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QUARTERLY

Cereal grain resorcinolic lipids inhibit H_2O_2-induced peroxidation of biological membranes*

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Cereal grain resorcinolic lipids (5-n-alk(en)ylresorcinols) at micromolar concentrations are able to protect the erythrocyte membrane against hydrogen peroxide-induced lipid oxidation. The antioxidative effect is dependent upon chain length of alkylresorcinol molecules. The C15 : 0 homolog (IC_{50} of 10 μM) exhibited strongest activity whereas for long chain homologs (C19 : 0 and C23 : 0) IC_{50} values were higher, 32.5 and 59 μM, respectively. The protective effect of alkylresorcinolic antioxidants was also dependent on their incorporation into the membrane, that is governed by their water-membrane partition coefficient. The results obtained show that alkylresorcinols should be recognized as hydrophobic, membrane-localised antioxidants.

Lipid peroxidation has been defined as oxidative deterioration of polyunsaturated lipids by free radical reactions [1]. This process is a widely accepted mechanism for cellular injury and death [2–4]. It was initially shown to represent the primary mechanism of cellular injury following ionizing radiation [5]. Later, increased lipid peroxidation has been associated with various toxic substances including drugs [6, 7], solvents [8], pesticides [9], ethanol [10] and certain transition metals [11, 12]. It has also been reported to occurr in a wide variety of human diseases and disorders including stroke [13], acute myocardial infarction [14], rheumatoid arthritis [15], diabetes mellitus [16], atherosclerosis [17] and cancer [2, 18].

Transition metals, such as iron, are involved in production of reactive oxygen species, such as the hydroxyl radical (•OH), lipid alkoxy radical (RO•) and the iron-oxygen species responsible for initiation of lipid peroxidation. Production of •OH in biological systems usually involves iron-ion-dependent decomposition of metabolically generated hydrogen peroxide (H_2O_2). Therefore, these radicals seem to play one of key roles in the lipid peroxidation-mediated cell damage. Although various protective systems (enzymatic and nonenzymatic) were shown to occurr in the cell (e.g., [19, 20]) any disturbances may lead to imbalance of these finely tuned systems resulting in increased production of active free radicals that affect the structural and metabolical cell integrity.

Among various systems protecting against increase of peroxidation processes above the physiological level, the lipid soluble chain-breaking antioxidants play a significant role. The best known natural lipophilic chain-breaking antioxidants are tocopherols (vit. E), their

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Abbreviations used: BHA, butylated hydroxyanisole; BHT, butylated hydroxytoluene,; TBRS, thiobarbituric acid-reacting substance.

analogs and synthetic phenolic antioxidants [21, 22]. Chemically, these compounds are phenolic derivatives with a long saturated isoprenoic side chain. Recently, other cellular phenolic compounds, e.g. ubiquinones, were found to show antioxidant activity [23]. In consequence, there is a growing nutritional demand for food protection against oxidation of fat and for supplementation of the daily food intake with antioxidative nutrients or additives. Although very effective synthetic antioxidants such as butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) are still in use, with their total consumption of 2.5–5 kg per year per average U.S. citizen [24], the reports on their toxic and carcinogenic effect (e.g., [25, 26]) resulted in a growing demands for other effective natural antioxidants. Resorcinolic lipids, the compounds which were demonstrated to occurr in cereal grains and related materials, e.g. bran products [27, 28], are similar to tocopherol phenolic compounds, except that they have straight aliphatic hydrocarbon side chain and a single phenolic ring. The structural similarity of these two groups of compounds suggested the possibility that resorcinolic lipids might have antioxidant properties.

We have shown previously that alkylresorcinols at micromolar concentrations are active antioxidants which protect both free fatty acids and phospholipids against ferrous ion-induced peroxidation [29]. They are also active in protection of unsaturated fatty acids and triglycerides against autooxidation [30]. In this report further experiments on antioxidative properties of cereal grain resorcinolic lipids are presented. We will show that these compounds are also effective in protection against hydrogen peroxide oxidation of lipids in natural membrane (erythrocyte plasma membrane).

