Effects of Flavonoids on the Resistance of Microsomes to Lipid Peroxidation *In Vitro* and *Ex Vivo*

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Incubation of rat liver microsomes with preparations of grape flavonoids, dihydroquercetin, and silibinin increased their resistance to lipid peroxidation induced by NADPH-Fe^{2+}. This was manifested in less pronounced accumulation of lipid peroxidation products and changes in activity of microsomal enzymes induced by lipid peroxidation. *In vitro* antioxidant activity of grape flavonoids markedly surpassed that of dihydroquercetin and silibinin. Addition of flavonoids into fodder led to moderate, statistically significant, and similar increase in the resistance of rat liver microsomes to *ex vivo* induced lipid peroxidation.

Key Words: *microsomes; lipid peroxidation; oligomeric proanthocyanidins; dihydroquercetin; silibinin*

The relationship between the imbalance between pro- and antioxidants, development of oxidative stress, and high risk of chronic and age-related degenerative diseases aroused considerable interest in natural antioxidants in the 1990s ("antioxidant boom"). Flavonoids (FL) are most abundant and widely distributed natural antioxidants. High activity of these compounds is related to their structural peculiarities [6].

Much attention is given to grape FL as a component of the "Mediterranean diet". It was hypothesized that these FL determine low risk of cardiovascular diseases in people inhabiting the Mediterranean region [5]. Grape FL are mainly presented by polymeric and oligomeric proanthocyanidins (OPC) of grape seeds, small content of constituting flavan-3-ol monomers (catechin and epicatechin), and anthocyans of grape skins.

Fruits, vegetables, and medicinal plants of more than 50 species are potential sources of FL. Flavonolignans were isolated from *Silybum marianum* plants extensively used as potent hepatoprotectors. Complex preparation Silimarin consisting of silibinin and small content of its stereoisomers and individual flavonolignans (silibinin) are used as medicinal agents and biologically active food additives [14].

FL dihydroquercetin was obtained from nontraditional plant sources (wood of Siberian and Dahurian larches). Biological activity of dihydroquercetin is poorly known. This compound possesses antioxidant properties, is characterized by low toxicity, and can be isolated from available sources in Russia. Therefore, dihydroquercetin is a promising natural antioxidant [1,3]. It should be emphasized that the flavonoid part of molecules in silibinin and other flavonolignans is presented by dihydroquercetin.

Here we studied the antioxidant effect of preparations from grape FL, silibinin, and dihydroquercetin on rat liver microsomes during *in vitro* and *ex vivo* induced lipid peroxidation (LPO).

MATERIALS AND METHODS

LPO in microsomal membranes was induced in the NADPH-Fe^{2+} system. Oxidative modification of lipids was estimated by accumulation of thiobarbituric acid (TBA)-reactive LPO products and changes in activity of enzymes having different localization in membranes of the endoplasmic reticulum (microsomes). Microsomes were isolated from the liver of male Wistar

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rats. We evaluated the influence of FL preparations on the rate of induced LPO. The suspension of microsomes in 40 mM Tris-HCl buffer (pH 7.4, 1 mg protein/ml) was incubated with FL in increasing concentrations (0.5-50.0 μg/ml) at 37°C for 10 min under constant shaking. NADPH in a final concentration of 0.025 mM, 12 μM $Fe(NH_4)_2(SO_4)_2 \times 6H_2O$, and 0.2 mM $Na_4P_2O_7 \times H_2O$ were added to the incubation medium. The mixture was repeatedly incubated at 37°C for 10 min under constant shaking. The reaction was stopped by adding 30% trichloroacetic (TCA) acid. The content of TBA-reactive compounds was measured.

We studied the effect of FL on LPO-induced changes in enzyme activity. Microsome suspension in 40 mM Tris-HCl buffer (pH 7.4, 1 mg protein/ml) was incubated with preparations of grape FL (10 μg/ml), dihydroquercetin, silibinin (50 μg/ml), or solvent at 37°C for 10 min under constant shaking. Oxidation was induced in the system containing 0.025 mM NADPH. The reaction was stopped by adding 30% TCA acid and 5 mM ethylenediaminetetraacetic acid to determine the content of TBA-reactive compounds and enzyme activity, respectively. To estimate the content of TBA-reactive compounds, the incubation mixture was centrifuged at 6000 rpm for 10 min. HCl (0.6 N, 0.2 ml) and TBA (0.12 M, 0.8 ml) were added to 1 ml supernatant. The mixture was heated at 100°C for 10 min. The content of malonic dialdehyde (MDA) was measured at 532 nm and estimated using molar extinction coefficient 1.56×10^5 $M^{-1}cm^{-1}$. UDP-glucuronosyl transferase (GlT) activity was measured in the reaction with *p*-nitrophenol as the substrate [7] in the absence of detergent. The rate of benz[a]pyrene hydroxylation (BPH) was measured as described elsewhere [8].

