CYP2C9, a Metabolic CYP450s Enzyme, Plays Critical Roles in Activating Ellagic Acid in Human Intestinal Epithelial Cells

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Source of support: This work was supported by Natural Science Foundation of Jiangsu province (Grant No. BK20180678)

Background: The metabolic processing of ellagic acid (EA) by cytochrome P450s (CYP450s) expressed in the intestines is unclear. This study aimed to investigate the effects of CYP450s that are highly expressed in HIEC cells on metabolic activity of EA.

Material/Methods: HIEC cell models expressing 2B6, 2C9, 2D6, and 3A4 were generated by stably transfecting with CYP450 genes using a lentivirus system. PCR and Western blot assay were used to detect expression of CYP450s. Cell Counting Kit-8 (CCK-8) assay was used to examine the cytotoxic effect of EA on CYP450s-expressing HIEC cells. Flow cytometry was employed to evaluate apoptosis of CYP450s-expressing HIEC cells after addition of EA. Metabolic clearance rate of EA in vitro by the constructed HIEC cell models was measured using UPLC-MS method.

Results: CYP450s expression HIEC cell models, including CYP2B6, CYP2C9, CYP2D6, and CYP3A4, were successfully established. EA treatment at different concentrations (10 μg/mL and 50 μg/mL) remarkably decreased cell viability of HIEC cells expressing CYP2C9 compared to the untreated control (p<0.01), in a concentration-dependent manner. Expression of CYP2C9 significantly increased the apoptosis rate of HIEC cells treated with EA compared to that in HIEC cells without any CYP450s expression (p<0.01). The clearance rate of EA in CYP2B6-expressing (p<0.05) and CYP2C9-expressing (p<0.001) HIEC cell models was remarkably reduced after 120 min.

Conclusions: Ellagic acid was effectively activated by CYP2C9 in HIEC cells and caused cytotoxicity and apoptosis of HIEC cells. Therefore, CYP2C9 is main metabolic enzyme of EA when compared to other CYP450 HIEC cell models.

MeSH Keywords: Cytochrome P-450 Enzyme System • Ellagic Acid • Epithelial Cells Metabolic Clearance Rate

Full-text PDF: https://www.medscimonit.com/abstract/index/idArt/923104
Background

Ellagic acid (EA) is a natural polyphenol compound that widely exists in different plants, mainly fruits such as nuts, pomegranate, and strawberry [1,2]. EA is slightly soluble in water and alcohol and is soluble in alkali and pyridine [3]. EA has anti-inflammatory effects on ulcerative colitis by reducing COX-2 and iNOS, blocking the p38 MAPK, NF-xB, and STAT3 signaling pathways [4]. EA also demonstrates anti-tumor effects as it blocks tumor angiogenesis by regulating the VEGFR-2 signal pathway in pathogenesis of breast cancer [5]. Additionally, EA can significantly enhance NK cell activity, therefore killing tumor cells [6]. Giorgio et al. [7] reported that EA inhibits proliferation of prostate cancer and colon cancer cells by metabolizing urostonin. Moreover, the antioxidant activity of EA and its corresponding mechanism may be directly associated with the removal and/or inhibition of active oxygen radicals [8]. Therefore, EA demonstrates extensive and promising clinical applications in future.

The development of Traditional Chinese Medicine in recent years has attracted attention of scholars all over the world. Previous studies [9,10] have focused on the extraction, synthesis, and development of EA, but none has tackled the metabolic activation of EA. Through studying the drug metabolism and activation, we sought to define the process of drug activation and toxicity, which is of great significance in identifying the mechanism of drug metabolism in vivo and ensuring drug safety. Therefore, it is necessary to study the metabolic activation of EA. EA is mainly detected using high-performance liquid chromatography (HPLC)-mass spectrometry (MS) [11–13].

