Higher Cardol Homologues (5-Alkenylresorcinols) from Rye Affect the Red Cell Membrane-Water Transport

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The influence of 5-heptadecenylresorcinol and total rye 5-alkenylresorcinols isolated from rye grains on the red blood cell water permeability was studied using osmotic shrinkage experiments performed in 300 mM sucrose. The studied compounds induced significant increase of erythrocyte water permeability. The threshold concentration needed for the increase of water permeability was in an order of 10^{-6} mol/l. The temperature dependence of the observed process showed the discontinuity which was related to the 5-alkenylresorcinol transition temperatures. It was shown also that alkenylresorcinols did not exert the biphasic action on hypotonic lysis of erythrocytes usually observed for water soluble surfactants. The specific lysine activity is postulated for the studied compounds.

Introduction

5-Alk(en)ylresorcinols are amphiphilic phenolic lipids mainly with 15 carbon atoms long aliphatic chain (cardol [1] and bilobol [2]) naturally occurring in plant materials from different families (Anacardiaceae, Proteaceae, Graminae). The presence of 5-alk(en)ylresorcinols in cereal grains was also noted [3–6]. The aliphatic chain of 5-alk(en)ylresorcinols is 15 to 31 carbon atoms long dependent on the species and variety of the source plant [3, 5–7]. The homologs with saturated chain are predominant in cereal materials however rye alk(en)ylresorcinols contain significant amounts of mono and di olefinic homologs [4, 8–10]. Phenolic lipids isolated from Anacardiaceae have well known skin irritating and sensitizing activities [11]. The role of phenolic lipids in cereal grain biology is not known as yet, however, their participation in grain antimicrobial resistance is suggested [5, 12].

The study of the possible antimicrobial activity and participation in "antinutritive" properties of rye grains [4] of cardol homologs bases on the amphiphilic character of these compounds. Hitherto obtained results show the high affinity of cereal resorcinol derivatives to biomembrane [13]. The incorporation of these water insoluble molecules significantly enhanced the permeability of both erythrocyte and liposome membrane for alkali cations and small solutes [14]. The derivatives with unsaturated aliphatic chains showed also strong haemolytic activity [13, 14] which was comparable to some known water soluble surfactants [13].

The lytic activity of known haemolytic agents, both water soluble and insoluble, is connected with the increase of the cell membrane permeability for electrolytes and small solutes [15, 16]. The activity of known lytic agents is based on the osmotic lysis. In this process the increase of the cell membrane permeability not only for small solutes but also for water is necessary for the cell swelling and subsequent disruption of the membrane [17]. On the other hand most of amphiphilic molecules incorporated into the red cell membrane in certain concentrations protected the cells against hypotonic lysis [18], which suggested that the alteration of the water transport through the membrane played significant role in the mechanism of the observed event [18].

In this paper the influence of main water insoluble amphiphiles present in cereal grains – 5-alkenylresorcinols and isolated 5-heptadecenylresorcinol on the erythrocyte membrane water permeability is studied with respect to their "detergent-like" activities.

Materials and Methods

Total 5-alkenylresorcinols and 5-heptadecenylresorcinol were isolated from acetone extracts of rye (*Secale cereale* L.) grains by chromatographic procedure [10, 14]. The composition and the purity of the preparations were determined with the pre-

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viously described methods [19–21]. For the permeability experiments used 5-alkenylresorcinols were dissolved in ethanol and 5 milimolar stock solutions were used. The concentration of the compounds was determined colorimetrically [22].

Fresh human blood (B, Rh+) was obtained from healthy volunteers, protected from the coagulation with acidic citrate/dextrose and centrifugated at $1000 \times g$ for 10 min. The erythrocytes were washed three times with 0.145 M NaCl in 10 mM Tris-HCl (pH 7.3) by suspending and centrifugation for 10 min at $600 \times g$. The washed erythrocytes were suspended in the same isotonic solution at a haematocrit of 50% and used within 12 h.

