Effects of cereal alkylresorcinols on human platelet thromboxane production

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An extraction, purification, and identification procedure was used to determine and isolate alkylresorcinol homologs in rye. The major alkylresorcinol homologs have saturated odd-numbered carbons with the predominant chain length of C15, C17, C19, C21, C23, and C25. Rye alk(en)ylresorcinol homologs of C17:0, C17:1, C19:0, C21:0, C23:0, and C25:0, including a C15:0 standard, were used to study their effects on thrombin-challenged human platelet thromboxane production. C15:0 at .02 μmol/L, C17:1 at .2 and 2 μmol/L, C19:0 at .02 μmol/L, C21:0 at 2 μmol/L, and C23:0 at .02 and 2 μmol/L, significantly stimulated thromboxane production approximately 30 to 65 percent. These stimulation effects of alk(en)ylresorcinol homologs can be interpreted via either their membrane disruptive or free radical scavenging properties.

Keywords: alkylresorcinols; cereals; thromboxane; human platelets

Introduction

The natural occurrences of 5-n-alkyl and 5-n-alkenylresorcinols (AR) in cereal grains has been well documented.[1-8] These compounds are present in the highest amount in rye, in lower amounts in wheat and triticale, and in small quantities in other cereals. ARs are thought to fulfill protective functions in plants during dormancy and germination. They were shown to be present quite early in kernel development and located mostly in the outer part of the grain kernel. Milling of wheat, rye, and triticale into bran, shorts, and flour indicated that bran contains the highest, shorts intermediate, and flour the lowest amount of ARs. The study of these compounds in human food and how they are affected by food processing is very limited.

ARs in cereal grains were shown to inhibit growth in young chickens, swine, and rats.[9-11] In a pair-feeding experiment on growth depressing effects of 5-n-pentadecylresorcinol, a model for cereal alkylresorcinols, approximately 70% of the growth depression was attributed to decreased food intake, and 30% to a direct "toxic" effect.[10]

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The biological activity of ARs in grain which may be responsible for the negative effects of the fed animal is still under investigation. Most of the studies have been conducted in model systems. Alkylresorcinols have amphophilic characteristics. They are difficult to disperse in water and have a hydrophobic-lipophilic balance of about two. The amphophilic character of alkylresorcinol indicates a possible action on membranes as these compounds disturb membrane organization and induce marked permeability changes.[12-16] The hemolytic activity of 5-n-alk(en)-ylresorcinols is temperature-dependent and is proportional to the side chain unsaturation and inversely proportional to the chain length. Mono- and dienoic homologs of 5-n-heptadecyl and 5-n-nonadecyl are the most active.[15,16] The threshold concentration needed for the increase of water permeability is in the order of 10^{-6} mol/L. This increase of biological membrane permeability can be a basis for the biological activity of phenolic lipids. Further experiments[17] showed that resorcinols can induce protein aggregration in membranes as seen with an electron microscope. This might be due to the interaction of resorcinols with membrane lipids or proteins causing the induction of permeability changes in natural membranes. It is possible that resorcinol increases membrane permeability by the formation of reversed micelles. An altered osmotic equilibrium and an increasing percentage of

non-bilayer structure can finally lead to disruption of the cell membrane.

Recent studies show the ability of alk(en)ylresorcinol homologs to inhibit phospholipid vesicles hydrolysis by phospholipase A2. These compounds inhibit the increase of the enzyme latency period.[18] The unsaturated homologs isolated from rye appear to be the most potent. Phospholipid hydrolysis is the rate-controlling step in eicosanoid biosynthesis, which is a very important biological activity. This hypothesis is supported by the fact that these compounds are positional homologs of alkylcatechols in poison ivy and cashew nutshells which exhibit inflammatory properties.[19]

So far, the knowledge of biological activity of these compounds is limited, primarily due to the absence of standards and an effective method to identify, isolate, and purify these compounds. Now, improved chromatographic techniques can be used to isolate 5-n-alkylresorcinols from cereal grains[20] and, therefore, research can progress on testing their biological actions.

