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A methoxyflavonoid, chrysoeriol, selectively inhibits the formation of a carcinogenic estrogen metabolite in MCF-7 breast cancer cells

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ABSTRACT

A 17β-estradiol (E_2) is hydrolyzed to 2-hydroxy-E_2 (2-OHE_2) and 4-hydroxy-E_2 (4-OHE_2) *via* cytochrome P450 (CYP) 1A1 and 1B1, respectively. In estrogen target tissues including the mammary gland, ovaries, and uterus, CYP1B1 is highly expressed, and 4-OHE_2 is predominantly formed in cancerous tissues. In this study, we investigated the inhibitory effects of chrysoeriol (luteorin-3′-methoxy ether), which is a natural methoxyflavonoid, against activity of CYP1A1 and 1B1 using *in vitro* and cultured cell techniques. Chrysoeriol selectively inhibited human recombinant CYP1B1-mediated 7-ethoxyresorufin-*O*-deethylation (EROD) activity 5-fold more than that of CYP1A1-mediated activity in a competitive manner. Additionally, chrysoeriol inhibited E_2 hydroxylation was catalyzed by CYP1B1, but not by CYP1A1. Methylation of 4-OHE_2, which is thought to be a detoxification process, was not affected by the presence of chrysoeriol. In human breast cancer MCF-7 cells, chrysoeriol did not affect the gene expression of CYP1A1 and 1B1, but significantly inhibited the formation of 4-methoxy E_2 without any effects on the formation of 2-methoxy E_2. In conclusion, we present the first report to show that chrysoeriol is a chemopreventive natural ingredient that can selectively inhibit CYP1B1 activity and prevent the formation of carcinogenic 4-OHE_2 from E_2.

1. Introduction

The prolonged exposure of estrogens, especially in postmenopausal women, is well known to play a role in breast cancer etiology [1]. The conceivable mechanisms that could partially play a part in the mutagenic effects of the endogenous metabolites of estrogens within the catechol estrogens (CEs) pathway are shown in Fig. 1. This pathway is initially promoted *via* cytochrome P450 (CYP) families, especially CYP1A1 and CYP1B1. 17β-Estradiol (E_2), one of the major estrogens, is hydrolyzed for CEs by both CYP1A1 and CYP1B1, and mainly converted to 2-hydroxy-E_2 (2-OHE_2) and 4-hydroxy-E_2 (4-OHE_2), respectively [2,3]. 4-OHE_2 was carcinogenic in the hamster kidney, whereas 2-OHE_2 did not induce any tumors [4]. However, in mouse uterine, both hydroxyl metabolites were carcinogenic, and 4-OHE_2 had a higher tumor incidence compared with 2-OHE_2 in mice [5]. Additionally, ratios of 4-OHE_2/2-OHE_2 formation in neoplastic tissue were higher than that in normal breast tissue [6]. Quinone intermediates derived by oxidation of 4-OHE_2 have been reported to react with purine bases of DNA to form depurinating adducts that generate highly mutagenic apurinic sites, although quinones from 2-OHE_2 produce less harmful and stable DNA adducts [7]. Furthermore, the metabolites of CEs may also generate potentially mutagenic oxygen radicals by metabolic redox cycling or other mechanisms [7]. Several types of indirect DNA damage are caused by estrogen-induced oxidants, such as oxidized DNA bases, DNA strand breakage, and adduct formation by reactive aldehydes derived from lipid hydroperoxides [8,9]. It was found that 4-OHE_2 was capable of causing a loss of heterozygosity at doses as low as 0.007 nM [10,11]. On the other hand, catechol-*O*-methyltransferase (COMT) is involved in methylating CEs such as 2-OHE_2 and 4-OHE_2 (Fig. 1), and decrease their detrimental effects [12]. This background information indicates that inhibition of hydroxylation of E_2 by the CYP family, especially CYP1B1, without affecting the methylation pathway has been postulated to be important for the estrogen related carcinogenesis such as breast cancer.

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Recently, many types of natural compounds have been reported to be chemopreventive ingredients that have selective inhibitory effects against the activity of CYPs. For example, some kinds of flavonoids, which are one of the polyphenol groups that exist abundantly in vegetables, fruits and teas [13], are able to modulate the effects of the CYPs system [14]. The flavonoid acacetin, a methoxyflavonoid, showed an extremely strong inhibitory effect for activation of the CYP enzymes [15]. Interestingly, methoxy flavonoids including acacetin, diosmetin, hesperetin, homoeriodictyol, and 2,3′,4,5′-tetramethoxystilbene have been reported to selectively inhibit the activation of CYP1B1 [15–17], indicating that the methoxy substituent may be important for inhibition of CYP activation, especially for CYP1B1. However, there has been no report about the inhibitory effects on estrogen metabolism activated by CYP1A1 or CYP1B1 in human breast cells or *in vivo*.