The changes of erythrocyte permeability to water were followed by the turbidimetric monitoring of the cell shrinkage. The shrinking experiments were performed in temperature controlled conditions. To 5 ml of preincubated and vigorously stirred 300 mM sucrose in 10 mM Tris-HCl, pH 7.3, the microliter amounts of ethanolic solution of studied compounds were added. The concentration of ethanol in the medium did not exceed 0.2% in any test. After 60 s 25 microliters of the erythrocyte suspension were rapidly injected and the changes in the transmittance were recorded at 600 nm (Specol 100) Zeiss, Jena, GDR (equipped with home-made thermostated cuvette adapter and connected to a chart recorder). Initial optical density of the erythrocyte suspension was determined by injection of the same amount of erythrocytes into isotonic buffered saline.

Determination of the influence of studied resorcinol derivatives on the human red cells osmotic fragility were performed in similar way with the use of different concentration of sodium chloride in the buffered medium. After 10 min of incubation the extent of haemolysis was determined spectrophotometrically at 540 nm in the supernatant obtained after centrifugation of 3 ml samples for 5 min at $3000 \times g$. 100% haemolysis was estimated after suspending the erythrocytes in distilled water.

Results

The analysis of the composition of the used total 5-alkenylresorcinols isolated from rye grains in order to determine the length and the unsaturation of the aliphatic chain shows its heterogeneity (Table I). The presence of six homologs with the chain length of 15 to 25 carbon atoms was demonstrated. Heptadecyl and nonadecyl resorcinols were the main constituents of the mixture (as monoene derivatives) therefore the further experiments were performed on both 5-heptadecenylresorcinol and total 5-alkenylresorcinols. The diene derivatives were also present at the amount of about 25%. For the calculation of the molar concentrations of total 5-alkenylresorcinols the average chain length of 18.04 carbons and molecular weight of 363 was used.

Table I. Analysis of the composition of 5-alkenylresorcinols.

Homolog (name of the aliphatic radical)	Relative amount [%]
Pentadecyl	7.6
Heptadecyl	42.6
Nonadecyl	31.6
Heneicosyl	11.8
Tricosyl	5.6
Pentacosyl	0.8
Monoenoic homologs	75.6
Dienoic homologs	24.4

The homologs were determined colorimetrically [22] after thin layer chromatographic separation according to chain length and unsaturation [19–21].

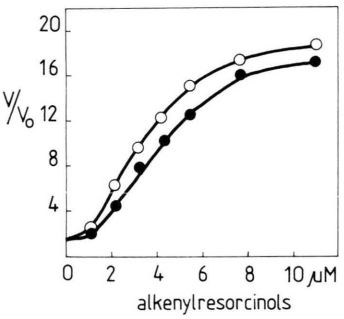

Fig. 1. Effect of increasing 5-alkenylresorcinols concentration on relative velocity of erythrocyte shrinkage. The experiments were performed at 37 °C. Alkenylresorcinols were added to the isotonic sucrose in the amounts of 2–20 microliters of 1 milimolar ethanolic solution. ○—○—○ 5-Heptadecenylresorcinol; ●—●—● 5-alkenylresorcinols.

Earlier results showed, that the unsaturated 5-alk(en)ylresorcinols isolated from rye grains were very active affecting biological membrane structure and permeability [13, 14, 23]. Fig. 1 shows the osmotic shrinkage experiments performed with human erythrocytes as a function of 5-heptadecenylresorcinol and 5-alkenylresorcinols concentrations.

The significant increase of erythrocyte shrinking rate indicating enhanced water permeability was observed for both studied compounds above the concentration of 2 micromolar. This increase was slightly higher for pure 5-heptadecenylresorcinol than for total mixture of homologs and stabilized above 10 micromolar 5-alkenylresorcinols present in the medium.