We determined *ex vivo* effect of FL on the resistance of microsomes to LPO. Experiments were performed on liver microsomes from male Wistar rats (6 animals per group) feeding a half-synthetic diet with OPC, dihydroquercetin, and Silimarin in daily doses of 150, 130, and 150 mg/kg, respectively, for 2 weeks.

We used standard preparation of OPC from grape seeds (43.2% OPC); standardized grape extract (GE) containing 20.3% proanthocyanidins, 4.8% anthocyans, and small content of resveratrol (La Gardonnenque); dihydroquercetin (Chemical Department, Moscow Pedagogic State University) with a purity of more than 96% (high-performance liquid chromatography); Silimarin (ICN Biomedicals); silibinin; TBA; NADPH; ascorbic acid; benz[a]pyrene (Sigma); and other reagents ("pure for analysis", Russia). OPC and GE were dissolved in water. An aqueous solution of ethanol (10%) and acetone served as solvents for dihydroquercetin and silibinin, respectively. The final concentration of solvents in experimental and control samples (without FL) did not exceed 1%.

The results of *in vitro* studies are presented as means determined in 3-4 independent experiments. The data were analyzed by Student's *t* test.

RESULTS

Incubation of microsomes with different FL improved their resistance to LPO induced by NADPH-Fe^{2+}. The decrease in the rate of LPO depended on the type and concentration of preparations (Fig. 1). OPC in a concentration of 0.5 μg/ml significantly inhibited the formation of LPO products. Generation of LPO products was inhibited by 14, 11, and 10% in the presence of OPC, GE, and dihydroquercetin in a concentration of 1 μg/ml, respectively. Silibinin markedly inhibited LPO only in a concentration of more than 5 μg/ml. OPC, GE, dihydroquercetin, and silibinin in maximum concentrations inhibited LPO by 100, 90, 63, and 59%, respectively. OPC, GE, dihydroquercetin, and silibinin in concentrations of 8, 10, 30, and 38 μg/ml, respectively, caused a 50% inhibition of NADPH-Fe^{2+}-induced LPO.

Our previous studies showed [2] that induction of LPO in microsomes was followed by suppression of BPH and increase in native GlT activity (without detergent). These changes directly depended on the concentration of LPO products (MDA). Therefore, variations in activity of microsomal enzymes during induced LPO and accumulation of LPO products adequately reflect structural and functional state of microsomes.

Preincubation of microsomes with 10 μg/ml OPC resulted in suppression of induced LPO. It was manifested in less intensive accumulation of MDA (by 2.2 times) and less pronounced changes in enzyme activity during induced LPO (Table 1). In the absence and presence of OPC the intensity of BPH in microsomes was

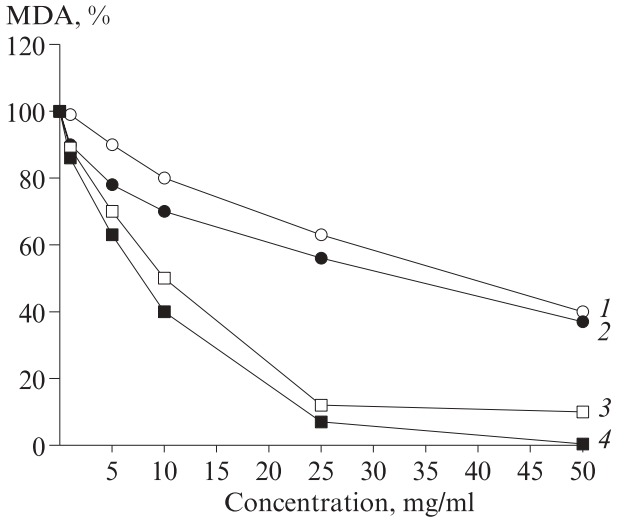

Fig. 1. Effects of silibinin (*1*), dihydroquercetin (*2*), grape extract (*3*), and oligomeric proanthocyanidins (*4*).