Cytochrome P450 (CYP450) is the main enzyme system involved in drug metabolism in vivo, which can catalyze about 75% of clinical drug metabolism [14]. There are 4 kinds of CYP450 enzymes (CYP3A4, CYP2D6, CYP2C9, and CYP2B6), which are highly expressed in the intestines and participate in metabolism of exogenous chemicals [15,16]. Previous studies mainly focused on the effects of EA on activity of metabolic enzymes [17,18], but the metabolic effects of CYP450 expression on EA in the intestinal tract are unclear. The metabolism of drugs in the body, especially in the intestines, is an important part of the pharmacokinetics (absorption, distribution, metabolism, exception, ADME) [19]. Therefore, in this study, HIEC cell lines with high expression of CYP3A4, CYP2D6, CYP2C9, and CYP2B6 were constructed, and the effect of EA treatment on different HIEC cell models expressing CYP450 enzymes was observed. The most potent CYP450 enzyme that metabolized EA was further screened by culturing and incubating cells in vitro and detecting by UPLC-MS method. Our results provide a theoretical basis for the biotransformation of EA in the intestine.

Material and Methods

Preparation of EA

The EA is natural plant phenol, used as a white crystalline powder in this study. EA (molecular formula: C14H6O6) with purity more than 95% (UPLC-MS) was purchased from Sigma-Aldrich (Cat. No. E2250, Sigma-Aldrich, St. Louis, MO, USA). EA was sealed and stored at 2–8°C in dry conditions. The working solution of EA was prepared before each experiment according to the directions of the manufacturer. Dimethyl sulfoxide (DMSO) was used to dissolve and prepare different EA concentrations and was assigned as the vehicle control. DMSO demonstrated no effects on the viability of HIEC cells.

Synthesis of lentivirus vectors

The lentivirus-expressing plasmids carrying CYP2B6, CYP2C9, CYP2D6, and CYP3A4 expression genes were sub-cloned into pYr-Lvsh vector (MiaoLing Bio. Sci. Tech. Co., Wuhan, China), while pYr-Lvsh-CYP450s were synthesized by Yingrun Tech. Co. (Guangzhou, China). The sequencing results for identification showed that the lentivirus expression system plasmids of CYP450, including pYr-Lvsh-CYP2B6, pYr-Lvsh-2C9, pYr-Lvsh-2D6, and pYr-Lvsh-3A4, were successfully constructed.

Cell lines and culture conditions

The HIEC cells used in this study were purchased from ATCC (Manassas, VA, USA). HIEC cells were cultured in the OPTI-MEM (Gibco BRL. Co., Grand Island, NY, USA), supplemented with 5% fetal bovine serum (FBS, Gibco BRL. Co.) and 1% penicillin-streptomycin (Gibco BRL. Co.) at 37°C and 5% CO₂. HIEC cells were seeded at a density of 1×10⁶ cells/well in 6-well plates (Corning, NY, USA). Wells that contained HIEC cells were assigned as control. The other wells contained different HIEC cell models (H-2B6, H-2C9, H-2D6, and H-3A4). All of the above control HIEC cells and HIEC cell models were then treated with EA for the following experiments.

The 293T cells were cultured at 37°C with 5% CO₂ and then digested with 0.25% trypsin (Beyotime Biotech., Shanghai, China) and then the single-cell suspension (all cells existing as single cells in medium) was prepared.