The alkenylresorcinols showed the thermotropic behaviour of the chains as was previously demonstrated by differential scanning calorimetry [23]. Therefore the temperature dependence of 5-alkenylresorcinols induced erythrocyte osmotic shrinkage was studied (Fig. 2). The shrinkage rate estimated for the control cells was changed in the presence of both unsaturated resorcinol derivatives and the break appeared (Fig. 2A). Arrhenius plots of the rates of erythrocyte shrinkage in the presence and absence of studied 5-alkenylresorcinols are shown in Fig. 2B. The plot for the control cells does not show any changes in the rate of water permeation. In erythrocytes in the presence of 5-alkenylresorcinols the increase in the rates of water transport both above and below the breaks appeared in the region of 25–35 °C was observed. The activation energies for water permeation were calculated from the slopes of the linear parts of the Arrhenius plots. The calculated values of activation energies for 5-heptadecenylresorcinol and 5-alkenylresorcinols were similar – 34.4 and 36.4 kJ/mol for the region above, and 187.1 and 168.4 kJ/mol for the region below the break of the plots. These values indicate that the different molecular events are rate-limiting steps in the water permeation below and above transition which are related to the conformational changes of 5-alkenylresorcinol molecules.

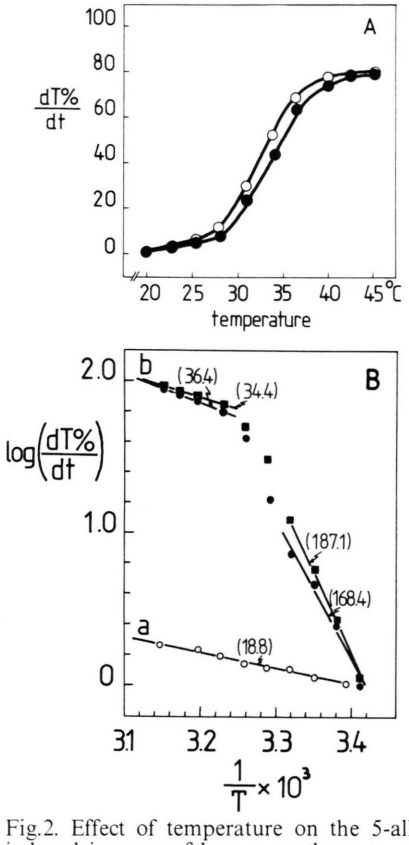

Fig. 2. Effect of temperature on the 5-alkenylresorcinols-induced increase of human erythrocyte osmotic shrinkage in 300 mM sucrose. The erythrocytes (25 microliters) were injected to 5 ml of buffered medium containing 2.5×10^{-6} M/l of studied alkenylresorcinols. A. Temperature dependence of the osmotic shrinkage. ○—○—○ 5-Heptadecenylresorcinol; ●—●—● 5-alkenylresorcinols. B. Arrhenius plots of the osmotic shrinkage of erythrocytes a – in absence ○—○—○, b – in presence of 2.5×10^{-6} M/l of alkenylresorcinols: ■—■—■ – 5-heptadecenylresorcinol, ●—●—● – 5-alkenylresorcinols. Numbers in brackets are activation energies (kJ/mol).

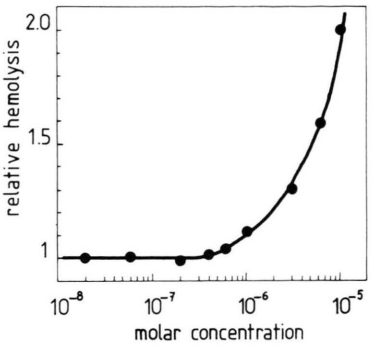

Fig. 3. Effect of 5-heptadecenylresorcinol on fragility of human erythrocytes upon hypotonic lysis. The alkenylresorcinol was added to 5 ml of 70 mM buffered NaCl solution from ethanolic solution of the appropriate concentrations (up to 10 microliters). A relative haemolysis of 1.0 indicates an absolute haemolysis of about 40%. The cell concentration was 8.5×10^7 cells/sample.

