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Stimulation of estradiol glucuronidation: A protective mechanism against estradiol-mediated carcinogenesis?

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17β-Estradiol (E2) and its catechol and methoxy metabolites are believed to play important roles in the mechanism of E2–mediated tumor formation. Because conjugation with glucuronic acid lowers tissue levels by facilitating excretion, we have determined the kinetic parameters of the glucuronidation of E2, estrone (E1), and seven phase I metabolites using human liver microsomes. The catechol estrogens 2- and 4-hydroxy-E2/E1 exhibited the highest clearance, exceeding that of E2, E1, and the methoxy metabolites by factors of 6–44. Homotropic activation kinetics were observed for the 3-glucuronidation of E2 but not for any of the metabolites. None of the metabolites affected the kinetics of the 3-glucuronidation of E2. In contrast, the isoflavone daidzein stimulated the formation of E2-3-glucuronide, as has been reported previously. This heterotropic activation by daidzein appears to be specific for the glucuronidation of E2 because daidzein did not affect the glucuronidation of the 2- and 4-hydroxy metabolites of E2. However, daidzein may lower the glucuronidation of 2-methoxy-E2 *in vivo* due to its preferential glucuronidation. The decreased tissue levels of E2 and increased concentrations of 2-methoxy-E2, as implied by this study and the previous one, may contribute to the protective effect of daidzein against breast and endometrial cancer.

Keywords: Daidzein / Estradiol / Glucuronidation / Oxidative metabolites

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1 Introduction

The endogenous steroid hormone 17β-estradiol (E2) is believed to play a major role in the etiology of breast and endometrial cancer [1, 2]. Although the mechanisms of E2-mediated carcinogenesis are still a matter of debate, there is general consensus that cancer risk increases when tissue levels of E2 are elevated over prolonged periods of time [3, 4]. A major determinant for the tissue level of E2 is its rate of metabolism, which comprises dehydrogenation to estrone (E1) and hydroxylation at various positions of E2 and E1 [5]. Major metabolic routes are the 2- and 4-hydroxylation, which lead to catechol estrogens, and their subsequent methylation (Fig. 1). Some of the hydroxylated and methoxylated metabolites exhibit biological activities and appear to be involved in E2-mediated carcinogenesis: 4-hydroxy-E2 is genotoxic and believed to initiate cancer, whereas 2-methoxy-E2 may have a protective effect [6, 7]. Conjugation of E2 and its oxidative metabolites with sulfate and glucuronic acid, catalyzed by sulfotransferases and uridine diphospho-glucuronosyl transferases (UGTs), gives rise to polar metabolites which lack biological activity and are more readily excreted [5]. However, only glucuronidation leads to a complete inactivation and excretion of E2 and its phase I metabolites, whereas sulfates can be cleaved by intracellular sulfatases and therefore serve as a depot of steroidal estrogens [8].

Due to its two hydroxyl groups, E2 is able to form two different glucuronides. In humans, the glucuronide at position 3 of E2 E2-3-glucuronide (E2-3-G) is specifically formed by UGT1A1 with an atypical enzyme kinetics. UGT1A1 undergoes homotropic activation by E2 [9]. In contrast, the formation of E2-17-glucuronide (E2-17-G) in humans is preferentially catalyzed by UGT2B7 with minor contributions of UGT1A3, 1A4, and 2B15, and follows the classical Michaelis–Menten kinetics [10]. The type of kinetics is important for the intrinsic clearance (CL), which

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Abbreviations: CL, clearance; **E1,** estrone; **E2,** 17β-estradiol; **E2-3-G,** E2-3-glucuronide; **E2-17-G,** E2-17-glucuronide; **HLM,** human liver microsomes; **UGT,** uridine diphospho-glucuronosyl transferase

is calculated from the kinetic parameters determined *in vitro* and which is a measure of the efficiency of the enzyme to handle a certain substrate under *in vivo* conditions where both the substrate and enzyme concentrations are much lower than *in vitro*. For E2, only the formation of the 3-glucuronide represents an inactivating pathway because the 17-glucuronide is biologically active and known to inhibit the bile flow, thereby causing cholestasis [11]. We have recently reported that the soy isoflavone daidzein and several of its phase I metabolites markedly stimulate the 3-glucuronidation in human hepatic microsomes, thereby enhancing the metabolic CL of E2 [12]. This was the first report of the heterotropic stimulation of a UGT by a nonsteroidal and naturally occurring compound, and we hypothesized that an enhanced rate of glucuronidation of E2 may contribute to the putative protective effect of soy isoflavones against hormonal cancer.