Construction of CYP450s-expressing HIEC cell models

The HIEC cells (4×10⁵/mL) were seeded in 6-well plates and cultured at 37°C with 5% CO₂, and 293T cells were also cultured at 37°C with 5% CO₂ until reaching 80% confluence. A mixture of pMD2G (0.1 μL, MiaoLing Bio. Sci. Tech. Co.), psPAX (0.1 μL, MiaoLing Bio. Sci. Tech. Co.), pYr-Lvsh-CYP450s (0.1 μL, pYr-Lvsh-CYP2B6, pYr-Lvsh-2C9, pYr-Lvsh-2D6 or
pYr-Lvsh-3A4), and Lipofectamine 2000 Transfection Reagent (0.2 μL, Invitrogen/Life Technologies, Carlsbad, CA, USA) was prepared in OPTI-MEM (Gibco BRL. Co., Grand Island, NY, USA). The mixture was kept at room temperature for 15 min, after which the above mixture was added to the 293T cells and cultured for another 6 h in fresh DMEM (Gibco BRL. Co.) supplemented with 10% fetal bovine serum (FBS, Gibco BRL. Co.). After 48 h, transfection was evaluated with fluorescence microscopy (Model: IX-70, Olympus, Tokyo, Japan). Then, the cell culture medium (containing virus solution) was collected and filtered with a 0.45-μm membrane. When the fusion degree of HIEC cells reached 80%, the collected virus solution was added to HIEC cells (MOI=10.0). HIEC cells were cultured in DMEM at 37°C and 5% CO₂ for 24 h, after which the infection process was repeated. The virus solutions in the supernatant of culture medium were discarded, and the HIEC cells were cultured for another 48 h. The same treatment and incubation conditions were employed for PCR and Western blot assay. Then, puromycin (Sigma-Aldrich, St. Louis, MO, USA) was added for cell screening to obtain the purified and high-CYP450s (CYP2B6, CYP2C9, CYP2D6 and CYP3A4)-expressing HIEC cell models. The expression of CYP450s was identified using PCR assay. The obtained RNA (1 μg) was subsequently used to reversely-synthesize the complementary DNA (cDNA) using the cDNA Reverse-Transcription Kit (Cat. No. 4374967, Invitrogen/Life Technologies). The obtained cDNA was used for amplifying PCR products. The primers for PCR assay are listed in Table 1. PCR assay was conducted using the FTC-3000P Real-time PCR device (Funglyn Biotech., Canada). The obtained PCR products were loaded onto 1.5% agarose gel for electrophoresis, using the method described in a previous study [20]. The agarose gel images were captured using the UVP Gel Image Scanning System Labworks 4.6 (Bio-Rad Laboratories, Hercules, CA, USA).

**Table 1. Description of PCR primers used for PCR assay.**

| Metabolic enzyme genes | Primers | Sequences (5’-3’) |
|------------------------|---------|-------------------|
| CYP2C9                 | PPL01624-4a-Forward | GAATTCGATATCTTGTGGTCTTTG |
|                        | PPL01624-4a-Reverse  | TCAGACAGGATGAGAAGCACAGC |
|                        | PPL01134-4a-Forward  | GAATTCCGGCCATGGACGCTTG |
|                        | PPL01134-4a-Reverse  | CTAGCGGGGCACAGCACAAA |
| CYP2D6                 | PPL01622-4a-Forward  | GAATTCGACTCATCCGCTCCC |
|                        | PPL01622-4a-Reverse  | TCACGGGGGCACAGAACG |
| CYP2B6                 | PPL01630-4a-Forward  | GAATTCGCTCATCCGACACTT |
|                        | PPL01630-4a-Reverse  | TCAGGGTCACCTACGGTTG |

Determination of CYP450s expression in HIEC cell models by Western blot

The expression of CYP450s was also examined using Western blot assay. HIEC cells were washed 3 times with PBS (Beyotime Biotech.), lysed with Tris lysate (Beyotime Biotech.) for 10 min, and then centrifuged at 10 000 rpm for 10 min to obtain the protein. The concentration of protein was measured with BCA protein assay kit (Cat. No. P0011, Beyotime Biotech). The protein was loaded to SDS-PAGE gel and transferred onto PVDF membranes (AB, Piscataway, NJ, USA). PVDF membranes were incubated with rabbit anti-human 3A4 antibody (Cat. No. AB1254, EMD Millipore Corporation, Temecula, CA, USA), rabbit anti-human 2D6 antibody (Cat. No. AB10081, Sigma-Aldrich, St. Louis, Missouri, USA), rabbit anti-human 2C9 (Cat. No. AP1181, Sigma-Aldrich), rabbit anti-human 2B6 (Cat. No. SAB4500588, Sigma-Aldrich), and rabbit anti-human GAPDH antibody (Cat. No. ABS156, Sigma-Aldrich) at 4°C overnight. Subsequently, PVDF membranes were incubated with HRP-conjugated goat anti-rabbit IgG (Cat. No. 074-1506, KPL, Gaithersburg, MD, USA) at room temperature for 2 h. The Western blot bands were imaged using electrochemiluminescence (ECL, Amersham Biosciences, Piscataway, NJ, USA) and the images were analyzed with UVP Gel Image Scanning System Labworks 4.6 (Bio-Rad Laboratories, Hercules, CA, USA).

