Letter to the Editor

Oxidative Metabolism of Estrone Modified by Genistein and Bisphenol A in Rat Liver Microsomes*

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Genistein, the main isoflavone from soy, and bisphenol A (BPA), a food contaminant, are considered ubiquitous xenoestrogens. Here we investigated the influence of genistein and BPA on estrone (E_1) metabolism in rat liver microsomes. Both substances inhibited the 2-hydroxylation and 16α-hydroxylation of E_1, but in different degrees, thereby reducing the 2-OH-E_1/16α-OH-E_1 ratio, a biomarker for the risk of breast cancer. Although the 2-OH-E_1/16α-OH-E_1 ratio was lower under BPA treatment than under the control condition, the difference was not statistically significant. The 2-OH-E_1/16α-OH-E_1 ratio was significantly reduced by genistein treatment (P<0.05), and this effect was more pronounced when using a combination of BPA and genistein (P<0.01). Our findings suggest that a synergistic inhibition effect of genistein and BPA on E_1 metabolism is associated with an increased risk of breast cancer.

The oxidative metabolism of estrone (E_1) leads to the formation of several metabolites with various estrogenic and genotoxic potentials. 2-Hydroxyestrone (2-OH-E_1) and 4-hydroxyestrone (4-OH-E_1) (in an average ratio of 3:1) account for more than 80% of the metabolites formed by the NADPH-dependent catalytic action of cytochrome P_{450}. The major isoenzymes responsible for this metabolic pathway are CYP3A4, CYP3A5[1], CYP1A2, CYP1A1, and CYP1B1[2]. Hydroxylation in 16α position of E_1 is a minor alternative metabolic pathway that involves many of the same isoenzymes (CYP3A4, CYP3A5, CYP2C19, and CYP1A1)[1-2]. Although 2-OH-E_1 is a weak estrogen, 16α-OH-E_1 has an important role in carcinogenesis, showing strong estrogenic effects and genotoxic properties[1]. 16α-OH-E_1 covalently binds to estrogen receptors, thus stimulating cell proliferation. The 2-OH-E_1/16α-OH-E_1 ratio has been proposed and studied as a biomarker for the risk of breast cancer[3].

A soy-rich diet can modulate E_1 metabolism, resulting in increased metabolism to 2-OH-E_1 and subsequent increase in the 2-OH-E_1/16-OH-E_1 ratio, thus providing a protective effect against breast cancer in young to middle-aged and premenopausal women. A recent meta-analysis of 35 studies reported in the literature found that soy isoflavone intake is associated with a reduction in the risk of breast cancer in both pre- and post-menopausal women. This effect is more evident in Asian than in Western countries, possibly related to the high and low intake levels of soy isoflavones, respectively[4]. In some studies, excessive soy consumption was associated with a negative impact on male fertility and reproductive hormones as well as disruption of the thyroid function, although these effects were inconsistent with those reported in other studies[5].

Genistein (G), the principal isoflavone present in soy, is a well-known phytoestrogen with an endocrine-disrupting potential. Because soybean consumption is now widespread, the exposure to G may be considered frequent in several population categories, from infants to the elderly. The therapeutic potential of G is yet to be clearly defined because various studies have reported conflicting results[6]. However, there is consistent evidence that G shows estrogenic and tyrosine kinase inhibitory activities[6].

Bisphenol A (BPA) is a xenobiotic with xenoestrogenic properties that may alter multiple endocrine-related pathways. It is produced in large quantities worldwide and is a monomer of

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polycarbonate and epoxy resins, which are widely used in the food and beverage packing industry. Under certain circumstances, particularly hot and acidic pH conditions, BPA can be released from packing to become a food contaminant. BPA is also a component of everyday products such as electronics, plastics, paper, and dental items. As such, BPA exposure can be considered ubiquitous[7].

Given their common biological effects (endocrine disruptors), common metabolic pathways (hydroxylation and glucuronidation), and the prevalence of daily co-exposure, many studies have addressed the interactions between G and BPA at various biological levels. G and BPA interactions are complex and depend on the biological target, doses, and species.

Isolated liver microsomes are useful for determining the metabolic pathways of xenobiotics and studying the possible interactions between chemicals that have affinity for the same metabolic enzymes. Here we aimed to determine the influence of G and BPA, alone and in combination, on the 2-OH-E_1/16α-OH-E_1 ratio in pooled rat liver microsomes and to identify any potential interactions that may occur between them. A liquid chromatographic tandem mass spectrometric (LC-MS/MS) method was used for the quantification of E_1 metabolites.