In order to determine if the 5-alkenylresorcinols induced water permeation increase affects the osmotic resistance of the cells, other words if studied compounds behave as non-specific detergents, the hypotonic lysis experiments were performed. As it can be observed in Fig. 3 the 5-heptadecenylresorcinol at the concentration range of $10^{-8} - 10^{-5}$ M do not protect the cells against hypotonic lysis. Essentially identical results were obtained for total 5-alkenylresorcinols (data not shown).

Discussion

The molecular mechanism of action of phenolic lipids on the cell function with respect to their biological activity is until now not known. The high hydrophobicity of these compounds however make the cellular membranes as the primary side of action very likely. The biological membranes despite maintained bilayer structure are very sensitive to the incorporation of foreign amphiphilic compounds. Alteration of the existing membrane balance leads to alteration of the biological properties *e.g.* changes of the barrier functions. Based on the shape concept [24, 25] one can conclude that only "cone" and "inverted-cone" shaped amphiphilic molecules will have the membrane perturbing properties which would be related to the dynamic shape balance between polar and hydrophobic parts of their molecules [25]. The molecules of 5-alkenylresorcinols due to their apparent noncylindrical shape should show strong affect on membrane structure [23] and permeability which would lead to cell lysis [13, 14, 26]. The differences in molecular shape between 5-alkyl and 5-alkenyl resorcinols could be one of the explanation of lower antimembraneous activities of saturated resorcinol derivatives, with their more cylindrical molecules.

Presented in this paper results indicate that in the 5-alkenylresorcinol induced lysis drastic increase of the cell membrane water permeability is also involved. The data obtained for total 5-alkenylresorcinols where long chained homologs are about 20% of the total amount suggest that the relatively short chained components of this mixture seems to be responsible for the observed water permeability increase. The results show that the water transport is primarily affected during the interaction of alkenylresorcinols with biological membrane as the threshold concentration needed for water transport increase is about ten times smaller than necessary for the lytic effect. The rate of the erythrocyte water transport is also higher than showed for other small solutes ([14] and Kozubek, in preparation) and ions [14]. The red cell swelling and lysis in isotonic sucrose observed above the certain level of 5-alkenylresorcinols concentration [26] suggests that these compounds are able to cause formation of membrane defects enough large to pass sucrose molecule the membrane barrier. The discontinuity showed in the temperature dependence of the relative rate of water permeability induced by studied compounds is consistent with the phase transitions observed by calorimetric scanning of these compounds [23]. The high mobility of the alkenyl chains of the compounds above transition temperature seems to be necessary for the effect on the erythrocyte membrane permeability and structure. The most significant changes in the rate of permeation observed in the transition region are in some similarities to the highest permeability changes observed for lipid bilayers on transition temperature of these lipids [27, 28].

The observed increase of membrane permeability could be facilitated *via* formation of a new water transducting pores or other structural changes within the membrane interior. This suggestion is supported by the fact that the formation of nonlamellar structures within lipid bilayer in the presence of alkenylresorcinols was observed earlier [23]. The high activation energy below the transition of studied compounds suggests the possible alteration also of membrane proteins properties. This is in accordance with the changes in distribution of protein particles on the erythrocyte membrane surface after the action of 5-alk(en)ylresorcinols [23].

In contradiction to other surface active agents 5-alkenylresorcinols do not show any protective activity on erythrocyte osmotic (hypotonic) lysis. For this reason these compounds could be accounted to the group of specific lysins as saponins [29]. This observation indicate the possibility of the existence of specific receptor sites on the membrane surface.

Presented in this paper data together with our previous findings [14, 26] support the suggestion that the increase of biological membrane permeability can be a basis of the biological activity of phenolic lipids not only present in cereal grains. The membrane properties alteration can be considered then as the primary effect of other known toxic cardols and cardanols present in cashew nutshell liquid or urushiols from poison ivy [11].

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