The aim of the present study was to elucidate the kinetics of the glucuronidation of major phase I metabolites of E2 in human hepatic microsomes and to disclose whether any of these E2 metabolites is able to stimulate the glucuronidation of E2 in the same manner as daidzein does. Furthermore, the effect of daidzein on the glucuronidation of the catechol metabolites of E2 has been investigated.

2 Materials and methods

2.1 Chemicals and materials

E2, E2-3-G, E2-17-G, E1, the four catechol estrogens 2- and 4-hydroxy-E2 and -E1, 2-methoxy-E2, daidzein, and all other biochemicals and reagents were from Sigma/Aldrich/Fluka (Taufkirchen, Germany), 2-methoxy-E1 from Paesel (Frankfurt, Germany), and 4-methoxy-E1 and 4-methoxy-E2 from Steraloids (Wilton, New Hampshire, USA). Human liver obtained from a 63-year-old White male was a kind gift from Dr. J. Weymann (former Knoll AG, Ludwigshafen, Germany).

2.2 Glucuronidation assay

Human liver microsomes (HLM) were prepared as previously described [12]. In the previous study, the activity of these HLM for the glucuronidation of E2 was found to be in the same range as that of commercial human hepatic microsomes pooled from 15 donors of diverse age and both genders [12]. For the kinetic studies, 0.05–0.1 mg microsomal protein and 25 μg alamethicin were mixed in 50 μL of 0.1 M potassium phosphate buffer pH 7.1 and placed on ice for 15 min. $MgCl_2$ (final concentration 10 mM), the β-glucuronidase inhibitor saccharolactone (5 mM), and the substrate at concentrations ranging from 5 to 200 μM dissolved in DMSO (final DMSO concentration was 1%) were added. After incubating the mixture at 37°C for 5 min, the reaction was started by adding uridine diphosphoglucuronic acid (UDPGA, final concentration 4 mM) to give a final volume of 200 μL, and further incubated for 10–30 min. The assays were terminated by adding 200 μL 0.7 M glycin/HCl buffer pH 1.2. The incubation mixture was extracted with 2 × 800 μL ethylacetate, the combined extract evaporated to dryness, and the residue dissolved in methanol and analyzed by HPLC. The HPLC conditions were the same as recently reported [12].

2.3 Enzyme kinetic analysis

Visual inspection of fitted functions (velocity as a function of substrate concentration) and Eadie–Hofstee plots (velocity as a function of velocity/substrate concentration) was used to select the best-fit enzyme kinetic models. These include the Michaelis–Menten model ($V = V_{max} \times S/K_m + S$), the substrate activation model (Hill equation, $V = V_{max} \times S^n/K_{0.5}^n + S^n$), and the substrate inhibition model ($V = V_{max} \times S/K_m + S \times (1 + S/K_s)$), where n is the Hill coefficient or the degree of sigmoidicity of the curve and K_s is an inhibition constant. For the Michaelis–Menten and substrate inhibition models, the estimated values of intrinsic CL (CL_{int}) were calculated based on the equation $CL_{int} = V_{max}/K_m$, whereas for the sigmoid model the maximal CL (CL_{max}) was calculated using the equation $CL_{max} = (V_{max}/K_{0.5}) \times [(n-1)/(n \times (n-1)^{1/n})]$ [13]. Three independent concentration curves were determined for each substrate, and the kinetic parameters were derived by nonlinear curve-fitting using the Origin program from Microcal Software.