In the present study, we investigated the effects of chrysoeriol (luteolin 3′-methoxy ether) on metabolism of E_2 *via* CYP1A1 and CYP1B1, because there has been no report concerning the inhibitory effects of CYPs by chrysoeriol. Additionally, this methoxy flavonoid has been reported to be abundantly available in our daily foods. First, we analyzed effects of chrysoeriol on 7-ethoxyresorufin *O*-deethylation (EROD) activity induced by human recombinant CYP1A1 and CYP1B1. Next, we evaluated the protective effects of chrysoeriol on the formation of individual CEs (2-OHE_2 and 4-OHE_2) from E_2, and also methylation of 4-OHE_2. Finally, these effects were evaluated using the human breast cancer cell line MCF-7. To our knowledge, this is the first report to find that physiological levels of chrysoeriol act as a selective inhibitor for CYP1B1 in human breast cancer cells.

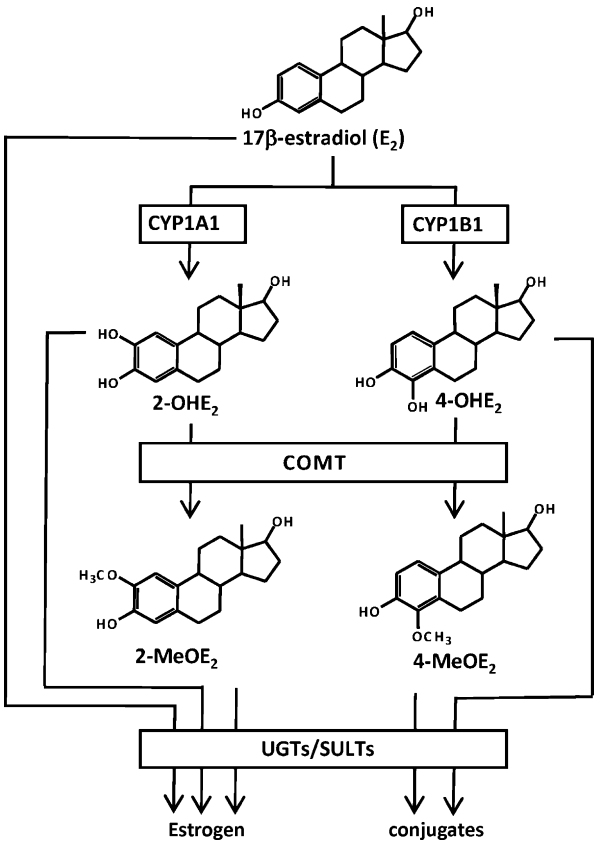

Fig. 1. Scheme of estrogen metabolism in breast cells.

2. Materials and methods

2.1. Chemicals

HPLC grade chrysoeriol was purchased from Extrasynthese (Genay, Cedex, France). Ethoxyresorufin, resorufin, 2-OHE_2 and 4-OHE_2 were obtained from Sigma Chemical Co. (St. Louis, MO). 2-Methoxyestradiol (2-$MeOE_2$) and 4-methoxyestradiol (4-$MeOE_2$) were purchased from Steraids Inc. (Newport, RI). β-NADPH, β-NADH and glucose-6-phosphate (Oriental Yeast Co., Ltd., Osaka, Japan), recombinant human CYP1A1 and CYP1B1 supersomes (Gentest Corporation *via* BD Biosciences, San Jose, CA), human liver cytosol (XenoTech, LLC, Lenexa, KS), *S*-adenosyl-L-methionine (SAM) (New England Biolabs, Inc., Ipswich, MA), and [methyl-^{3}H] SAM (PerkinElmer Life and Analytical Sciences, Boston, MA) were used in this study. All the other chemicals and reagents were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

2.2. 7-Ethoxyresorufin O-deethylation (EROD) enzyme assay

The EROD enzyme assay was employed as a method for evaluation of CYP1A1 and 1B1 activity with slight modifications [18]. The reaction mixture (200 μL), which was composed 4 mM NADPH, 4 mM NADH, 20 mM $MgCl_2$, 0.1 M potassium phosphate buffer (pH 7.4), individual CYP supersomes and various concentrations of chrysoeriol was incubated at 37 °C for 5 min, and then the reaction was initiated by the addition of 40 μL of ethoxyresorufin. After incubation at 37 °C for 10 min, the formation of resorufin was determined fluorometrically (530 nm excitation and 590 nm emission) with a spectrofluorometer (Thermo Fisher Scientific Inc., Worcester, MA). IC_{50} values were determined graphically by plotting percent of control enzyme activity versus inhibitor concentration.