The relative expression of CYP450s by the different HIEC cell models was represented as the ratio of Western blot band grey density CYP450s normalized to grey density of GAPDH protein.
HIEC cells at logarithmic growth stage were seeded into the 96-well plates (Corning, NY, USA), adjusted to 1×10^4 cells/mL, and cultured at 37°C and 5% CO₂ for 24 h. Cells of the different HIEC models were treated with different concentrations of EA (0 μg/mL, 2 μg/mL, 10 μg/mL, 50 μg/mL). After 24 h of HIEC culture, 20 μL CCK-8 was added into each well and cultured for another 2 h. The optical density (OD) value of each well was measured at a wavelength of 450 nm with a microplate reader (Model: 680, Bio-Rad Laboratories, Hercules, CA, USA). Cell viability was represented as the ratio of OD_{450 nm} obtained in different experimental wells containing different concentrations of EA (2 μg/mL, 10 μg/mL, 50 μg/mL) to that obtained in control wells (0 μg/mL).

### Determination of apoptotic flow cytometry

The apoptosis of HIEC cells was evaluated using the Annexin V-APC/7-ADD staining kit (Cat No. KGA1026, KeyGen Biotech. Co., Nanjing, China) according to the instructions of the manufacturer. The HIEC cells were digested, collected, and washed with ice-cold PBS. Supernatants were discarded and then HIEC cells were re-suspended using 200 μL binding buffer. Subsequently, total of Annexin V-APC (5 μL) and 7-ADD (1 μL) were added into the HIEC cells and incubated for 15 min in the dark at room temperature. HIEC cells were incubated using binding buffer for the Annexin V-APC and were analyzed using an FCAS Calibur flow cytometer (BD Biosciences, San Jose, CA, USA) to measure the fluorescence emitted at the wavelengths of 530 nm and 488 nm. The "percentage of apoptotic cells (%)" was represented as the percentage of "early apoptotic cells (%)" plus "late apoptotic cells (%)".

### Determination of in vitro metabolism and clearance rate of EA by UPLC-MS

We recorded the metabolism level at 0 min and 120 min after administrations in each HIEC cell model. The constructed HIEC cells were designated as H-2B6, H-2C9, H-2D6, and H-3A4 groups expressing CYP2B6, CYP2C9, CYP2D6, and CYP3A4, respectively. The control HIEC wells contained HIEC cells treated with PBS. The reaction system (total volume of 100 μL) contained the following: EA (final concentration 1 mg/mL, Gibco BRL. Co. Ltd.), CYP450s (Sigma-Aldrich), NADPH (final concentration 1 mg/mM Sigma-Aldrich), and EDTA (final concentration 1 mM, Sigma-Aldrich). Then, the HIEC cells were incubated with the above reaction system and cultured at 37°C for 120 min, then the reaction was directly terminated using methanol (Beyotime Biotech.). The content of wells containing HIEC cell models and control HIEC cells were centrifuged at 15 000×g at 4°C for 10 min to harvest the supernatant.

### Statistical analysis

All data in this study were analyzed using SPSS software 20.0 (SPSS Inc., Chicago, IL, USA). The measurement data were recorded as mean±standard deviation (SD). The Tukey’s post hoc test-validated ANOVA was used to compare the differences among HIEC cell models from at least 6 trials. p<0.05 was regarded as statistically significant.

### Results

**PCR analysis demonstrated mRNA expression of HIEC-CYP450 cell lines**

The PCR findings showed that a 1.5 kb target band was amplified in H-2B6, H-2C9, H2D6, and H-3A4 HIEC cell models, but without a corresponding band in the control HIEC model (Figure 1). The results indicate that mRNA (2B6, 2C9, 2D6, and 3A4) was highly expressed in H-2B6, H-2C9, H2D6, and H-3A4 HIEC cell models (Figure 1). Therefore, HIEC-CYP450 cell lines were successfully constructed.