3 Results

The glucuronidation of E2 and E1 as well as their catechol metabolites and some of their 2- and 4-methoxy metabolites (Fig. 1 and Table 1) was carried out with HLM, and the glucuronides were separated by HPLC. While E2 gave rise to two glucuronides, identified as E2-3-G and E2-17-G by cochromatography with authentic standards, only one glucuronide was detected with all the oxidative E2 metabolites. For metabolites with more than one hydroxyl group, the exact chemical structure, *i.e.*, the position of glucuronidation, could not be determined due to the lack of reference compounds. In order to obtain the kinetic parameters of the glucuronidation reaction, various substrate concentrations ranging from 5 to 200 μM were used and the rates of glucuronide formation determined. This is exemplified in Fig. 2 for E2 and its catechol metabolites. After selecting the best-fit enzyme kinetic model, the kinetic data were calculated using the appropriate equations (see Section 2). The results

Table 1. Kinetic parameters for the glucuronidation of E2 and its metabolites by HLM

Substrate	Kinetic model	V_{max} (pmol · min^{-1}mg^{-1})	K_m ($K_{0.5}$) (μM)	n	K_s	Clearance (μL · min^{-1}mg^{-1})
E2 (3-G)	A	1100	29	1.7	–	20 ± 4.7
E2 (17-G)	MM	180	11	1	–	17 ± 3.5
E1	I	170	18	1	120	8 ± 1.0
2-HO-E2	I	5150	20	1	150	258 ± 13.0
2-HO-E1	I	3400	17	1	95	263 ± 93.5
4-HO-E2	MM	4400	35	1	–	125 ± 6.0
4-HO-E1	MM	23500	29	1	–	882 ± 314.2
2-MeO-E2	MM	6400	195	1	–	33 ± 2.6
2-MeO-E1	MM	1300	76	1	–	17 ± 1.8
4-MeO-E2	I	1150	70	1	29	16 ± 4.0
Daidzein	MM	2200	25	1	–	88[a)]

a) Mean of two independent experiments.
A, activation; MM, Michaelis–Menten; I, inhibition; $K_{0.5}$, apparent K_m, and n, sigmoidicity, for A kinetics; K_s, inhibition constant for I kinetics. Kinetic parameters were determined as described in 2.3 and are the mean values of three independent experiments. CL was calculated for each kinetic experiment and is given as mean ± SD.

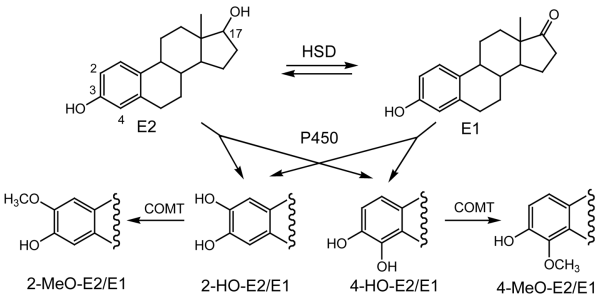

Figure 1. Catechol metabolites of E2 and E1 and their methylation products. HSD, 17–hydroxysteroid dehydrogenase; P450, cytochrome P450; COMT, catechol-*O*-methyltransferase; MeO, methoxy.

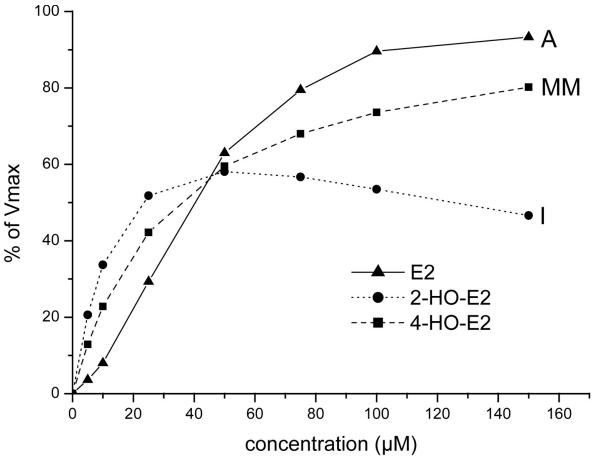

Figure 2. Glucuronidation kinetics of E2 and its catechol metabolites. A, activation; MM, Michaelis–Menten; I, inhibition; HO, hydroxy.

obtained with E2 and its oxidative metabolites are listed in Table 1. The most important kinetic parameter is the CL, which is a measure for the metabolic glucuronidation and

