

Coffee Inhibits the Glucuronidation of 1-Naphthol in Rat Syncytiotrophoblast Cells

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The placenta acts as a barrier that protects the fetus from xenobiotics in the maternal blood. Phase II conjugation reactions in the placenta are considered to play an important role in this protective process by transferring polar hydroxyl groups and thereby imparting water solubility that facilitates the excretion of xenobiotics. Since coffee consumption during pregnancy is reported to affect fetal growth and development, we examined its effects upon the conjugation reactions in the rat syncytiotrophoblast cell line TR-TBT 18d-1. We detected a high level of glucuronidation for 1-naphthol, a model compound, in both a dose- and culture time-dependent manner in the TR-TBT 18d-1 cells. However, no sulfation was detected in any of our analyses. Coffee was found to inhibit the glucuronidation of 1-naphthol with an IC_{50} of 4.5 % (v/v). In contrast however, caffeine, which is a major bioactive constituent of coffee, did not show any inhibition at doses of up to 100 μ M. Coffee was also found to inhibit UDP-glucuronosyl transferase (UGT) activity towards 1-naphthol *in vitro* to a similar extent [IC_{50} = 1.5 % (v/v)] as in intact cells. Moreover, the expression of the UGT 1A6, which is known to mainly catalyze the glucuronidation of 1-naphthol, was not affected by coffee. If coffee inhibits placental glucuronidation during pregnancy, its consumption would increase the fetal plasma concentrations of harmful xenobiotics, potentially affecting normal fetal growth and development.

Key words— coffee, glucuronidation, 1-naphthol, placenta

INTRODUCTION

The placenta separates the blood supplies of the mother and fetus, but at the same time is being simultaneously perfused by both.¹⁾ The major function of the placenta is to transfer nutrients and oxygen to the fetus and to assist in the removal of waste products to the mother. In addition, it acts as a barrier against xenobiotics circulating in the maternal blood.^{2,3)} Many such compounds, including a number of drugs in clinical use, are known to cause unwanted effects in the fetus, including in utero death, the initiation of birth defects, and the production of functional abnormalities. Drug metabolizing enzymes including the cytochrome P450s (CYPs) and phase II conjugation enzymes play a major role in xenobiotic metabolism and detoxification. Many studies have now indicated that the constituents of common foods and beverages, such as flavonoids and catechins, affect the activities of hepatic and intestinal drug metabolizing enzymes. However, little is currently known about the effects of such agents upon xenobiotic metabolizing enzymes in the placenta.

Syncytiotrophoblasts, which form a continuous barrier between maternal and fetal circulating blood, play an essential role in the restriction of xenobiotics, including drugs and food ingredients, through the blood-placental barrier (BPB). Kitano *et al.* have previously established syncytiotrophoblast cell lines (TR-TBTs) from a transgenic rat harboring a temperature-sensitive simian virus 40 large T-antigen (Tg-rat).^{4,5)} The conditionally immortalized TR-TBT cells that were derived display a syncytium-like morphology and express several syncytioblast-specific markers and polarized glucose transporters.^{4,6)} Previously also, we have shown that several CYP isoforms and phase II enzymes are expressed, and that bisphenol A is metabolized, in TR-TBT 18d-1 cells.^{7,8)} To further investigate the effects of different foods and beverages

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on placental xenobiotic metabolism in our current study, we have employed the TR-TBT 18d-1 cell line as a placental barrier model. We tested the effects of coffee upon conjugation reactions in these cells, using 1-naphthol as the model compound, because of its worldwide consumption and its reported adverse effects upon fetal development.^{9–11)}

MATERIALS AND METHODS

Reagents—All chemicals were obtained from Wako Chemicals (Tokyo, Japan). Blended coffee powder was obtained from Brooks Co., (Yokohama, Kanagawa, Japan). Coffee extracts were prepared by extracting 8 g of powder with 140 ml water at 95°C. After sterile filtration, aliquots were frozen at –80°C and stored until use. The dry weight of 1 ml coffee is 8.4 mg. The original concentration of the extract was assigned a value of 100 % (v/v). UDP-[U-¹⁴C]glucuronic acid (300 mCi/mmol) was purchased from American Radiolabeled Chemicals, Inc. (St. Louis, MO, U.S.A.).

Cell Culture—TR-TBT 18d-1 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % fetal bovine serum, 10 U/ml penicillin and 10 U/ml streptomycin at 33°C in a humidified atmosphere containing 5 % CO₂. The cells were then seeded in 12-well plates at a concentration of 5 × 10⁵ cells/ml and cultivated for 4–5 days prior to use.

Analysis of 1-Naphthol Conjugation in TR-TBT Cells—To analyze 1-naphthol metabolism in TR-TBT cells, this compound was added to the culture medium at a concentration range of 0–100 μM and the cells were further incubated at 37°C. