Effects of Combined Treatment with Resveratrol and Indole-3-Carbinol

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Male Wistar rats received a semisynthetic diet with resveratrol (100 mg/kg), indole-3-carbinol (20 mg/kg), or a mixture of these compounds in the same doses for 1 week. Activities of ethoxyresorufin dealkylase (EROD), methoxyresorufin dealkylase (MROD), pentoxyresorufin dealkylase (PROD), and 6β-testosterone hydroxylase (6β-TH) and the content of mRNA for *CYP1A1*, *CYP1A2*, and *CYP3A1* were elevated in the liver of rats receiving indole-3-carbinol. These changes were accompanied by an increase in activity of phase II xenobiotic metabolism enzymes (quinone reductase, hemoxygenase-1, glutathione transferase, and UDP glucuronosyl transferase). Resveratrol did not modify activity of these enzymes. After combined treatment with the test compounds, resveratrol suppressed the indole-3-carbinol-induced increase in activities of EROD, MROD, PROD, and 6β-TH, and expression of the corresponding genes. Combined treatment was characterized by potentiation of the antioxidant effects of these compounds.

Key Words: *resveratrol; indole-3-carbinol; xenobiotic metabolism enzymes; antioxidant state*

Studying the functional role of dietary biologically active substances (BAS) showed that the majority of these compounds are involved in a complex regulatory system of the defense and adaptive response to stress exposure. Among a variety of mechanisms underlying the protective effect of BAS, much attention is paid to the following two processes: AhR (aryl hydrocarbon receptor) transcription factor-regulated activation of gene expression for the enzymes that provide detoxification of xenobiotics and electrophilic metabolites; and Nrf2 transcription factor-regulated activation of gene expression for antioxidant enzymes and enzymes for the synthesis of other antioxidant agents [4,13,15].

Recent studies showed that AhR regulates the expression of at least three genes of the cytochrome P-450 family (CYP1; *CYP1A1*, *CYP1A2*, and *CYP1B1*) and phase II enzymes of xenobiotic detoxification, including UDP glucuronosyl transferase (UDP GT) GT1 (GT1A1 and GT1A6), glutathione transferase, and quinone reductase. Nrf2 regulates the expression of genes for hemoxygenase-1, γ-glutamyl cysteine synthetase (key enzyme of glutathione synthesis), glutathione reductase, and glutathione peroxidase GPx2. Moreover, Nrf2 regulates the expression of genes for phase II xenobiotic metabolism enzymes (quinone reductase, glutathione transferase, and GT1A6) playing a role in the antioxidant defense of cells [3,13].

In vitro experiments showed that various natural BAS, including flavonoids (resveratrol, RES; lycopene; and sulforaphane) can activate Nrf2. Some of them (indole-3-carbinol, I-3-C; and RES) serve as AhR ligands [4,15]. RES and I-3-C attract much attention of investigators.

Polyphenol RES (3,5,4'-trihydroxystilbene) found in grapes, red wine, berries, rhubarb, and peanut acts as a strong antioxidant. RES actively traps superoxide anion radicals and hydroxyl radicals in various model systems of oxidation. Similarly to α-tocopherol, RES inhibits lipid peroxidation (LPO) due to trapping of lipid peroxide radicals [8]. Apart from antiradical ef-

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fect, RES increases activity of factor Nrf2 and antioxidant and cytoprotective enzymes in various cells [14]. The so-called "French paradox" (relatively low incidence of cardiovascular disease in the Mediterranean population) is associated with high consumption of RES. RES holds much promise as an anticarcinogenic compound (chemoprotective agent) in relation to polycyclic aromatic carbohydrates. They serve as AhR ligands and induce the synthesis and activity of cytochromes P-450 CYP1. These cytochromes are involved in metabolic activation of carbohydrates. In cell culture experiments, RES acted as an antagonist of AhR and suppressed activation of the expression of *CYP1A1* and *CYP1A2* genes induced by various xenobiotics [4-6,11].

I-3-C is one of the major BAS from vegetables of the Cruciferae family (all species of cabbage plants, black radish, garden radish, and mustard) constituting 10-15% of consumed vegetables. I-3-C is one of the rare BAS with high anticarcinogenic activity (proved in studies on model systems and animal experiments) [10]. *In vitro* and *in vivo* studies showed that I-3-C is a ligand of AhR, which can induce activity of cytochromes P-450 (CYP1A1 and CYP1A2), quinone reductase, and glutathione transferase in the liver and other organs [7,10]. Little is known about the antioxidant properties of this compound. Our previous study showed that I-3-C *in vitro* and *ex vivo* inhibits microsomal LPO [1]. Dietary I-3-C dose-dependently increased antioxidant capacity of blood plasma and decreased accumulation of LPO products in rats

These data indicate that biological activity of RES and I-3-C is mediated by common mechanisms. Moreover, both compounds may be consumed with food products. However, the effect of combined treatment with RES and I-3-C remains unknown. Here we studied combined action of RES and I-3-C on the antioxidant status and activity of xenobiotic metabolism enzymes in the liver of rats.