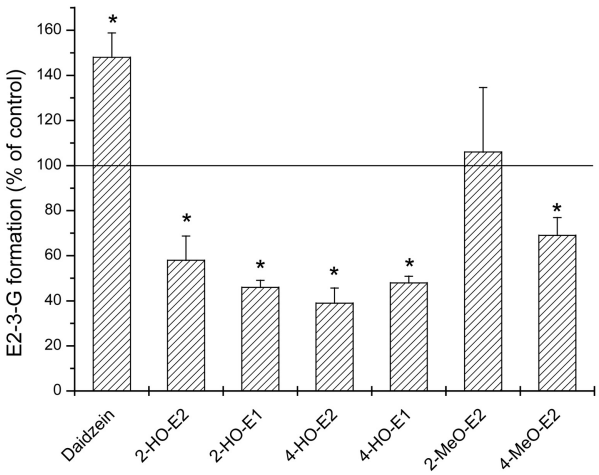

Figure 3. Effect of various metabolites of E2 and E1 and of daidzein on the formation of the 3–glucuronide of E2 in HLM. Concentration of E2 and of the other compounds was 25 μM. Data represent the mean ± SD of three independent experiments. MeO, methoxy. *, $p < 0.001$ (Student's *t*-test).

therefore inactivation without saturation of the enzyme, and which allows to predict the *in vivo* CL of the respective compound [13]. It is obvious from Table 1 that the CL of the methoxy metabolites is about the same as that of E2, with the exception of 4-methoxy-E1, for which no glucuronide formation was observed under our experimental conditions. E1 itself was a poorer substrate for glucuronidation than E2. In contrast, all the catechol estrogens proved to be excellent substrates for glucuronidation by HLM, their CL exceeding that of E2 by a factor of at least 6 and up to 44 in the case of 4-hydroxy-E1 (Table 1). Of all the compounds studied, only the 3-glucuronidation of E2 exhibited activation kinetics, whereas the E2 metabolites followed either Michaelis–Menten or inhibition kinetics (Table 1).

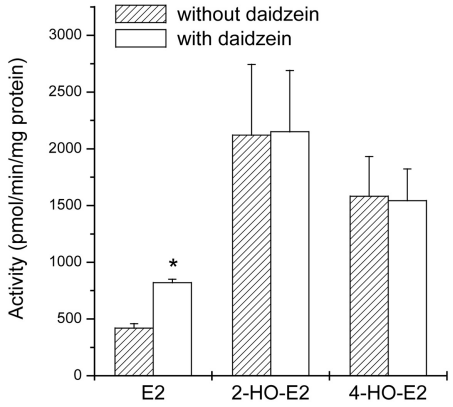

Figure 4. Effect of daidzein on the 3–glucuronidation of E2 and of the catechol metabolites of E2 in HLM. Concentration of each compound was 25 μM. Data represent the mean ± SD of three independent experiments. *, $p < 0.001$ (Student's *t*-test).

In order to clarify whether the E2 metabolites are able to modulate the 3-glucuronidation of E2, the formation of E2-3-G from E2 in the presence of the various metabolites was determined and expressed as percent of the respective glucuronidation of E2 in the absence of metabolite. Daidzein was included in this experiment because it is known to stimulate the formation of E2-3-G [12]. As depicted in Fig. 3, daidzein but none of the catechol or methoxy metabolites of E2 had a stimulating effect on the 3-glucuronidation of E2. To the contrary, all of the E2 metabolites inhibited this glucuronidation, with the exception of 2-methoxy-E2 which was without effect.

When the glucuronidation of E2 and its 2- and 4-hydroxy metabolite was measured in the absence and in the presence of an equimolar concentration of daidzein, a stimulatory effect of daidzein was noted for the glucuronidation of E2 but not of either of the two catechol metabolites (Fig. 4).