The experimental protocol was approved by the Ethics Committee of the Iuliu Haţieganu University of Medicine and Pharmacy, Cluj-Napoca (Romania), being in compliance with the European guidelines for the use of animals for scientific purposes. Two male Wistar albino rats (approximately 200 g) supplied by the Practical Skills and Experimental Medicine Centre of Iuliu Haţieganu University of Medicine and Pharmacy, Cluj-Napoca (Romania), were treated daily for enzyme induction with phenobarbital (Sicomed S.A., Romania) in doses of 100 mg/kg bw/day by gastric intubation for 7 d. Fraxiparine (GlaxoSmithKline) was administered under anesthesia (with ketamine and xylazine), and the whole liver was excised after being washed through the portal vein with Buffer 1 [50 mmol/L Tris buffer (pH 7.4) containing 25 mmol/L sucrose and 1 mmol/L EDTA]. Microsomes were isolated by differential centrifugation using a Sorvall WX 100 ultracentrifuge (Thermo Fisher Scientific). Each liver was homogenized in Buffer 1 and centrifuged at 13,000 ×g for 30 min at 4 °C. The obtained supernatants were centrifuged at 105,000 ×g for 60 min at 4 °C. Microsomal pellets were suspended by homogenization in 100 mmol/L Tris buffer (pH 7.4) containing 1 mmol/L EDTA and 20% glycerol (v/v), pooled, snap frozen, and stored at -80 °C. For the experimental protocol, the pooled microsomal preparation was defrosted and diluted in Buffer 2 [50 mmol/L Tris buffer (pH 7.4) containing 100 mmol/L KCl] to obtain a final concentration of 0.5 mg microsomal proteins/mL.

Total proteins were quantified by the Bradford method using bovine serum albumin (2 mg/mL) as the standard for calibration and Coomassie Brilliant Blue G as color reagent. The absorbance was measured at 595 nm using a UV/Vis spectrophotometer (Jasco V-530).

Stock solutions of E_1 (50 mmol/L), BPA (25 mmol/L), and G (25 mmol/L) in dimethyl sulfoxide were prepared. Pooled microsomal preparations (0.5 mg microsomal proteins/mL) were marked with different concentrations of substrate (100, 200, 400, 600, and 800 µmol/L E_1) and preincubated in a water bath at 37 °C for 5 min, with or without G (100 µmol/L) and/or BPA (100 µmol/L). The tested concentrations of BPA and G were selected on the basis of previously reported IC_{50} or K*i* values for the CYP isoforms to ensure adequate inhibitory potency[8-9]. Reactions were initiated by the addition of an NADPH-generating system (10 mmol/L NADP, 50 mmol/L G6P, 10 U/mL G6P-DH-ase, and 50 mmol/L $MgCl_2$). After various incubation periods (0, 5, 10, 15, 20, and 30 min), the reactions were stopped by the addition of ice-cold acetonitrile and centrifugation (10 min at 1398 ×g using a Sigma 2-16 centrifuge). The supernatant (50 µL) was analyzed by LC-MS/MS chromatography. All samples were analyzed in triplicates.

A HP 1100 Series Agilent liquid chromatographic system coupled with an Agilent Ion Trap SL mass spectrometer was used. Chromatographic separation was performed on a Gemini-NX-C18 column (50×2.0 mm i.d., 3 µm) on-line equipped with a 0.2-µm filter and a mobile phase comprising a mixture of 2 mmol/L ammonium formate in water and methanol (35:65, v/v), at a flow rate of 0.8 mL/min and a column temperature of 40 °C. The detection was performed by MS/MS fragmentation, positive ionisation mode, using an atmospheric pressure chemical ionisation source (dry temperature: 400 °C; nebulizer: nitrogen, 60 psi; dry gas nitrogen, 7 L/min, 350 °C); the m/z 269.2 ion obtained from m/z 287 was monitored for 2-OH-E_1 detection, whereas the m/z (199.2+251.3) ions obtained from m/z 287 were monitored for

16α-OH-E_1 detection. Calibration curves were obtained within a concentration range of 0.4-4.4 ng/mL for both of the E_1 metabolites analyzed.

The results are expressed as mean±standard deviation and statistically analyzed by one-way analysis of variance (ANOVA). This was followed by a Fisher's protected least significant difference test as a post hoc test for comparison between the two groups. Statistical analyses were performed using the Origin 4.1 and GraphPad softwares. *P*-values< 0.05 were considered statistically significant.

Chromatographic separation conditions were optimised to obtain the best signal and selectivity. The LC-MS/MS method had a good linearity over the range of concentrations studied (r^2>0.9967). Moreover, this approach is relatively fast and simple; sample preparation requires only a single protein precipitation and centrifugation step and the instrumental method is time efficient, the run time of an assay being 2 min.