The purpose of this experiment was to assess the effect of cereal 5-n-alk(en)ylresorcinols on platelet thromboxane (TX) production. The platelet was selected as the bioassay system because the anti-thrombotic and anti-inflammatory action of polyphenols has been attributed to inhibition of the arachidonic acid cascade (TX and eicosanoid biosynthesis) pathway.[21,22] The action of ARs could be the disruption of membranes and/or phospholipase activity. Since alkylresorcinols possess a phenolic structure, they might act as antioxidant or free radical scavenger. Free radicals are important in controlling prostaglandin and thromboxane biosynthesis. TX biosynthesis involves several enzymatic steps that require a free radical activator but can be attacked by high concentrations of radicals. Therefore, it was postulated that alkylresorcinols at a certain concentration level would either activate or inhibit TX production due to their antioxidant properties.

Materials and methods

Isolation of alkylresorcinol homologs by HPLC

Alkylresorcinols in 1000 g rye (cultivar Bonel) were extracted and purified by the method described by Hengtrakul et al.[20] The brownish-yellow oil obtained amounted to approximately 1% of the starting grain weight. This oil was stored under nitrogen until use.

Alyklresorcinol homologs were separated by HPLC. Fractions which had been identified by mass spectroscopy included C17:1, C17:0, C19:0, C21:0, C23:0, and C25:0. The amount of each homolog was quantitated using C15:0 (Aldrich, Milwaukee, WI) for constructing a standard curve. The solvent was removed by evaporation under a stream of nitrogen. To the dried alkylresorcinol homolog, a small amount of dimethyl sulfoxide (DMSO) was added. Then, 4% aqueous solution of bovine serum albumin (BSA) was added to give an initial concentration of 100 μmol/L. The ratio of DMSO and 4% BSA solution was maintained at 1:9. The solution was stirred vigorously and left in the refrigerator overnight before dilutions were made.

Sample collection and challenging TX production by human whole blood

Eight adult males participated in the experiment. All subjects were healthy. They were instructed to refrain from use of aspirin, indomethacin or other anti-inflammatory agents for at least 10 days prior to their involvement with the sample collection. Four and one-half mL of blood was collected into siliconized glass vacutainers (No. 6418, Becton-Dickinson, Rutherford, NJ) containing 0.5 mL 0.129 mol/L citrate. Four containers were needed for each experiment. Each vacutainer was filled and then inverted gently to mix the anticoagulant without damage to the erythrocytes (RBC) and platelets. Vacutainer contents were then pooled into a plastic tube. Five microliters of buffer control, containing an equivalent amount of DMSO, or seven alkylresorcinol homologs, C15:0 (Aldrich, Milwaukee, WI), C17:1, C17:0, C19:0, C21:0, C23:0, and C25:0 at three concentration levels of 1, 10, and 100 μmol/L in triplicate were placed into siliconized cuvettes (7 × 45 mm) containing siliconized stirring bars (1 × 5 mm) (Sienco, Inc., Morrison, CO). Then, 200 μL aliquots of blood were added to the tubes. The cuvettes were preincubated in a multicavity stirrer (Sienco, Inc., Morrison, CO) for 5 minutes at 310°K and 700 rpm. After the preincubation period, the contents were recalcified with 10 μL of calcium chloride solution to a final concentration of 5 mmol/L. The samples were challenged immediately with 25 μL containing 1 unit of human thrombin (Sigma Chemical Co., St. Louis, MO). The final concentration of ARs were 0.02, 0.2, and 2.0 μmol/L. The incubation was continued for 4 minutes and the TX production was stopped with 25 μL of aspirin at a final concentration of 4.2 mmol/L. The samples were allowed to sit at room temperature for at least 1 hour in order to ensure adequate clotting. After this, the serum was separated by centrifuging the samples at 100*g* for 5 minutes at 277°K.