4 Discussion

The tissue level of the endogenous estrogen E2 is considered an important determinant for the development and progression of breast and endometrial cancer [1, 2]. In addition to the rate of formation, the rate of inactivation by conjugation and subsequent excretion determine the tissue and plasma concentrations of E2. As sulfate conjugates represent a form of storage from which E2 can be released by intracellular hydrolysis, only the glucuronides of E2 are considered terminal cellular excretion products [8]. In 2000, Fisher *et al.* [9] reported that the 3-glucuronidation of E2, which is specifically mediated by UGT1A1 in the human liver, is stimulated by E2 following an autoactivation enzyme kinetics described by the Hill equation. Our laboratory has recently disclosed that the isoflavone daidzein and several of its phase I metabolites stimulate the formation of E2-3-G in HLM and recombinant UGT1A1 *in vitro* [12]. We have hypothesized that this stimulation, if it occurs in the cells of estrogen target tissues, should lower the intracellular concentration of E2 and may thus contribute to the putative protective effects of soy isoflavones against breast and endometrial cancer.

In addition to the tissue level of E2 itself, which is important for cell proliferation in estrogen target tissues, certain E2 metabolites appear to play important roles in E2-mediated carcinogenesis. Currently, much attention is focused on the genotoxic metabolite 4-hydroxy-E2 as well as the "protective" metabolite 2-methoxy-E2 [6, 7]. In our present study, we have determined the kinetic parameters of the glucuronidation of E2 metabolites using microsomes from human liver. We noted a high regioselectivity of the glucuronidation of the catechol estrogens 2- and 4-hydroxy-E1/E2 and their methylated metabolites, as only one glucuronide was obtained with each of these substrates. We also observed that 2- and 4-hydroxy-E1/E2 exhibited by far the highest CL, exceeding that of E2 and the methoxy metabolites by a factor of at least 4 and up to 44 (Table 1). With HLM, homotropic activation kinetics were only observed for the 3-glucuronidation of E2 but not for any of the metabolites (Table 1). Lepine *et al.* [14] have recently determined the kinetic parameters of the glucuronidation of various phase I metabolites of E2 and E1 with 16 recombinant UGTs, including 1A1, 1A3, 1A9, and 2B7 which are expressed in liver. In contrast to our findings, sigmoid profiles indicative of homotropic activation were observed not only for the 3-glucuronidation of E2 but also for 2-hydroxy-E1 and 4-hydroxy-E1 by UGT1A1 [14]. 2-Hydroxy-E1 was shown to be preferentially glucuronidated by 1A1 but also to some extent by 1A3 and 1A9, whereas the most active UGT for 4-hydroxy-E1 was 2B7. The presence of several UGTs with different kinetics in HLM should result in a "mixed" kinetic profile depending on the amounts and apparent K_ms of the individual isoenzymes. This may explain why we did not observe homotropic activation of the catechol metabolites of E1 with HLM. Likewise, the pattern of UGTs in other tissues should affect the kinetics of E2 metabolites and thus determine their tissue concentrations.

One of the major goals of this study was to disclose whether any of the phase I metabolites of E2 modulates the 3-glucuronidation of E2. As shown in Fig. 3, none of the E2 metabolites had an activating effect. On the contrary, all of them, except 2-methoxy-E2, significantly inhibited the formation of E2-3-G. The most pronounced inhibitory effects were noted for the four catechol estrogens. However, as these are excellent substrates for glucuronidation and also subjected to methylation by catechol-*O*-methyltransferase (COMT) in intact cells, their intracellular concentrations must be

expected to be low. Another E2 metabolite implicated in the mechanism of E2-mediated carcinogenesis is 16α-hydroxy-E1, which should also be studied with respect to its glucuronidation.

Finally, it was of interest to see whether daidzein, which is known to stimulate the 3-glucuronidation of E2 [12], is able to modulate the glucuronidation of the E2 metabolites, in particular of the potentially dangerous catechol estrogens. However, no effect of daidzein was observed on the glucuronidation of 2- and 4-hydroxy-E2 (Fig. 4), indicating that the heterotropic activation of UGT1A1 by daidzein is specific for the glucuronidation of E2.

In conclusion, our study indicates that the phase I metabolites of E2 do not stimulate their own glucuronidation nor that of E2 in human liver, whereas daidzein stimulates the 3-glucuronidation of E2 but not of the catechol estrogens. However, due to its preferential glucuronidation over 2-methoxy-E2, daidzein must be expected to lower the glucuronidation of this protective metabolite *in vivo*. The decreased cellular and tissue levels of E2 and increased concentration of 2-methoxy-E2, as suggested from this study and the previous one [12], may contribute to the protective effect of daidzein against E2-mediated tumor formation.

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