MATERIALS AND METHODS

The homologs of 5-n-alk(en)ylresorcinol were isolated chromatographically from rye grain by the method described earlier [31]. For experiments the stock 5 mM ethanolic solutions of homologs studied were used. The remaining reagents used were of the highest available purity.

Fresh blood (B, Rh+) was obtained from the local Blood Bank. Erythrocytes were isolated by 10 min centifugation at 1000 × *g* and then washed twice with 0.14 M NaCl in 0.05 M phosphate buffer, pH 7.4. Final, third washing was done with the same solution supplemented with sodium azide (2 mM) to inhibit catalase activity. Washed erythrocytes were suspended in the azide-containing buffer to get a suspension of 2.5% hematocrit.

Peroxidation of erythrocyte membrane lipids

The experiments were performed in 4 ml volumes of the suspension. Resorcinolic lipids were injected in microliter amounts into test samples whereas controls contained the same volumes of ethanol. The samples were thoroughly mixed and incubated for 10 min at the temperature indicated. Peroxidation of membrane lipids was initiated by injection of freshly diluted hydrogen peroxide (100 µl) to obtain a final concentration of 20 mM. The reaction was carried out for 60 min. The amount of products of lipid peroxidation was determined by the method of Stocks & Dormandy [32] and expressed as the amount of thiobarbituric acid reacting substances (TBRS). In brief, 2 ml of 28% (w/v) aqueous trichloroacetic acid-sodium arsenite (0.1 M) was added to each sample, mixed and centrifuged 10 min at 1000 × *g*. An aliquot (4 ml) of the supernatant was transferred into Pyrex boiling tube and 1 ml of 1% (w/v) thiobarbituric acid solution in 0.05M NaOH was added. The tubes were closed by glass marbles against evaporation, placed in a boiling-water bath for 15 min and then immediately cooled under tap water. Absorbancies of the samples were read at 532 nm in a Hitachi 100-60 spectrophotometer against a reagent blank. The amount of thiobarbituric acid-reacting peroxidation products was expressed in nanomoles using the molar absorption coefficient of $1.56 \times 10^5\ M^{-1}\ cm^{-1}$ for malonyldialdehyde [32].

RESULTS AND DISCUSSION

Preliminary experiments on erythrocyte membrane lipid peroxidation by hydrogen peroxide under conditions when catalase activity was not inhibited showed very low reproducibility in agreement with the results of other authors [32]. Inhibition of catalase, one of the cellular enzymes participating in antioxidative

defence, allowed to obtain reproducible action of hydrogen peroxide on the lipids. The extent of oxidation measured after 1 h was in our case over three times as high as reported by Stocks & Dormandy [32]. The estimated level of peroxidation products expressed as malondialdehyde was 3804 nanomoles per 1 mg of protein. Incubation of the cells with C15 : 0 alkylresorcinol injected into the suspension resulted in a significant decrease of peroxidation products formed during the reaction with hydrogen peroxide. The extent of the inhibition was dependent on the time of erythrocyte preincubation with resorcinolic lipid (Fig. 1). When the peroxidation was initiated 30 s after injection of alkylresorcinol the extent of inhibition was less than 40%. Prolongation of preincubation of the cells with resorcinolic lipid over 60 s resulted in a further increase of antioxidant effectivity. Longer preincubation times were without marked effect, and after 10 min further decrease of oxidation was less than 20%. This time-dependence of antioxidative properties of resorcinolic lipids suggests the importance of incorporation of these compounds into the lipid bilayer. Thus, resorcinolic lipids appear to act in a way similar to other lipid soluble antioxidants. The short half-time for maximal membrane protection (approximately 30 s) suggests fast incorporation of resorcinolic lipids into the lipid core of the membrane. This observation is in good agreement with very high membrane-buffer partition coefficients of resorcinolic lipids, demonstrated in our previous paper [33].