2.3. Enzyme inhibition kinetics

The enzyme kinetics for CYP1B1-catalyzed EROD was measured at increasing concentrations of ethoxyresorufin (0, 0.19, 0.37, 0.75, 1.5, 3.0, and 6.0 μM), or chrysoeriol (0, 25, 50 and 75 nM). The reaction mixtures were preincubated at 37 °C for 5 min, and the reactions were initiated by addition of recombinant microsomes. Incubations were performed in a shaking water bath at 37 °C for 10 min. The formation of resorufin was determined as described above. For the inhibition kinetics studies, V_{max} and K_m values were determined by the nonlinear regression curve fit using the Michaelis–Menten equation by GraphPad Prism 4 (GraphPad Software, Inc., San Diego, CA).

2.4. Hydroxylation of E_2 by recombinant CYP1A1 and 1B1

The CYP1A1 and 1B1-mediated hydroxylation of E_2 was carried out by a modified EROD enzyme assay. Briefly, the reaction mixture, described above but with ethoxyresorufin substituted for 20 μM E_2, was incubated at 37 °C for 40 min, and then immediately extracted with 5 mL of dichloromethane. After centrifugation at 3000 rpm for 10 min, the sodium sulfate was added to the lower fraction, then filtered through an Ekicrodisc (0.2 μm, PTFE, Gelman Sciences, Tokyo, Japan), and finally concentrated to ca. 1 mL by nitrogen gas. After 50 μL of bistrimethylsilyltrifluoroacetamide (BSTFA) was added to the solution, the mixture was kept at room temperature for 60 min, and the solvent was used for the quantitative analysis.

The reactant mixtures were analyzed on a Hewlett-Packard (HP) model 6890 gas chromatograph -HP 5972A mass spectrometry (GC/MS) system (Agilent Technologies, Palo Alto, CA). The GC/MS instrument was equipped with an automated sample introduction system and a splitless injector. A DB-5 capillary column was used (5% phenylmethylpolysiloxane, 60 m × 0.320 mm × 0.25 μm,

J&W Scientific). Helium was used as a carrier gas at a flow rate of 1 mL/min. The oven temperature was kept at 100 °C for 5 min, increased from 100 to 300 °C at a rate of 15 °C/min, and then kept at 300 °C for 20 min. The temperature of the injector and the GC/MS transfer line was kept at 300 °C. The MSD was run in electron impact ionization mode, and the electron energy was 70 eV. Quantifications of the metabolites were performed in selected-ion monitoring (SIM) mode. The following ions were used by the SIM program for qualification and identification of the metabolites (m/z): 432 and 504 for trimethylsilylated 2-OHE_2 (retention time, 23.95 min); 432 and 504 for trimethylsilylated 4-OHE_2 (retention time, 24.59 min); and 340 for Equilin (retention time, 22.42 min) as internal standard.

2.5. Methylation of 4-OHE_2 by human hepatic cytosolic COMT

The catechol-*O*-methyltransferase (COMT)-mediated *O*-methylation of estrogens was carried out as described previously [19]. The reaction was initiated by the addition of human liver cytosolic protein (0.5 mg) in to the reaction mixture consisted with 1.2 mM $MgCl_2$, 250 μM SAM (containing 0.5 μCi [methyl-^{3}H]SAM), 1 mM dithiothreitol, and 10 μM 4-OHE_2 in 125 μL of Tris–HCl buffer (10 mM, pH 7.4) and incubated at 37 °C for 20 min. After the incubation, the reaction mixture was immediately cooled on ice, 500 μL of ice-cold distilled water was added, and extraction with 3 mL of ice-cold *n*-heptane was performed. After centrifugation at 1000 × *g* for 10 min, the organic fractions were dissolved in 5 mL of Ultima Gold scintillation cocktail (PerkinElmer, Japan) and the radioactivity was measured by a liquid scintillation analyzer (LSC-5100; Aloka, Co., Ltd., Tokyo, Japan).

2.6. Cell culture and treatment

Human breast cancer MCF-7 cells kindly provided by Dr. H. Hagenmaier (University of Tuebingen, Germany) were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum, 0.1 mg/mL kanamycin and 0.1 mg/mL ampicillin at 37 °C under 5% CO_2. When cells were 90% confluent in Ø 100 mm dishes, the medium was changed to estrogen free DMEM including 50 or 100 nM chrysoeriol. After 15 min of treatment, 1 μM E_2 was added into the dishes and then incubated again for 24 h. Dimethyl sulfoxide (DMSO; 0.1% of final volume) was used as a vehicle control in the experiment. The media were isolated by centrifugation at 3000 rpm for 10 min and stored at −20 °C. To evaluate gene expression of CYP1A1 and 1B1, sub-confluent cells were incubated with estrogen free DMEM including 100 or 500 nM chrysoeriol for 15 min, and then 10 nM E_2 was added into the dishes. Two micromolar of benzo[*a*]pyrene (BaP) was used as positive control to induce CYP1A1 and 1B1. The cells were collected, and then RNA extraction was immediately carried out using the method described below.