TX radioimmunoassay

Platelet derived thromboxane TXA_2 was measured as the production of its spontaneous degradation product, TXB_2. All radioimmunoassays were conducted under equilibrium conditions at 277°K in phosphate buffered (pH7.0) saline containing merthiolate (PBS) and 0.05% gelatin. Samples were incubated with the presence of tritiated TXB_2 (New England Nuclear, Boston, MA) and TXB_2 specific rabbit antiserum (in PBS-gel plus 0.01 mol/L EDTA) for 24 hours.[23] Anti-rabbit gamma globulin was then added to the medium to precipitate bound constituents. Following a second incubation, the mixtures were centrifuged to separate the bound from the free radiolabeled TXB_2. The unbound TXB_2 was poured off and the remaining bound radioactivity was counted using liquid scintillation solution (Budget Solve, RPI, Mount Prospect, IL) and

counter (Model Packard 4450, United Technologies, Downers Grove, IL). A computer program developed by Duddleson et al.[24] was used for logit transformation, curve fitting, weighted averaging, and correction for dilution.

Statistical design and analysis

Paired *t* test by SAS (SAS Institute Inc., Cary, NC) was used to compare buffer control with treatments (each alkylresorcinol homologs at each dose). Thus, each subject served as his own control. When inhibition or stimulation was observed, it did not appear linear. Therefore, correlations were not calculated.

Results

The effects of cereal AR homologs on platelet TX production by each subject are shown in *Table 1*, and since each subject's platelets possessed different capability for TX synthesis, the effect was expressed as a percentage response and as a mass difference response relative to the subject's buffer control sample (i.e., no ARs added) (*Table* 2). Concentration of ARs (i.e., 0.02, 0.2, and 2.0 μmol/L final) affected the response. Inhibitory and stimulatory responses by AR homologs obviously exist (*Table* 2). Besides the above-mentioned in vitro variability, subject variability was also considerable (*Table* 1). For example, in subjects No. 7 and 8, the effect of increasing concentration from 0.02 to 2.0 μmol/L of the C15:0 homolog shows the opposite results. In subject No. 7, as concentration of C15:0 homolog increases, the TX production decreases, but in subject No. 8, as concentration of C15:0 homolog increases, the TX production also increases.

AR homologs that showed significant stimulatory responses ($P < 0.05$ by paired *t* test, *Table* 2) are 0.02 μmol/L of C15:0 (40.5%), 0.2 μmol/L of C17:1 (42.4%), 2.0 μmol/L of C17:1 (48.5%), 0.02 μmol/L of C19:0 (7.0%); 2.0 μmol/L of C21:0 (6.0%), 0.02 μmol/L of C23:0, (50.7%), and 2.0 μmol/L C23:0 (45.2%). All showed activation effects on TX production.

Table 2 Thromboxane synthesis responses to alkylresorcinols. Response is expressed relative to control

		Percentage response[1] (%)	Mass response[2] (ng/ml serum)	Prob > [T][3]
C15:0	.02 μmol/L	40.54	17.27 ± 6.04	.0243*
	.2 μmol/L	9.42	3.69 ± 4.39	.4282
	2 μmol/L	59.35	22.58 ± 13.84	.1468
C17:0	.02 μmol/L	42.75	17.95 ± 13.88	.2368
	.2 μmol/L	12.73	4.02 ± 4.33	.3482
	2 μmol/L	4.38	2.57 ± 4.51	.5861
C17:1	.02 μmol/L	28.32	8.23 ± 10.00	.4378
	.2 μmol/L	42.35	16.76 ± 6.51	.0369*
	2 μmol/L	48.48	20.54 ± 6.42	.0150*
C19:0	.02 μmol/L	26.97	9.34 ± 3.47	.0311*
	.2 μmol/L	1.33	.43 ± 5.91	.9447
	2 μmol/L	36.81	14.91 ± 8.31	.1157
C21:0	.02 μmol/L	14.48	3.71 ± 7.30	.6261
	.2 μmol/L	14.85	3.80 ± 7.90	.6449
	2 μmol/L	65.99	26.71 ± 8.88	.0197*
C23:0	.02 μmol/L	50.68	24.98 ± 9.18	.0297*
	.2 μmol/L	−3.92	−1.76 ± 6.97	.8077
	2 μmol/L	45.24	19.89 ± 5.68	.0100*
C25:0	.02 μmol/L	48.31	17.45 ± 8.18	.0704
	.2 μmol/L	22.36	10.65 ± 5.58	.0982
	2 μmol/L	24.21	13.49 ± 11.14	.2653