The investigations on E_1 metabolism were conducted at high concentrations of substrate, at which the maximum reaction rate is practically equal to the initial rate. Initial formation rates of the 2-OH-E_1 and 16α-OH-E_1 metabolites were calculated for all substrate concentrations tested in the presence and absence of 100 μmol/L G (G100) and 100 μmol/L BPA (B100). The E_1 metabolite levels did not continuously increase with increasing concentrations of substrate, confirming that at high concentrations of E_1, a substrate-mediated inhibition of its metabolizing enzymes occurs, as originally reported by Zhu et al.[10]. Both tested substances showed an inhibitory effect on the oxidative metabolism of E_1. The degrees of inhibition by G100 and B100 were calculated both as averages and individually for each tested E_1 concentration (Table 1). The formation of 2-OH-E_1 was significantly inhibited by both G (46.90%±8.40%) and BPA (57.84%±4.71%), with the greatest inhibition observed when they were applied in combination (71.16%±8.22%; *P*<0.05 *vs.* G100). 16α-OH-E_1 formation was significantly decreased with BPA (48.02%±8.52%) than with G, which had a minor effect on this pathway (8.20%±14.31%; *P*<0.01 *vs.* B100). Although G reduced the inhibitory potential of BPA when applied together (31.94%±21.87%), the difference was not statistically significance.

Table 1. The Inhibition Degree of 100 μmol/L Genistein (G100), 100 μmol/L Bisphenol A (B100), or Their Combination on the Formation Rate of 2-hydroxyestrone (2-OH-E_1) and 16α-hydroxyestrone (16α-OH-E_1) at Various Concentrations of Substrate

E_1 concentration (μmol/L)	Inhibition Degree *vs.* E1		
	E_1+G100	E_1+B100	E_1+G100+B100
For 2-OH-E_1			
100	43.13	59.12	68.66
200	44.11	60.35	71.24
400	40.65	50.35	60.21
600	61.64	62.68	72.61
800	44.96	56.7	83.07
	Mean±SD		
	46.90±8.40	57.84±4.71	71.16±8.22*
For 16α-OH-E_1			
100	−5.94	38.13	1.98
200	11.31	42.87	17.66
400	4.03	45.29	39.04
600	23.39	56.56	44.5
800	−13.06	57.26	56.51
	Mean±SD		
	8.20±14.31	48.02±8.52**	31.94±21.87 **

Note. *n*=3; *P<0.05 *vs.* E_1+G100; **P<0.01 *vs.* E_1+G100.

Figure 1 shows the levels of 2-OH-E_1 and 16α-OH-E_1 in rat liver microsomes after 30 min of incubation at different concentrations of substrate in the presence and absence of the tested inhibitors. The mean values were determined for all E_1 concentrations. The means of the 2-OH-E_1/16α-OH-E_1 ratio calculated in the presence and absence of G100 and/or B100 are shown in Figure 2.

The same isoenzymes of cytochrome P_{450} are involved in the metabolism of E_1 (mainly CYP3A4, CYP3A5, CYP1A2, CYP1A1)[1-2] as well as in the metabolism of G and BPA. This explains the metabolic interactions observed between E_1, G and BPA. G forms 8-hydroxy, 6-hydroxy, and 3'-hydroxilated metabolites in rat and human liver microsomes, with the principally responsible isoenzymes being CYP1A2, CYP1A1, CYP2E1, and CYP1B1[8]. BPA also forms several hydroxylated metabolites in the presence of microsomal cytochrome P450, mainly under the catalytic action of CYP3A4, CYP3A5, and CYP2D6*1. BPA metabolism can generate oxidative stress by BPA-3,4-quinone that is capable of forming covalent bonds with DNA. BPA, the hydroxylated metabolites of BPA and G, and E_1 can then undergo conjugation reactions forming glucuronides and sulfates[7].

The different degrees of inhibitory action of the two E_1 metabolic pathways have caused a reduction