2.7. Deconjugation, extraction and HPLC analysis

To determine total estrogen (unconjugated and conjugated metabolites), 2 mL aliquots from the media were incubated with 1000 U of β-glucuronidase/sulfatase at 37 °C for 17 h after addition of 0.5 mL of 2 M ammonium acetate buffer. The mixtures were then extracted 2 times with 2.5 mL of dichloromethane. The combined dichloromethane extracts were evaporated to dryness and then assayed by a CoulArray HPLC system as described below. Rates of metabolite formation were normalized to cellular protein content, which was determined by the BCA Protein Assay Kit (Pierce, Rockford, IL).

HPLC analysis was carried out according the method reported by Lavigne et al. [20] with some modifications. Briefly, the HPLC system employed in this study was analyzed chromatographically by a CoulArray HPLC system (ESA, Inc., Chelmsford, MA) equipped with 5600A CoulArray detector set at 440 mV. The column, Capcell Pak C18-UG120 column (250 mm × Ø 4.6 mm, S-5, 5 μm, Shiseido Co. Ltd., Tokyo, Japan) was used at 40 °C. Linear gradient elution was performed with solution A (0.1 M ammonium acetate, 15% acetonitrile, 5% methanol) and solution B (0.1 M ammonium acetate, 50% acetonitrile, 20% methanol) delivered at a flow rate of 1.0 mL/min as follows: initially 100% of solution A; for the next 50 min, 10% A; and finally, 10% A for 10 min. The injected volume of the extract was 50 μL.

2.8. Gene expression of CYP1A1, 1B1, and COMT

Total RNA was extracted from MCF-7 cells treated with chrysoeriol using the RNA Protect Cell Reagent (Qiagen Inc., Valencia, CA) and the RNeasy Plus Mini Kit (Qiagen Inc.) according to the protocol included. Following isolation, RNA quantity, purity, and concentration were determined using a Gene Quant pro spectrophotometer (Amersham Biosciences, Foster City, CA). Gene expression of CYP1A1 and 1B1 were measured using quantitative RT-PCR method as described previously [21]. Briefly, the RNA sample (300 ng) was added to 20 μL of reaction mixture containing random hexamers, MuLv Reverse Transcriptase, RNase inhibitors, 25 mM MgCl2, 10 × PCR Buffer II (Applied Biosystems), and 10 mM dNTP mix (Promega Co., Madison, WI). Synthesis of cDNA was performed at 42 °C for 60 min, and the reverse transcription reaction was stopped by heating to 95 °C for 7 min followed by chilling on ice. The cDNA was stored at −20 °C until further use. A total of 2 μL of cDNA was added to the 18 μL of PCR mixture containing 10 μL Taq Man Gene Expression Master Mix (Applied Biosystems), 6 μL distilled water DNase RNase Free (Invitrogen Corp., Carlsbad, CA), 1 μL house-keeping gene solution (glyceraldehyde-3-phosphate dehydrogenase; GAPDH), and 1 μL individual target gene expression reagents: CYP1A1, Assay ID, Hs00153120_m1; CYP1B1, Assay ID, Hs00164383_m1; COMT, Assay ID, Hs00984971_m1. Quantitative RT-PCR was performed on a 7500 Real-Time PCR System (Applied Biosystems). The samples were amplified by incubation for 2 min at 50 °C, then 10 min at 95 °C, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The relative expression level of the target gene product was calculated by the comparative automatic threshold cycles method, using the house-keeping gene, GAPDH, as a calibrator. The relative differences in expression between groups were expressed using cycle time values and the relative differences between groups were expressed as relative increases, setting the control as 100%.

2.9. Statistical analysis

Results were expressed as mean ± standard deviation (SD) for experiments performed at least in triplicate. Statistical significance of differences was evaluated using two-tailed unpaired ANOVA followed by Dunnett's multiple comparison test.

3. Results

3.1. Inhibitory effects of chrysoeriol on EROD activity induced by CYP1A1 or 1B1

The inhibitory effects of chrysoeriol were determined from EROD activities induced by human recombinant CYP1A1 and CYP1B1. As shown in Table 1, chrysoeriol dose-dependently decreased EROD activity induced by each CYP. The individual inhibitory activities (IC_{50} values) of CYP1A1 and 1B1 were 94.7 and 19.7 nM, respectively.

A kinetic study of formation of resorufin from 7-ethoxyresorufin catalyzed by human recombinant CYP1B1 was analyzed in the presence of chrysoeriol (0, 25, 50, and 75 nM) using a Lineweaver–Burk

Table 1
Effects of chrysoeriol on human recombinant CYP1A1- and CYP1B1-mediated EROD activity.