MATERIALS AND METHODS

Experiments were performed on male Wistar rats (initial body weight 150-155 g). The animals were divided into 4 groups (6 animals per group). Control rats received a semisynthetic diet including casein, cornstarch, vegetable oil and lard, vitamin-salt mixture, and microcrystalline cellulose. Group 1-3 animals received the same diet supplemented with RES (100 mg/kg, DSM), I-3-C (20 mg/kg, Sigma), and RES+I-3-C (similar doses), respectively. The duration of our study was 1 week.

Activity of phase I xenobiotic metabolism enzymes was measured in liver microsomes. Activities of ethoxyresorufin dealkylase (EROD, for CYP1A1), methoxyresorufin dealkylase (MROD, for CYP1A2), pentoxyresorufin dealkylase (PROD, for CYP2B1/2), and 6β-testosterone hydroxylase (6β-TH, for CYP3A) were measured as described elsewhere [2].

The expression of mRNA for *CYP1A1*, *CYP1A2*, and *CYP3A1* was studied by reverse transcription-PCR. mRNA was isolated with TRI reagent (Sigma) according to manufacturer's recommendations. Total RNA concentration was estimated from optical density at 260 nm. cDNA was obtained in the reverse transcription reaction with synthetic hexanucleotides. The reaction was performed with 4 μg total RNA at 37°C for 1 h. The sample was maintained at 94°C for 10 sec for enzyme inactivation. cDNA was used in PCR. The reverse transcription reaction with water (instead of RNA) served as the control. PCR for the studied genes was conducted as follows: denaturation at 94°C for 1 min; annealing of primers at 60°C for 10 sec (60°C for 30 sec for the *CYP1A2* gene; 62°C for 10 sec for the *CYP1A1* gene); product synthesis at 72°C for 10 sec; and maintenance at 72°C for 3 min. The number of cycles varied from 22 to 30, which depended on the nature of the studied gene. RNA was used as the control (instead of cDNA). Reverse

TABLE 1. Primers and Size of Amplified Fragments

Gene	Primer nucleotide sequence	Amplicon size, b.p.
β-Actin	5'-TGCAGAAGGAGATTACTGCC-3'	212
	5'-GCAGCTCAGTAACAGTCCG-3'	
CYP1A1	5'-CCATGACCAGGAACTATGGG-3'	341
	5'-TCTGGTGAGCATCCAGGACA-3'	
CYP1A2	5'-TAGTGAAGCAGGGGGATGAC-3'	431
	5'-GACCGGAAAGAAGTCCACAG-3'	
CYP3A1	5'-GGAAATTCGATGTGGAGTGC-3'	329
	5'-AGGTTTGCCTTTCTCTTGCC-3'	

transcriptase, M-MuLV, and Taq SE DNA polymerase (Sibenzim) were used. Hexanucleotides and primers were manufactured by Litekh. Reverse transcription and amplification were performed on a Tertsik device. Table 1 shows the sequence of primers and size of amplified fragments.

Reverse transcription-PCR products were separated electrophoretically in 2.5% agarose gel at 180 V. Electrophoretograms were subjected to densitometry. The gels were analyzed by means of Quantity One 4.5 software.

Activities of hemoxygenase-1 and UDP GT in liver microsomes were measured using *p*-nitrophenol as a substrate. Activities of quinone reductase and glutathione transferase were evaluated in the cytosol [2]. They serve as antioxidant enzymes and phase II xenobiotic metabolism enzymes.

The antioxidant status of rats was evaluated from the total antioxidant capacity and plasma reducing activity. The study was conducted with the test system of hemoglobin, hydrogen peroxide, and luminol and test system of FRAP [1].

Liver microsomes from group 1-3 rats were isolated to study the *ex vivo* effect of I-3-C, RES, and I-3-C+RES on the resistance of microsomes to NADPH-Fe^{2+}-induced LPO [1].

The results were analyzed by means of ANOVA (Statgraphics software). The differences were significant at $p<0.05$.