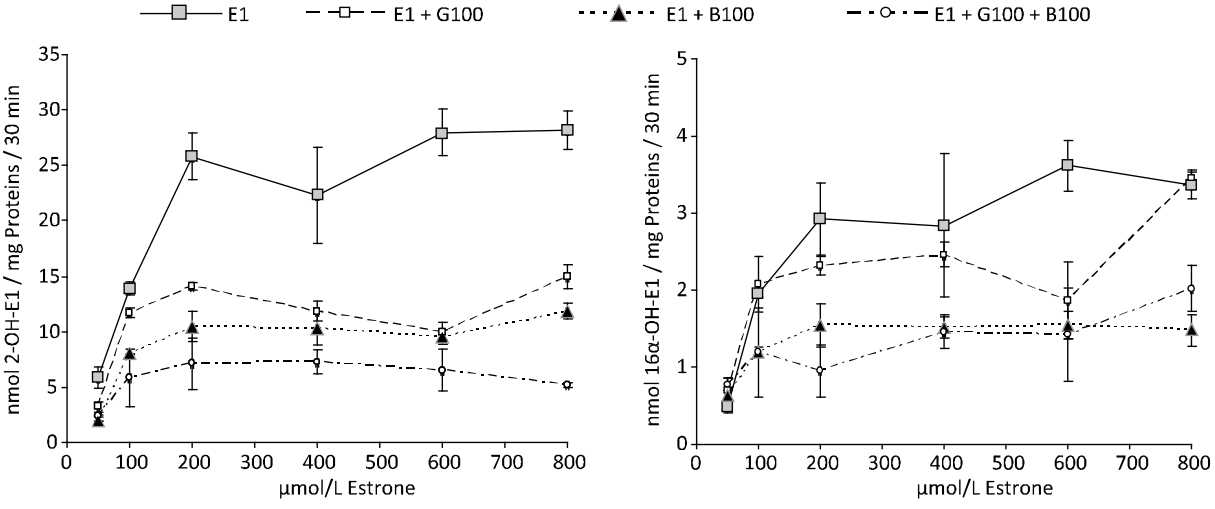

Figure 1. The formation of estrone hydroxylated metabolites in the liver microsomes depending on substrate concentration (after 30 min of incubation) in the presence or absence of 100 µmol/L genistein (G100) and 100 µmol/L bisphenol A (B100) (*n*=3).

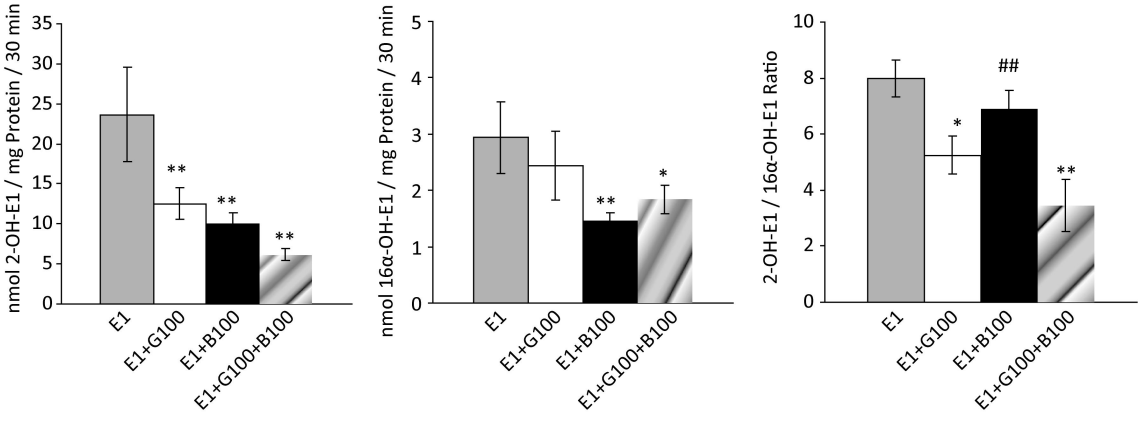

Figure 2. The mean values of the two estrone hydroxylated metabolites, 2-OH-E_1 and 16α-OH-E_1, determined for all estrone concentrations and the means of 2-OH-E_1/16α-OH-E_1 ratio, respectively, calculated in the presence and absence of 100 µmol/L genistein (G100) and 100 µmol/L bisphenol A (B100) after 30 min of incubation ($^{*}P$<0.05 *vs.* E_1; $^{**}P$<0.01 *vs.* E_1; $^{\#\#}P$<0.01 *vs.* E_1+G100+B100).

in the 2-OH-E_1/16α-OH-E_1 ratio, which was minor in the presence of BPA, statistically significant in the presence of G (P<0.05 *vs.* E_1), and still greater in the presence of a combination of G and BPA (P<0.01 *vs.* E_1)(Figure 2), suggesting a synergistic effect of the combination. To the best of our knowledge, this is the first study aimed at identifying a possible interaction between G and BPA on E_1 metabolism in rat liver microsomes. Our findings suggest that the changes induced in E_1 metabolism by BPA and G contribute to the increased risk of breast cancer associated with co-exposure to these endocrine disrupters.

In conclusion, the changes induced by G and/or BPA on the 2-OH-E_1/16α-OH-E_1 ratio, a biomarker for the risk of breast cancer, were studied *in vitro* in pooled rat liver microsomes. G and BPA inhibit E_1 metabolic pathways but in different manners, inducing a reduction of the 2-OH-E_1/16α-OH-E_1 ratio, which was mild in the presence of BPA, statistically significant in the presence of G, and most pronounced when G and BPA were used in combination. Our findings highlight the inhibitory potential of G and BPA on the oxidative metabolism of E_1 and their synergistic effect on the 2-OH-E_1/16α-OH-E_1 ratio.

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