Chrysoeriol (nM)	CYP1A1	CYP1B1
0	100.0 ± 7.9	100.0 ± 3.7
10	95.0 ± 1.9	66.3 ± 2.8**
40	72.2 ± 3.8**	36.2 ± 5.1**
100	41.7 ± 2.2**	17.9 ± 2.9**
IC_{50} values[a]	94.7 ± 5.7	19.7 ± 1.5

Microsomes containing individual CYP1A1 and 1B1 were incubated with increasing concentrations of chrysoeriol for 5 min, and then the reaction was started with the ethoxyresorufin substrate. The appearance of resorufin was measured over 10 min by a fluorescence plate reader (530 nm excitation and 590 nm emission) at 37 °C. The data was indicated as a percentage compared with controls that were treated with vehicle solvent but without chrysoeriol (mean ± SD, $n = 3$).

[a] Each IC_{50} value was calculated by plotting the suppression of EROD activity against the dose, indicating that the amount (nM) required for 50% suppression of EROD activity.

** Significant differences *vs.* control ($p < 0.01$) by ANOVA and Dunnett's multiple comparison test.

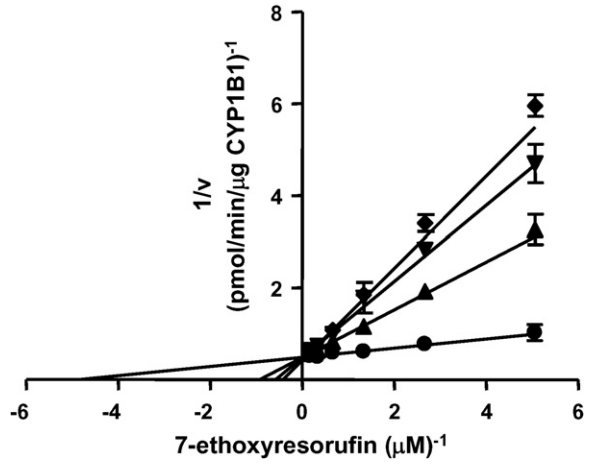

Fig. 2. Lineweaver–Burk plots showing inhibition kinetics of CYP1B1-catalyzed EROD activity by the presence of chrysoeriol. Human recombinant CYP1B1 was incubated with 7-ethoxyresorufin in the absence (●), or presence or various concentrations of chrysoeriol 25 nM (▲), 50 nM (▼), 75 nM (♦) at increasing concentration of 7-ethoxyresorufin from 0 to 6 μM. Resorufin formation was determined fluorometrically.

plot (Fig. 2). The V_{max} and K_i values were estimated at 2.08 pmol/μg protein/min and 8.3 nM, respectively. The plot also showed that chrysoeriol acts as a competitive inhibitor against CYP1B1-catalyzed conversion from 7-ethoxyresorufin to resorufin.

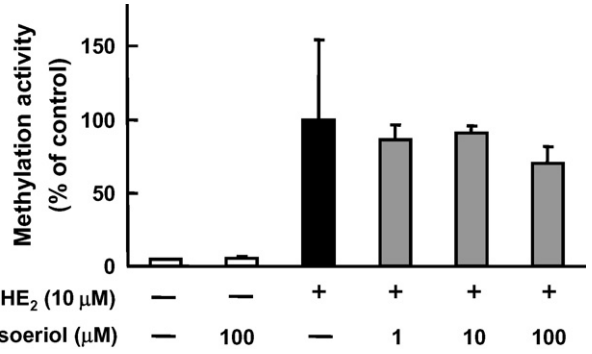

Fig. 4. Effects of chrysoeriol on the reaction of human hepatic COMT-mediated *O*-methylation of 4-OHE_2. The incubation mixture consisted of 10 μM 4-OHE_2, 250 μM SAM (containing 0.5 μCi [*methyl*-^{3}H]), 0.5 mg/mL human cytosolic protein, individual amounts of chrysoeriol, 1 mM dithiothreitol, and 1.2 mM $MgCl_2$ in a final volume of 1.25 mL adjusted using Tris–HCl buffer (10 mM, pH 7.4). Incubations were carried out at 37 °C for 10 min. Data are presented as the mean ± SD ($n = 3$). The resulting methylated estrogens were measured as radioactivity content using a liquid scintillation analyzer.

3.2. *In vitro effects of chrysoeriol on hydroxylation of E_2 by CYP1A1 and 1B1*

There was no inhibitory effect of chrysoeriol to the formation of 2-OHE_2 by CYP1A1 up to 100 nM (Fig. 3A), whereas chrysoeriol significantly inhibited the formation of 4-OHE_2 catalyzed by CYP1B1 at 50 and 100 nM (Fig. 3B).