RESULTS

The consumption of dietary RES (group 1) had little effect on activities of EROD and 6β-TH, but slightly increased activities of MROD and PROD (by 42%; Table 2). As differentiated from RES, feeding of I-3-C (group 2) was followed by an increase in activities of EROD (by 6 times), MROD (by 10.6 times), PROD (by 5.3 times), and 6β-TH (by 2.2 times). After combined treatment with RES and I-3-C (group 3), activities of EROD, MROD, and PROD decreased by 2 times. Under these conditions, activity of 6β-TH decreased by 30% (as compared to that in animals receiving I-3-C).

The relative content of mRNA for *CYP1A1*, *CYP1A2*, and *CYP3A1* in the liver of RES-receiving rats was reduced by 50, 25, and 25%, respectively. However, the amount of these mRNAs in the liver of animals was elevated after consumption of I-3-C (by 3.8, 2.2, and 1.8 times, respectively; Fig. 1). Combined consumption of RES and I-3-C was followed by a decrease in the concentration of mRNA for *CYP1A1*, *CYP1A2*, and *CYP3A1* (by 15, 25, and 6%, respectively, compared to group 2 rats).

RES had little effect on activities of phase II xenobiotic metabolism enzymes (Table 3). However, consumption of dietary I-3-C (group 2) was followed by a moderate increase in hemoxygenase-1 activity (by 18%) and significant increase in activities of quinone reductase (by 2 times), glutathione transferase (by 65%), and UDP GT in the liver (by 45%). Activity of hemoxygenase in group 3 animals (RES+I-3-C) was 11% higher than in group 2 specimens. Activities of quinone reductase and glutathione transferase remained unchanged in group 3 rats. However, UDP GT activity decreased by 10% (statistically insignificant).

Reducing activity and total antioxidant capacity of blood plasma serve as the integral criteria for the antioxidant status (Fig. 2). Feeding of RES or I-3-C was followed by a significant increase in reducing activity (by 14% in both groups) and antioxidant ca-

TABLE 2. Activity of Various Isoforms of Cytochrome P-450 in the Liver of Rats Receiving a Diet with RES (Group 1), I-3-C (Group 2), or RES+I-3-C (Group 3; *M*±*m*)

Enzyme	Group			
	control (*n*=6)	1 (*n*=6)	2 (*n*=6)	3 (*n*=6)
EROD (CYP1A1), pmol/mg protein/min	20.2±3.0	24.5±2.8	117.1±7.4*	64.4±5.2*+
MROD (CYP1A2), pmol/mg protein/min	66.0±11.7	93.8±19.2	700.0±66.0*	356.0±37.0*+
PROD (CYP2B1/2), pmol/mg protein/min	15.5±1.3	22.0±2.9	82.2±8.5*	45.2±5.7*+
6β-TH (CYP3A), nmol/mg protein/min	0.85±0.08	0.97±0.09	1.88±0.13*	1.49±0.16*

Note. Here and in Table 3: $p<0.05$: *compared to the control; +compared to groups 1 and 2.

