their alkyl chain (hydrophobicity) and follows the sequence PPME-16 > PPME-14 > PPME-12 > PPME-10 > PPME-8.

Table II. Polarization coefficient of the erythrocyte membrane vs. the time of erythrocyte membrane oxidation in the presence of the antioxidant.

Compounds	Polarization coefficient P						
	Time of erythrocyte membrane oxidation [min]						
	0	10	20	30	45	60	90
Control	0.365	0.372	0.389	0.397	0.398	0.412	0.418
PPME-8	0.373	0.380	0.387	0.402	0.406	0.412	0.415
PPME-10	0.370	0.369	0.379	0.388	0.398	0.410	0.414
PPME-12	0.368	0.370	0.375	0.383	0.394	0.400	0.408
PPME-14	0.362	0.365	0.369	0.380	0.382	0.389	0.390
PPME-16	0.376	0.373	0.375	0.380	0.385	0.383	0.382

Values represent the mean of 3 experiments. Standard deviation was ± 0.004.

Also, fluorimetric experiments showed that membrane fluidity depends, beside concentration, on the chain length of the compound. Such results can be explained by different abilities of the studied salts – cationic detergents – to incorporate into the erythrocyte membrane. This ability increases with alkyl chain length, as it was shown earlier (Kleszczyńska *et al.*, 1986; Kleszczyńska *et al.*, 1990). The concentration of a compound in the erythrocyte membrane should depend on its hydrophobicity and on the compounds partition coefficient between membrane and aqueous milieu. In the case of an antioxidant of long enough alkyl chain its effective concentration in the membrane is high and this ensures effective protection of membrane lipids against oxidation. The dependence of the partition coefficient on chain length was established by our earlier measurements for homologoues series of some quaternary ammonium cationic detergents (not yet published).

The localization of an antioxidant fragment in the membrane also may influence the antioxidant activity of the compound. In the case of a salt with longer alkyl chain the compound incorporates into the membrane deeper than that of shorter chain. It means that the polar head with the antioxidant fragment is located closer to the membrane surface. This reasoning is confirmed by results of fluorimetric experiments showing that a compound with a longer alkyl chain gave higher effective concentration in the membrane. Fluorimetric measurements showed also (Table II) that after oxidation the erythrocyte membrane became more rigid because its polarization coefficient P increased. This is due to the destruction of the hydrophobic part of unsaturated lipids and introduction of oxygen function into the alkyl chain of unsaturated fatty acids. The changes in rigidity of the membrane are smaller for more effective antioxidants. It can thus be inferred that concentration of the antioxidants studied within membrane is proportional to their alkyl chain length, since they can easily enter the lipid phase of the erythrocyte membrane and thus ensure effective protection of membrane lipids against oxidation.

That the compounds under study are more effective antioxidants for biological membranes than the well-known antioxidants like BHT, and have similar activity as a natural flavonoid.

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