3.3. *Effects of chrysoeriol on methylation of 4-OHE_2 by human hepatic cytosolic COMT*

The effects of chrysoeriol on *O*-methylation of 4-OHE_2 were evaluated using human hepatic cytosolic COMT, which catalyzes an inactivation pathway for CE as shown in Fig. 1. The incubation of 4-OHE_2 with COMT increased methylation activity, while addition of 1, 10, and 100 μM chrysoeriol indicated no significant inhibitory effect against this methylation activity (Fig. 4).

3.4. *Effects of chrysoeriol on E_2 hydroxylation and gene expression of CYP1A1, 1B1, and COMT in MCF-7 cells*

E_2 introduced in human breast cancer MCF-7 cells could be hydrolyzed to 2-OHE_2 and 4-OHE_2 by endogenous CYP1A1 and CYP1B1, respectively, as shown in Fig. 1. Following such metabolic processes, *O*-methylation, glucuronidation and sulfation for these 2-OHE_2 and 4-OHE_2 occurred in the cells. Indeed, when human breast cancer MCF-7 cells were exposed to 1 μM E_2 for 24 h, both 2-$MeOE_2$ and 4-$MeOE_2$ were observed in the medium after treat-

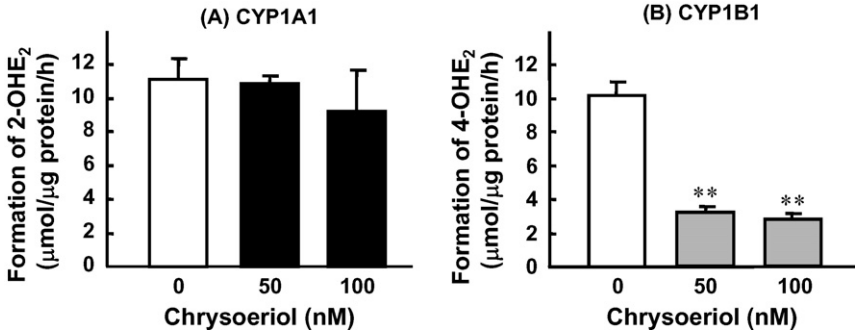

Fig. 3. Effects of chrysoeriol on catechol estrogen formation catalyzed by recombinant human CYP1A1 or CYP1B1. E_2 (20 μM) was incubated with recombinant human CYP1A1 or CYP1B1 microsomes in the presence or absence of chrysoeriol at 37 °C for 30 min, and the resulting catechol estrogens were detected by GC/MS analysis. Data are presented as the mean ± SD ($n = 3$). Static analysis for single comparison was performed using Dunnett's test (**$P < 0.01$ *vs.* control).

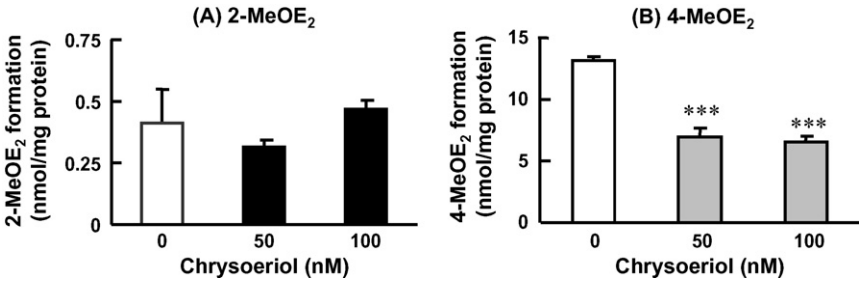

Fig. 5. Effects of chrysoeriol on estrogen metabolites in MCF-7 cells. Cells were treated with chrysoeriol for 24 h. Media was incubated with β-glucuronidase/sulfatase for 12 h, and then extracted according to the method described in Materials and methods. Concentrations of 2-$MeOE_2$ and 4-$MeOE_2$ were determined using a CoulArray HPLC system. Each preparation of these metabolites was normalized to the cellular protein concentration. Data presented as the mean ± SD ($n = 3$). Statistical analysis for single comparisons was performed using Dunnett's test (***$P < 0.001$ *vs.* vehicle control).

ment with β-glucuronidase/sulfatase (Fig. 5). Furthermore, the cells were co-treated with chrysoeriol and E_2, which resulted in the formation of 4-$MeOE_2$ at concentrations of 50 and 100 nM, the effects of chrysoeriol treatment alone was significantly inhibited (Fig. 5B), but not for 2-$MeOE_2$ (Fig. 5A). The effects of chrysoeriol on constitutive CYP1A1, CYP1B1 and COMT mRNA expression were investigated in MCF-7 cells using quantitative RT-PCR analysis. BaP significantly increased the gene expression of CYP1A1 and CYP1B1 (Fig. 6A and B) in a similar manner to our previous data [21]. On the other hand, chrysoeriol (100 and 500 nM) in the presence of E_2 did not affect gene expression of either CYP. Moreover, chrysoeriol showed no effect on the genetic expression of COMT (Fig. 6C).