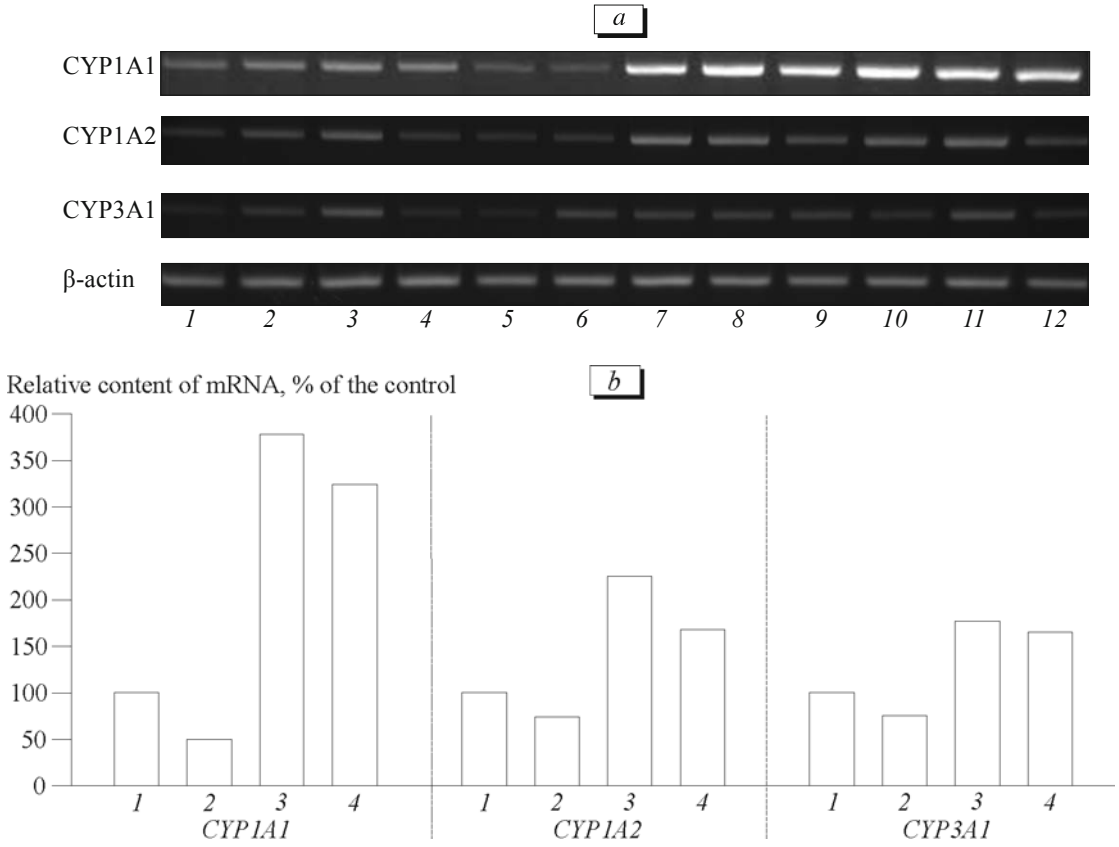

Fig. 1. Relative expression of mRNA for *CYP1A1*, *CYP1A2*, and *CYP3A1*. (*a*) Electrophoretograms of PCR gene products: control (*1-3*); RES (*4-6*); I-3-C (*7-9*); RES+I-3-C (*10-12*). (*b*) mRNA content: control (*1*); RES (*2*); I-3-C (*3*); RES+I-3-C (*4*).

pacity of blood plasma (by 10% for RES; and by 20% for I-3-C). After combined feeding of RES and I-3-C, these parameters increased by 28 and 25%, respectively.

We evaluated the *ex vivo* resistance of rat liver microsomes to induced LPO. The degree of LPO was significantly reduced in liver microsomes from rats receiving a diet with RES or I-3-C (Fig. 3). The rate of induced LPO in liver microsomes from group 3 rats (RES+I-3-C) was lower compared to the control (by 60%) and group 1 and 2 animals.

Our results indicate that I-3-C and RES serve as AhR ligands and have various effects on enzyme activity and expression of AhR-regulated genes. The consumption of dietary I-3-C (20 mg/kg) was followed by a sharp increase in EROD and MROD activities and amount of mRNA for *CYP1A1* and *CYP1A2*. The observed changes were accompanied by a slight increase in 6β-TH activity and *CYP3A1* gene expression. Our findings are consistent with the results of previous experiments with I-3-C in various doses. It was shown that CYP1A1, CYP1A2, and CYP2B1/2

TABLE 3. Activity of Phase II Xenobiotic Metabolism Enzymes in the Liver of Rats Receiving a Diet with RES (Group 1), I-3-C (Group 2), or RES+I-3-C (Group 3; *M*±*m*)

Enzyme	Group			
	control (*n*=6)	1 (*n*=6)	2 (*n*=6)	3 (*n*=6)
Quinone reductase, nmol/mg protein/min	148±21	161±17	289±59*	285±56*
Hemoxygenase, pmol/mg protein/min	12.5±1.2	12.8±1.8	14.8±0.7	16.2±1.9
Glutathione transferase, μmol/mg protein/min	1.01±0.09	0.89±0.04	1.67±0.13*	1.64±0.13*
UDP GT (GT1A6), nmol/mg protein/min	29.9±3.0	29.1±2.6	43.1±2.4*	39.1±2.1*

are most sensitive to the *in vivo* inducing effect of I-3-C. Published data [9] show that treatment with I-3-C in a dose of 25 mg/kg or higher is followed by an increase in activities of EROD, MROD, and PROD in the liver of rats. The content of mRNA for *CYP1A1* and *CYP1A2* was elevated only under the influence of I-3-C in a dose of 250 mg/kg. Chronic administration of I-3-C in a dose of 50 mg/kg produced an increase in EROD activity and CYP3A content (by 8.4 and 2.8 times, respectively) [8].

Published data and result of our study show that I-3-C induces activity of AhR-regulated enzymes for phase II xenobiotic metabolism, including quinone reductase, glutathione reductase, and UDP GT. Besides detoxification, these enzymes play an important role in the maintenance of antioxidant defense [3,13]. Therefore, the observed changes in enzyme activity for phase I and II xenobiotic metabolism are mediated by the I-3-C-induced activation of factor AhR.