**Western blot analysis demonstrated protein expression by HIEC-CYP450 cell lines**

In this study, we also used Western blot assay to verify the 4 kinds of HIEC-CYPs (Figure 2A). The results showed that there was no CYP450 expression in the control HIEC model. Compared to control HIEC, CYP2B6, CYP2C9, CYP2D6, and CYP3A4 protein in the HIEC cell models were expressed at significantly higher levels (Figure 2B, p<0.001).
Metabolism of EA by CYP2C9 in H-2C9 model cytotoxicity

Cytotoxicity of HIEC cells upon administration of different concentrations of EA was evaluated with the CCK-8 assay. Our results showed that high concentrations of EA (10 μg/mL and 50 μg/mL) caused no remarkable cytotoxicity in H-2B6, H-2D6, and 3A4 HIEC cell models (Figure 3A, all p >0.05). However, the administration of EA at the same concentrations as mentioned above remarkably decreased the cell viability of CYP2C9-expressing HIEC cells when compared to control HIEC (Figure 3A, p<0.01). At the same time, the cytotoxic effect of EA on HIEC cells expressing CYP2C9 was in a concentration-dependent manner (Figure 3A, p<0.05). Therefore, CYP2C9 expression can induce the cytotoxicity of HIEC cells. Moreover, EA treatment (10 μg/mL) also significantly decreased the cytotoxicity of H-2C9 compared to the HIEC cells without any treatment, after 24 h, 48 h, and 72 h (Figure 3B, p<0.01). Meanwhile, cytotoxicity of EA to CYP2C9-expressed HIEC cells occurred in a time-dependent manner (Figure 3B, p<0.05).

CYP2C9 induced apoptosis of EA-treated HIEC cells

The apoptosis of different HIEC cell models was determined by flow cytometry assay (Figure 4A). The results indicated that the treatment of different HIEC cell models with EA had no effect on the apoptosis rate of those expressing H-2B, H-2D6, and 3A4 when compared to control HIEC cells (Figure 4B, p>0.05). However, the expression of H-2C9 significantly increased the apoptosis rate of EA-treated HIEC cells when compared to control HIEC (Figure 4B, p<0.01).

CYP2C9 exhibited a higher clearance rate of EA

UPLC-MS findings showed that the peak of EA was obvious and with no interfering peaks; therefore, the clearance rate was analyzed accurately. The clearance rate for the EA administration in CYP450s enzymes-expressed HIEC cell models was evaluated. The data indicated that the clearance rate of EA in CYP2B6-expressing (p<0.05) and CYP2C9-expressing (p<0.001) HIEC cell models was remarkably reduced after 120 min (Figure 5). However, there were no significant differences in clearance rates of EA in the CYP2D6-expressed and CYP3A4-expressed HIEC cells at 120 min after treatment compared to those at 0 min (Figure 5, p>0.05).

Figure 2. Identification of CYP450 proteins by Western blot. (A) Western blot images. (B) Relative expression of CYP450s in different HIEC cell models. *** p<0.001.

Figure 3. Effect of different concentrations of EA on the cell viability of different HIEC cell models, as determined by CCK-8 assay. (A) Effect of different concentrations of EA on the cell viability of different HIEC cell models, as determined by CCK-8 assay. (B) Effect of time on the cell viability of HIEC cell models treated with 10 μg/mL EA. ** p<0.01.
Discussion

In recent years, many studies reported that EA inhibits colon cancer [22], liver cancer [23], lymphoid cancer [24], and breast cancer [25]. EA has good pharmacological effects and a wide range of biological effects. However, several shortcomings, such as different metabolic forms, lower bioavailability in vivo, and unclear metabolic effects have been reported [10]. Therefore, long-term research is needed before clinical use of EA. EA is generally considered to be non-toxic, but it has also been reported that EA has cytotoxic effects [26], which may be caused by toxic damage to cell DNA; however, it may ignore the cytotoxic effect caused by metabolic activation of EA in vivo levels. Most of the drug studies [27,28] are carried out on human colon cancer cells and experimental animals, and there has been little research using HIEC cells. HIEC cells retain good tissue characteristics, making it a good cell model; therefore, HIEC can largely simulate the metabolic process of drugs in the intestinal tract [29].