[1] Response expressed as percentage calculated from means in *Table 1*.
[2] Response expressed as mass difference via subtracting control value. Mean ± SEM.
[3] Paired *t* test. Stars (*) represent significant difference Prob < .05.

Table 1 Alkylresorcinol homologs alter TX production from challenged whole blood (ng/mL serum)

Subject no.		1	2	3	4	5	6	7	8	Average[1]
Control[2]		33.10	42.49	33.20	70.32	30.03	22.01	70.27	57.00	44.80 ± 18.76
C15:0	.02 μmol/L	47.74	36.16	64.27	101.87	57.47	24.85	108.34	55.90	62.08 ± 29.40
	.2 μmol/L	39.30	38.77	42.73	97.61	21.08	31.07	58.64	58.75	48.49 ± 23.57
	2 μmol/L	35.61	88.18	112.53	55.33	36.23	24.60	53.07	133.53	67.39 ± 39.65
C17:0	.02 μmol/L	51.66	19.16	72.11	81.21	46.93	22.74	50.42	157.78	62.75 ± 43.89
	.2 μmol/L	28.75	46.53	39.32	67.93	39.16	29.68	55.18	84.00	48.82 ± 19.28
	2 μmol/L	28.09	35.14	47.97	77.80	24.66	21.50	60.23	83.61	47.38 ± 24.24
C17:1	0.2 μmol/L	65.50	31.24	16.08	86.44	60.87	36.97	29.95	97.21	53.03 ± 29.07
	.2 μmol/L	44.15	49.32	71.10	102.23	37.18	33.77	57.24	97.51	61.56 ± 26.39
	2 μmol/L	58.37	101.27	60.27	91.10	34.25	23.67	88.48	65.33	65.34 ± 27.43
C19:0	.02 μmol/L	49.16	48.33	31.24	87.20	42.84	38.50	61.94	73.90	54.14 ± 18.90
	.2 μmol/L	14.84	56.52	22.16	53.66	42.09	24.23	62.69	85.63	45.23 ± 24.03
	2 μmol/L	40.10	60.39	40.38	46.18	35.97	39.53	99.46	115.68	59.71 ± 30.76
C21:0	.02 μmol/L	42.86	45.81	36.67	27.65	47.56	24.11	78.66	84.86	48.52 ± 22.17
	.2 μmol/L	44.96	34.27	46.72	35.24	39.09	25.60	63.07	99.88	48.60 ± 23.48
	2 μmol/L	32.84	83.60	70.53	62.85	51.67	41.66	99.17	129.76	71.51 ± 31.98
C23:0	.02 μmol/L	28.74	70.02	62.08	74.66	49.49	22.38	137.68	113.22	69.78 ± 39.50
	.2 μmol/L	16.38	50.17	26.09	41.18	26.16	26.64	63.01	94.69	43.04 ± 25.84
	2 μmol/L	34.30	73.00	50.03	73.90	45.20	32.77	103.23	105.09	64.69 ± 28.81
C25:0	.02 μmol/L	52.73	43.03	83.05	49.19	38.10	36.80	92.33	102.80	62.25 ± 26.30
	.2 μmol/L	37.00	29.74	35.67	76.83	49.81	26.76	110.07	77.72	55.45 ± 29.72
	2 μmol/L	33.22	52.41	17.34	76.45	26.32	41.71	157.45	61.45	58.29 ± 44.42

[1] Mean ± SEM.
[2] Buffer contaiing equivalent amount of DMSO and BSA.