Resorcinolic lipids present in most natural sources including cereal grains and the bran milling fraction are mixtures containing various homologs. Most of them are saturated-chain homologs of C15–C27 (e.g., [34]). The average chain length, calculated from their composition, varies from 18.5 to almost 20 for rye and wheat, respectively. To determine the role of aliphatic chain in the observed antioxidative properties of resorcinolic lipid, the effect

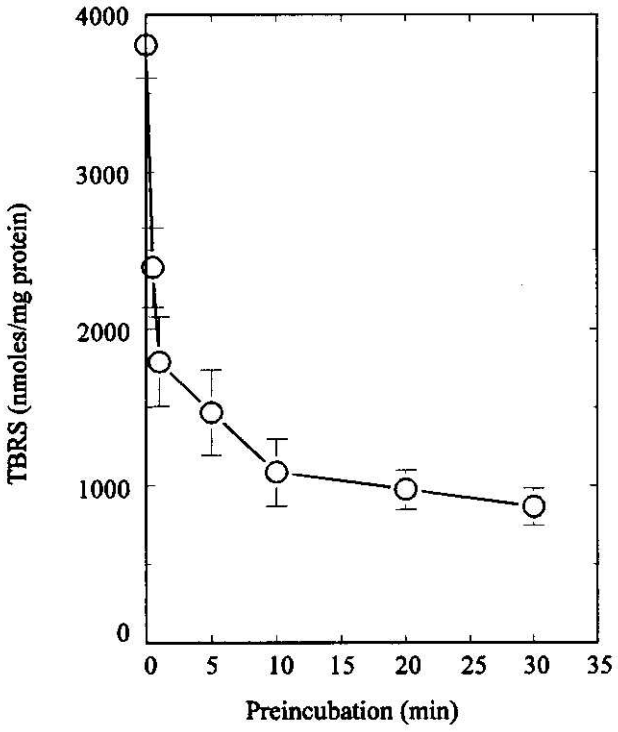

Fig. 1. Time dependence of the amount of TBRS formed in erythrocyte membrane incubated with 5-n-pentadecylresorcinol (hydrocardol) (12.5 μM) and subsequently with hydrogen peroxide for 60 min at 37°C.

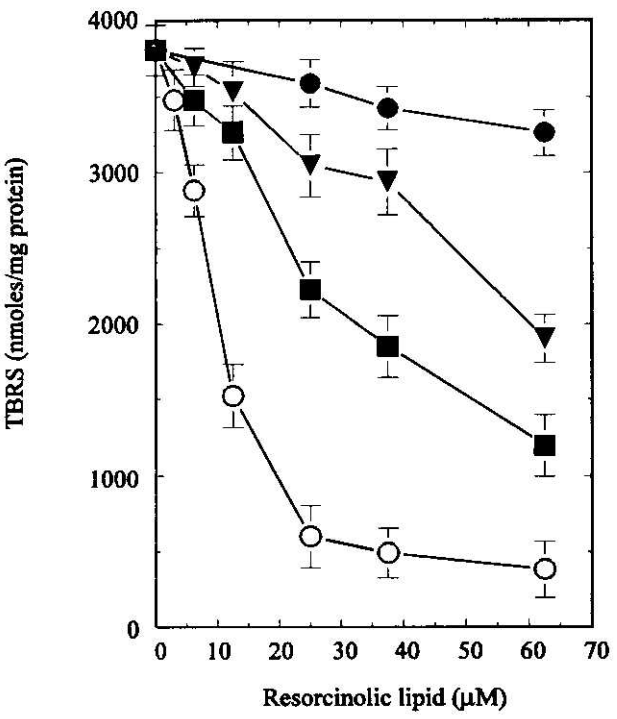

Fig. 2. The effect of various alkylresorcinol homologs on hydrogen peroxide-induced peroxidation of erythrocyte membranes.

Peroxidation was initiated after 5 min preincubation of the erythrocyte suspension with the compound studied, and the amount of thiobarbituric acid- reacting substances formed during 60 min incubation at 37 °C was estimated. ●, 5-n-Amylresorcinol (olivetol); ▲, 5-n-tricosylresorcinol; ■, 5-n-nonadecylresorcinol, ○, 5-n-pentadecylresorcinol (hydrocardol).