Drug metabolic enzymes mainly include class I and class II metabolic enzymes. The cytochrome P450 (CYP450) is a phase I metabolic enzyme and participates in the metabolism of many drugs [30]. Drug metabolism is the premise for the CYP450 metabolizing enzyme [31]. P450 metabolizing enzymes are involved in the metabolism of endogenous and exogenous compounds [31]. In the catalytic reaction of CYP450 to external sources, some are detoxification processes and some are toxic activation processes [32]. However, the metabolic effect of CYP450 expressed in the gut on EA is still unclear and requires further study. Therefore, based on the effect of intestinal cytochrome P450 metabolism on EA, the metabolic activation of EA was studied in this experiment. CYP450s with metabolic effect on EA was screened out to provide a basis for the biotransformation of EA.

Lentivirus-mediated cell transfection can greatly improve the efficiency of gene transfection, with higher and more stable gene expression, and the fragments are not easily lost [33]. In this study, the HIEC-CYP450 cell lines were successfully constructed by transfecting lentivirus to the HIEC cells.

Figure 4. Effects of CYP2C9 on apoptosis of EA-treated HIEC cells and HIEC cell models according to flow cytometry assay. (A) Flow cytometry images. (B) Comparison of apoptotic cells percentages in different HIEC cell models. ** p<0.01.

Figure 5. In vitro metabolism and clearance rate of EA by the different HIEC cell models determined by UPLC-MS. ** p<0.01.
a direct relationship between drug toxicity and cell activity; therefore, we can judge drug toxicity by cell activity. CCK-8 was employed in this study, as it can accurately evaluate the growth of cells with higher efficacy [34]. The HIEC cells were administered with EA at concentrations of 0 μg/mL, 2 μg/mL, 10 μg/mL, and 50 μg/mL. In the H-2C9 HIEC cell model, when the EA concentration was greater than 2 μg/mL, the measured cell activity was significantly decreased compared to that in HIEC cells without CYP450 enzyme expression. This result showed that EA demonstrates a significantly higher inhibitory effect on the H-2C9-expressed HIEC cells, which is also in a concentration-dependent manner. This phenomenon may be due to the toxicity caused by metabolic activities or the toxicity caused by metabolites, resulting in decreased cell activity. Therefore, it appears that CYP2C9 has a certain metabolic effect on EA. We also found that the administration of H-2C9 significantly increased the apoptosis rate of EA-treated HIEC cells compared to that in HIEC cells without any CYP450s expression. This result suggests that the expression of CYP2C9 activated the cytotoxic effect of EA on the HIEC cells, which might be associated with the apoptosis-associated signaling pathways. The metabolic clearance rate of the drug can be detected by the elimination of the original drug and the generation of metabolites [35,36]. The elimination of the original drug is more intuitive. In the metabolic clearance rate experiment of EA, there was a significant reduction of EA prototypes generated HIEC cell models that express 4 CYP450 enzymes, and it is the first study focused on the metabolism of EA by CYP2C9 in HIEC cells, and causes the cytotoxicity to HIEC cells through triggering the apoptosis-associated signaling pathway. Therefore, CYP2C9 is the main metabolic enzyme for EA in HIEC cells. Our findings may provide a theoretical basis for studies of toxicity theory and functions of CYP2C9 and EA in the gut.

Conclusions

To the best of our knowledge, this is the first study that generated HIEC cell models that express 4 CYP450 enzymes, and it is also the first study focused on the metabolism of EA by CYP450s. Our findings proved that EA can be effectively activated by CYP2C9 in HIEC cells, and causes the cytotoxicity to HIEC cells through triggering the apoptosis-associated signaling pathway. Therefore, CYP2C9 is the main metabolic enzyme for EA in HIEC cells. Our findings may provide a theoretical basis for studies of toxicity theory and functions of CYP2C9 and EA in the gut.

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