It should be emphasized that I-3-C has an inducing effect on PROD and 6β-TH. These enzymes characterize cytochrome P-450 isoforms, whose expression is not directly related to AhR. It is probably associated with a wide (overlapping) range of substrate specificity of various isoform, interaction between transcription factors (*e.g.*, AhR with PXR that regulates 6β-TH gene expression), or effect of unknown or unstudied metabolites of I-3-C.

RES had little effect on activity of the studied enzymes. After combined treatment with the test compounds, RES produced a selective inhibitory effect on the I-3-C-induced increase in activities of EROD, MROD, PROD, and 6β-TH. Less significant changes were observed in the content of mRNA for *CYP1A1*, *CYP1A2*, and *CYP3A1*. *In vitro* experiments showed that RES increases the expression of the *Nrf2* gene and activities of quinone reductase, glutathione transferase, and some antioxidant enzymes [14]. In our experiments, RES had no effect on activity of these enzymes (including Nrf2-regulated enzyme hemoxygenase-1). We found no published data on combined action of I-3-C and RES on AhR-regulated genes. However, numerous experiments on cultured cells showed that RES completely or partially inhibits xenobiotic-induced increase in the expression and enzyme activity of *CYP1A* genes, but did not suppress induction of phase II enzymes [4,6,11].

The absence of the effect of RES on I-3-C-induced activation of phase II enzymes can be explained by the fact that I-3-C can modulate expression of these enzymes not only as a ligand of AhR, but also as Nrf2 activator [12]. This hypothesis is confirmed by the fact that gene promoter regions have regulatory sites for AhR (XRE) and Nrf2 (ARE).

Speaking about antioxidant efficiency of combined action of I-3-C and RES we should note the expected potentiation of their effects on the antioxidant status of rats. Moreover, combined feeding of I-3-C and RES produced the inhibitory effect on induced LPO in rat liver microsomes.

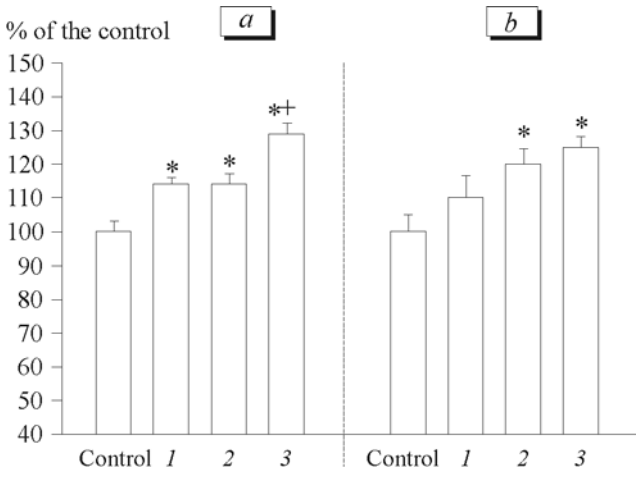

Fig. 2. Reducing activity (*a*) and antioxidant capacity (*b*) of blood plasma from rats receiving a diet with RES (*1*), I-3-C (*2*), or RES+I-3-C (*3*). Here and in Fig. 3: $p<0.05$: *compared to the control; $^{+}$compared to *1* and *2* (individual administration of RES and I-3-C).

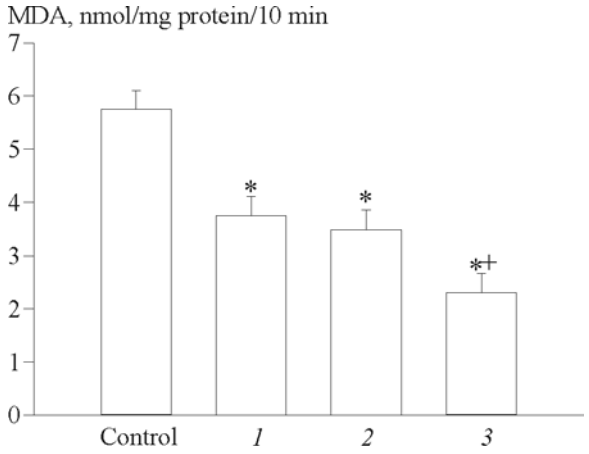

Fig. 3. *Ex vivo* effect of RES (*1*), I-3-C (*2*), and RES+I-3-C (*3*) on NADPH-Fe^{2+}-induced LPO in rat liver microsomes.

Our results indicate that the combined effect of various BAS should be taken into account. Many BAS are empirically and groundlessly used in the composition of functional food products and biologically active additives. Moreover, the ratio of these products in the dietary regimen of people increases progressively.

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