of several saturated alkylresorcinol homologs with alkyl chains of C15 (hydrocardol), C19 (nonadecylresorcinol) and C23 (tricosylresorcinol) was studied. For comparison, the C5 homolog (amylresorcinol, olivetol) was used. The dependence of the protective effect of these compounds upon erythrocyte membrane peroxidation is shown in Fig. 2. In the concentration range of 6–60 μM all long chain homologs inhibited peroxidation of membrane lipids. The extent of inhibition, was however dependent on aliphatic chain length of the compound studied. The values of IC_{50} were between 10 μM for hydrocardol, 32.5 μM for nonadecylresorcinol and 58.7 μM for tricosylresorcinol. Amylresorcinol was shown to be practically inactive in antioxidative protection (only approximately 15% inhibition at the highest concentration studied). The decrease of the antioxidation efficiency observed for the longest chain homolog together with the low activity of amylresorcinol confirmed the importance of the incorporation of resorcinolic lipids into the membrane for their antioxidative activity. Penetration of the short chain into the lipid bilayers and its anchoring is not efficient enough, whereas very long chains might be responsible for location of the antioxidant molecule too far from the membrane interface. This suggests that the peroxidation process, at least in part, is an interfacial phenomenon requiring penetration of the oxidation inducer below the level of the glycerol backbone. On the other hand, recent data on infrared analysis of alkylresorcinol-phospholipid bilayers show the existence of alkylresorcinol-phospholipid hydrogen bonds that stabilize the bilayer [35]. The stabilization of the bilayer structure, the decrease of alkylresorcinol-induced phospholipid mobility in liposomal and erythrocyte membranes [36] and the presence of phenolic moiety would be a prerequisite for antioxidative properties of alkylresorcinol.

Our previous reports showed that the effect of alkylresorcinols altering liposomal and biological membranes was higher at the temperatures above thermotropic transition regions of alkylresorcinol molecules [37, 38]. Therefore it was interesting to check whether temperature-related alteration of the membrane mobility of alkylresorcinol molecules might play a role in their antioxidative properties. The plot of the relationship between the extent of the decrease of peroxidation products *versus* time of preincubation of erythrocytes with hydrocardol (12.5 μM) is shown in Fig. 3. Although the increase of temperature resulted in increased peroxidation of the membrane lipids (additional oxidation with air oxygen cannot be excluded), the extent of this process is lowered in the presence of the compound studied. As the measure of the antiperoxidative activity we used the differences between the level of thiobarbituric acid-reacting substances determined at the preincubation time of erythrocytes with alkylresorcinol equal to zero minutes and the level of TBRS in samples that were preincubated with hydrocardol for 30 min prior 60 min oxidation with hydrogen peroxide. This difference increased from 1300 nmoles of TBRS at 17°C to almost 2900 nmoles at 42°C. These results show that the mobility of the alkylresorcinol molecules within the membrane hydrophobic core might be another factor determining their antioxidative properties. It is also possible that at higher temperatures free radi-

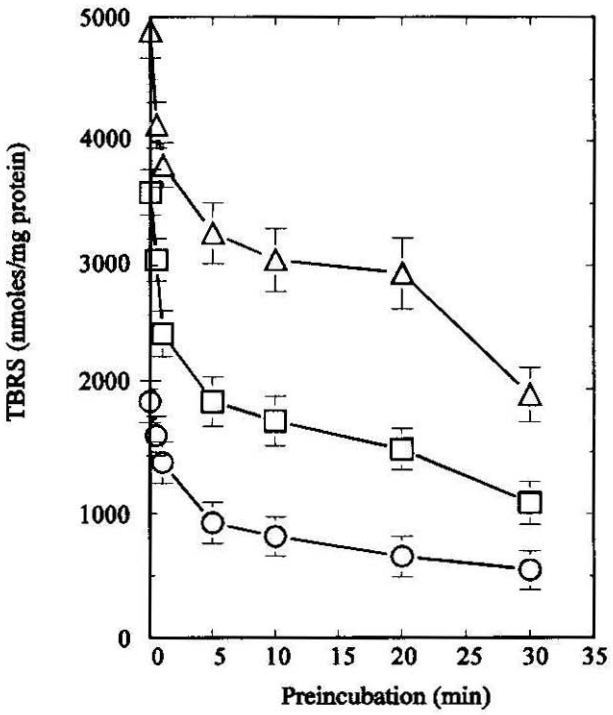

Fig. 3. Inhibition of hydrogen peroxide-induced peroxidation of erythrocyte membranes by resorcinolic lipid.