4. Discussion

Endogenous and/or exogenous estrogens are metabolized by CYP oxidation, glucuronidation by UDP-glucuronosyl transferase, sulfation by sulfotransferase, and *O*-methylation by COMT under physiological conditions (Fig. 1). Finally, estrogens are eliminated from the body as urine and/or feces after these metabolic transformations to estrogenically inactive metabolites. Hydroxylation, which is the first step in the metabolism of estrogens, is initiated by CYP enzymes. The hydroxides of estrogen were mainly found in liver. In liver, approximately 80% of E_2 is biotransformed to 2-OHE_2 and 20% to 4-OHE_2 [2,3,22]. On the other hand, in human cancerous breast tissues and cells, CYP1B1 is highly expressed, and therefore, formation of 4-OHE_2 was more dominant compared with 2-OHE_2 [6,23,24]. As we stated in the Introduction, 2-OHE_2 and 4-OHE_2 are carcinogenic, and especially, 4-OHE_2 has a higher tumor incidence compared with 2-OHE_2 [4,5]. Additionally, 4-OHE_2 generates free radicals from the reductive-oxidative cycling with the corresponding semiquinone and quinone forms, which cause cellular damage [7–9,25,26]. On the other hand, 2-OHE_2 is methylated by COMT, followed by transformation into an inactive form, 2-$MeOE_2$, at a faster rate than 4-OHE_2 [27]. Also, 2-$MeOE_2$ has an inhibitory effect on cell proliferation [28], indicating that CYP1A1 is important for detoxification of estrogen. Therefore, the compounds, which selectively inhibit CYP1B1 but not CYP1A1, would be considered to be useful for the chemoprevention of estrogen related carcinogenesis, as typified by breast cancer.

Several kinds of flavonoids, which exist widely in our foods, vegetables, fruits and teas [13], have been reported to affect CYP enzymes [14]. Among the flavonoids, myricetin, apigenin, kaempferol, quercetin, amentoflavone, quercitrin and rutin were slightly more selective for a CYP1B1 EROD inhibitor compared with CYP1A1 [18]. Another study has indicated that isorhamnetin was the most potent CYP1B1 inhibitor compared with major flavonoids, quercetin and kaempferol [29]. In contrast, flavonol glucoside, quercetin-3-*O*-rutinoside, kaempferol-3-*O*-rutinoside, and isorhamnetin-3-*O*-rutinoside did not inhibit CYP1 enzymes [29]. Interestingly, methoxy flavonoids, acacetin, diosmetin, hesperetin, homoeriodictyol, and 2,3′,4,5′-tetramethoxystilbene are more selective inhibitors for CYP1B1 than CYP1A1 [15–17], indicating that the methoxy substituents may be important for selective inhibition of CYP1B1.

Chrysoeriol (luteolin-3′-methoxy ether), found in Rooibos tea, celery, Chinese celery, celery seed, and leaves of *Digitalis purpurea* [30–33], is a bioactive flavonoid known for antioxidant, anti-inflammatory [30], bronchodilatory [31], antimutagenic [33], free radical scavenging activities [34], and the inhibition of 17β-hydroxysteroid dehydrogenase type 1 [35]. As described in the Introduction, inhibitory effects of methoxy flavonoids on estrogen metabolism are activated by CYP1A1 and CYP1B1 in breast cells in spite of many reports on their inhibitory effect against CYP 1 enzyme activity. Therefore, in the present study, we focused on chrysoeriol and investigated its effects on the metabolism of E_2 *via* CYP1A1 and CYP1B1 in *in vitro* enzyme and cell systems.

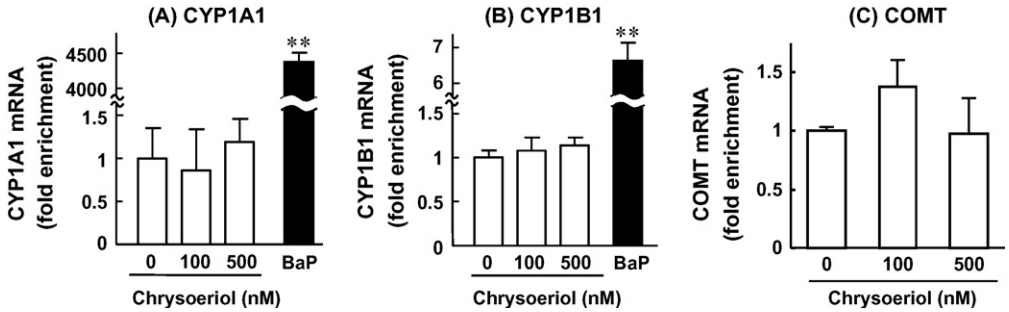

Fig. 6. Effects of chrysoeriol on CYP1A, 1B1, and COMT mRNA expression in MCF-7 cells. Cells were treated with 100 or 500 nM chrysoeriol with 10 nM E_2 for 12 h. Benzo[*a*]pyrene (BaP) was used as a positive control for the induction of CYP1A1 and 1B1. Levels were measured by quantitative RT-PCR and normalized to those of controls (10 nM E_2). Each column represents the mean of at least three independent experiments ± SD. Statistical analysis for single comparisons was performed using Dunnett's test (***$P < 0.001$ *vs.* vehicle control).