Discussion

It is interesting how AR homologs at different concentrations can alter platelet thomboxane production. In order to simplify their effects, the responses affected by all AR homologs at each concentration level were plotted and shown in *Figures 1* and *2*.

Knowledge of the biochemical control of the TX biosynthesis pathway involves several enzymatic steps that are positively and negatively modified, and helps in understanding these dissimilar responses. Arachidonate is an essential fatty acid substrate for TX production derived from the 2-position of phospholipids in cellular membranes as a result of phospholipase activities. Therefore, the inhibition of phospholipases can inhibit eicosanoid production because of the unavailability of the substrate. Cereal ARs are claimed to inhibit phospholipid vesicle hydrolysis by phospholipase A_2, an enzyme that catalyzes the liberation of free arachidonic from membrane phospholipid.[18,25] 5-n-Alk(en)ylresorcinols have been shown to cause an increase in membrane permeability to small solutes, RBC water permeability, and RBC hemolysis.[4,14,15,16,17] Divalent cations such as Ca^{2+} and Mg^2 had been shown to pass through AR modified membranes.[14] All of these effects could possibly liberate intracellular components. Disruption of platelets also shows an enhanced TX synthesis in vivo. Therefore, the hemolytic properties of 5-n-alk(en)ylresorcinol could cause an increase in TX biosynthesis.

The second key enzyme, PG endoperoxide synthase, possesses two separate enzyme activities—cyclooxygenase and peroxidase. The enzymatic conversion of arachidonic acid to PGG_2 by cyclooxygenase requires an active peroxide to stimulate. Thus, an antioxidant can slow down this process, which in turn would inhibit TX production. Peroxidase activity of

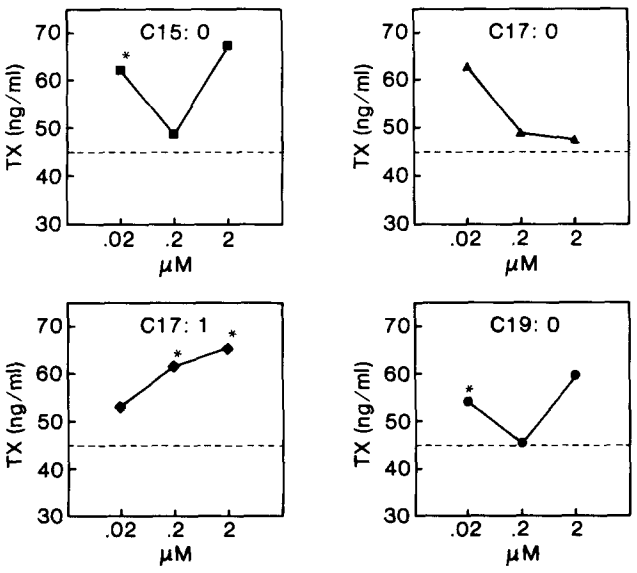

Figure 1 Effects of C15:0 standard, C17:0, C17:1, and C19:0 homologs from rye on platelet thromboxane production. Dashed lines represent control level. Stars represent significant difference by paired *t* test ($P < .05$).

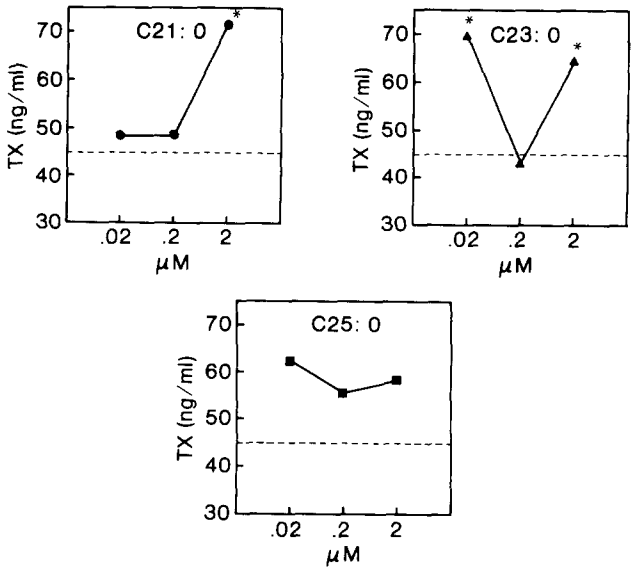

Figure 2 Effects of C21:0, C23:0, and C25:0 homologs from rye on platelet thromboxane production. Dashed lines represent control level. Stars represent significant difference by paired *t* test ($P < .05$).