Dependence of the extent of inhibition at various temperatures on time of preincubation of erythrocytes with 5-n-pentadecylresorcinol (12.5 μM). TBRS levels were determined after 60 min peroxidation. ○, 20°C; □, 37°C; △, 42°C.

cals prefer the interaction with resorcinolic lipids rather than with membrane lipids, and this would account for the observed higher antioxidative efficiency of alkylresorcinols.

The chain-breaking antioxidants compete with lipids for radicals forming stable antioxidant radicals, that in turn become transformed into nonactive associates, e.g., dimers terminating the oxidation chain. Therefore, in the environment in which an excess of radicals is present and the molecules that could regenerate the antioxidant are lacking, the effective number of antioxidant molecules will decrease. Incubation of alkylresorcinol with hydrogen peroxide prior to addition of erythrocytes (Fig. 4) resulted in the loss of antioxidant properties of the compound studied. After initial strong inhibition of the hydrogen peroxide-induced peroxidation (first 30 s) a relatively fast loss of antioxidative protection, reaching a plateau after 15 min, was observed. This shows that free alkylresorcinol, not incorporated into the membrane alkylresorcinol, significantly loses its antioxidant properties. When alkylresorcinol was first incubated with an oxidation inducer and then incorporated into the membrane, no further loss of its antioxidant properties was observed. The experiments that can give the answer to the question whether partially oxidized alkylresorcinol might itself act as a prooxidant, are during the course.

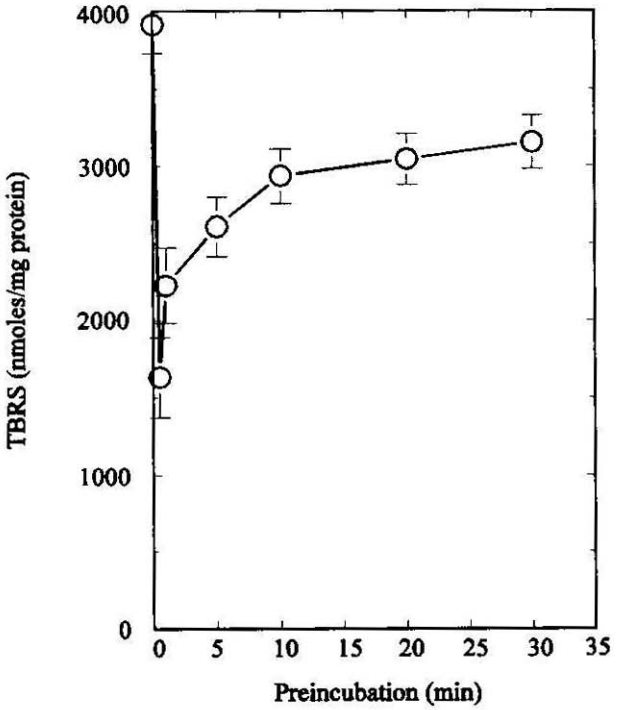

Fig. 4. The effect of preincubation with hydrogen peroxide on antioxidant efficiency of 5-n-pentadecylresorcinol.
Resorcinolic lipid (12.5 μM) was incubated at 37°C with inducer prior to addition of cellular suspension, then the reaction was continued for 60 min and the level of TBRS was determined.

The presented above and earlier results [29, 30] show that resorcinolic lipids, present in cereal grain materials (whole grain bread or bran products, that are now strongly recommended in human nutrition), can be recognized as another type of natural antioxidants with both chain-breaking and preventive properties. The ability of resorcinolic lipids to modulate also other cellular oxidation-related processes, such as thromboxane synthesis [39] and lipoxygenase-induced oxidation of unsaturated fatty acids (Kozubek & Deszcz, unpublished), as well as the activities of oxidoreductase enzymes [40, 41] and respiratory chain enzymes [42], clearly indicates that these compounds are of biochemical and nutritional importance and that many of their properties are related to their phenolic and amphiphilic nature.

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