Chrysoeriol inhibited human recombinant CYP1A1- and CYP1B1-mediated EROD activity with an IC_{50} value of 94.7 and 19.7 nM, respectively (Table 1), indicating a 5-fold selectivity in its inhibition of CYP1B1 rather than CYP1A1, in a competitive manner (Fig. 2). Chrysoeriol inhibited E_2 hydroxylation catalyzed by CYP1B1 at a lower concentration (Fig. 3B), but not hydroxylation *via* CYP1A1 (Fig. 3A). Our results strongly suggest that chrysoeriol is a selective inhibitor against CYP1B1 activity and prevents the formation of carcinogenic 4-OHE_2 from E_2.

On the other hand, 2-OHE_2 and 4-OHE_2 are rapidly *O*-methylated to form monomethyl ethers catalyzed by COMT as shown in Fig. 1, which is thought to be a detoxification process. COMT is an important enzyme that protects cells from the genotoxicity and cytotoxicity of catechol estrogens, by preventing their conversion to quinones by CYP1. The low activity allele of COMT confers an increased risk for developing breast cancer in certain women [36]. Human COMT has been shown to have a higher catalytic activity toward 2-OHE_2 compared with 4-OHE_2, which may contribute to the comparatively stronger carcinogenicity of 4-OHE_2 [19]. Inhibition of the COMT-mediated *O*-methylation of endogenous 2- and 4-hydroxylated estrogens by these catechol-containing dietary polyphenols is expected to result in an increase in the tissue levels of the procarcinogenic 4-OHE_2 plus a decrease in the tissue levels of the anticarcinogenic 2-$MeOE_2$. Zhu et al. have previously shown that quercetin, fisetin, catechin, epicatechin and (−)-epigallocatechin-3-*O*-gallate (EGCG) are substrates and also strong inhibitors for the *O*-methylation of catechol estrogens [19,37]. However, our results indicated that chrysoeriol, which does not have catechol structure due to a methoxy substitution at the 3′ position in the flavone structure, showed no effect on the COMT-mediated *O*-methylation of 4-OHE_2 until the amounts exceeded 100 μM (Fig. 4).

Furthermore, we found that chrysoeriol significantly inhibited the formation of 4-$MeOE_2$ but not 2-$MeOE_2$ at concentrations under 50 nM in human breast MCF-7 cells (Fig. 5). On the other hand, the same amount of chrysoeriol had no effects on gene expression of CYP1A1, 1B1 or COMT (Fig. 6). Chrysoeriol could not disrupt the AhR/ARNT pathway at such a concentration, and furthermore, it could not inhibit the methylation of 4-OHE_2 as described above. Hence, these results indicate that chrysoeriol can inhibit the enzymatic activity of CYP1B1, and consequently decrease the formation of 4-$MeOE_2$ in the cells.

Total daily intake of dietary flavonoids may be as high as 1 g [38,39]. Unfortunately, the serum concentration of methoxyflavonoids including chrysoeriol in humans is unknown as far as we can tell. On the other hand, some other flavonoids, such as quercetin and genistein, are well known to be absorbed into our bodies and circulate in the blood, although their plasma levels have been recognized less than one micromolar [40,41]. This background suggests that physiological levels of chrysoeriol may be on the order of less than one micromolar. In this study, we showed that chrysoeriol inhibited CYP1B1 activity less than one micromolar. Therefore, the physiological levels of chrysoeriol may accumulate to near effective levels, such as 19.7 nM, to inhibit CYP1B1as described above.

In conclusion, we provide the first report that chrysoeriol is a chemopreventive natural ingredient that can selectively inhibit CYP1B1 and prevent the formation of carcinogenic 4-OHE_2 from E_2 at physiological concentrations using *in vitro* and MCF-7 cell lines. Consequently, chrysoeriol may be a potent chemoprotectant in human mammary carcinogenesis by selective inhibition of the enzymatic activity of CYP1B1. Further studies are required to determine whether chrysoeriol inhibits DNA damage and estrogen related cancer *in vitro* and *in vivo*.

5. Conflict of interest

The authors declare that there are no conflicts of interest.

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