PG endoperoxide synthase consumes peroxide. During enzymatic conversion of PGG_2 to PGH_2 by peroxidase, a highly reactive intermediate [O_x] is also released. If [O_x] is not scavenged, it can attack cyclooxygenase and subsequent enzymes in the PG and TX synthesis pathway. Therefore, antioxidants can conversely stimulate PG and TX synthesis by scavenging [O_x]. In this case, antioxidants could play a double role in either activating or inhibiting TX biosynthesis. This double role can be distinguished definitely by the fact that lipid hydroperoxides or cellular peroxide tone effects are concentration dependent.[26] At low concentration (0.01–0.1 μmol/L), they activate cyclooxygenase to produce PGG_2. However, at higher concentrations they inhibit. Cereal ARs could have antioxidant properties because their phenolic structure is like other well-known antioxidants such as butylated hydroxy anisole, butylated hydroxy toluene, and flavonoids.[21,22,27]

In this study, the effects of cereal 5-n-alkylresorcinols at concentration levels of 0.02, .2, and 2.0 μmol/L show different responses among homologs studies. The effects of C15:0, C19:0, and C23:0 were biphasic. At concentrations of 0.02 and 2.0 μmol/L levels of these homologs, TX production is higher than that of the 0.2 μmol/L level where TX production is close to that of the control. This phenomenon could be best explained by the factors that have been discussed previously.

First, at low concentration of AR homologs (0.02 μmol/L), activation of TX snythesis is due to the antioxidant properties of ARs in scavenging [O_x] or free radicals that cause inactivation of cyclooxygenase and other enzymes in PGs and TX biosynthesis. When the concentration of ARs was increased to 0.02 μmol/L, they might again act as antioxidants which trap all free

radicals including the micro amount (i.e., 0.01 μmol/L) required to activate cyclooxygenase. Another possibility is that ARs at this concentration start to inhibit the enzyme phospholipase A_2. Therefore, TX production decreases. However, at the highest concentration of ARs studied (2.0 μmol/L), TX synthesis increases. This high concentration of ARs is consistent with concentrations that disrupt RBC membrane and thus increase membrane permeability. This phenomenon was observed visually but not documented chemically. It is well known that disrupted platelets show enhanced TX synthesis in vitro. For this reason, ARs at 2.0 μmol/L could activate TX synthesis by their hemolytic properties.

C17:1 is the only unsaturated homolog studied. The statistically significant activation effect of this homolog tended to increase as its concentration increased. Unsaturated species of ARs were shown previously to have strong hemolytic properties.[4,17] Therefore, as concentrations increase, the membrane disruptive effect could overwhelm the antioxidant property.

Some AR homologs such as C17:0, C21:0, and C25:0 did not show a biphasic response. This could be due to differences in either membrane permeability or variation among subjects which masked the response. In conclusion, AR homologs at particular concentrations significantly affect TX production by their stimulation effects, which could be explained by their antioxidant and their membrane-altering properties.

It is obvious that the end-products of arachidonic acid metabolism are pharmacologically active substances which are involved in many pathological and physiological conditions. The knowledge that ARs affect arachidonic acid metabolism contributes to a better understanding of nutritional effects of cereal ARs. Thus, further investigation into more specific effects of ARs on eicosanoid synthesis could clearly define the biochemical properties of these compounds. The increased consumption of cereal brans and foods containing bran, which results in higher intakes of ARs, suggests further investigation in determining these compounds in food products. Animal feeding studies with AR homologs, which may explain their effects on human nutrition, should be performed as well.

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