TABLE 1. Effect of FL Preparations on Changes in the Rate of Benz[a]pyrene Hydroxylation and GlT Activity during LPO Induced in Rat Liver Microsomes (*M*±*m*)

Treatment	MDA, nmol/mg protein/10 min	BPH, arb. units/mg protein	GlT, nmol/mg protein/min
OPC			
control (without prooxidant)	0.23±0.05	5.25±0.75	3.50±0.70
NADPH (25 μM)	9.20±0.10*	1.90±0.10*	12.6±0.81*
NADPH (25 μM) and OPC (10 μg/ml)	4.14±0.18*+	3.68±0.38+	6.65±0.35*+
Dihydroquercetin			
control (without prooxidant)	0.55±0.04	7.46±0.24	11.3±1.2
NADPH (25 μM)	10.40±0.13*	3.33±0.18*	31.4±0.5*
NADPH (25 μM) and OPC (10 μg/ml)	3.71±0.10*+	5.70±0.70+	11.9±0.5+
Silibinin			
control (without prooxidant)	0.25±0.04	7.52±0.09	6.25±1.90
NADPH (25 μM)	9.52±0.10*	3.20±0.10*	23.60±2.00*
NADPH (25 μM) and OPC (10 μg/ml)	3.89±0.04*+	4.85±0.19*+	8.75±0.65+

Note. *$p<0.05$ compared to the control (without prooxidant); +$p<0.05$ compared to the effect of prooxidant (NADPH).

36 and 70% of the control, respectively. Therefore, the degree of GlT activation decreased from 360 to 190%.

The content of MDA decreased by 2.8 times in the presence of 50 μg/ml dihydroquercetin. Under these conditions the degree of BPH suppression decreased more than by 1.7 times. GlT activity returned to normal (Table 1).

Silibinin in a concentration of 50 μg/ml suppressed production of MDA and reduced changes in BPH and GlT by 2.4, 1.5, and 2.7 times, respectively (Table 1).

Changes in the resistance of macromolecules (lipids, proteins, and DNA) to *ex vivo* oxidative modification serve as a marker for *in vivo* antioxidant activity of food components. The addition of FL into fodder increased the resistance of rat liver microsomes to *ex vivo* induced LPO. The intensity of MDA generation decreased in rats receiving OPC, dihydroquercetin (by 26%), and silimarin (by 32%, Table 2).

TABLE 2. Effect of FL Preparations on the Resistance of Rat Liver Microsomes to *ex Vivo* LPO (*M*±*m*, *n*=6)

Preparation	Group	Rate of NADPH-induced LPO, MDA (nmol/mg protein/10 min)
OPC	Control	8.16±0.41
	Experiment	6.03±0.45*
Dihydroquercetin	Control	10.55±0.99
	Experiment	7.85±0.56*
Silibinin	Control	8.16±0.41
	Experiment	5.59±0.63*

Note. *$p<0.05$ compared to the control.

Therefore, the studied preparations of FL exhibited high antioxidant activity and markedly increased the resistance of microsomes to adverse effects of LPO *in vitro*. It should be emphasized that preparations of grape FL were more potent than silibinin. No differences were revealed in activity of silibinin and dihydroquercetin.

Interestingly, antioxidant activity of FL directly depends on the number of hydroxyl groups in their structure and ability to reduce free radicals (*i.e.*, play a role of the hydrogen donor) with the formation of stable flavoxyl radicals. These changes abolish initiation or break chain reaction of free radical oxidation [6]. Higher efficiency of preparations from grape FL *in vitro* can be explained by the presence of OPC. Polymeric polyphenol structure and the presence of numerous hydroxylic groups determine high antioxidant activity of OPC. Previous studies on model systems of oxidation showed that OPC interact with the superoxide anion radical and hydroxyl radical and surpass vitamins C and E and β-carotene in antiradical activity [4]. OPC not only trap free radicals and block chain free radical reactions, but are adsorbed on membranes, bind to polar hydrophilic heads of phospholipids, and reduce access to the lipid bilayer for oxidants [13]. Increasing the degree of polymerization in polyphenol compounds is accompanied by the increase in antioxidant activity. Antioxidant activity decreases in the following order: trimers> dimers>monomers (catechin). Anthocyans entering the composition of GE *in vitro* display high antioxidant activity. Their ability to interact with peroxyl radicals 3-fold surpasses that of quercetin and catechin [15].

Published data show that radical-trapping activity of silibinin and dihydroquercetin is equal to or lower than that of vitamin E. As differentiated from OPC and anthocyans, these compounds act as weak chelators of iron ions [12]. L. Cavallini *et al* [10] demonstrated that dihydroquercetin and silibinin has similar antioxidant activity in various model systems of oxidation.

No differences were found in the influence of individual FL on the resistance of microsomes to *ex vivo* induced LPO, which contradicts the results of *in vitro* studies. Probably, these peculiarities reflect the biological availability and metabolism of compounds in the organism. Monomers and several dimers were found in the blood of humans and rats after administration of preparations containing grape OPC. These components *in vitro* have the lowest antioxidant activity [11]. Moreover, intestinal microorganisms can metabolize OPC to simple phenol acids. They possess lower antioxidant activity compared to OPC [9].

It should be emphasized that the system of induced LPO in microsomes allows estimating antioxidant activity of FL preparations not only *in vitro*, but also *in vivo*.

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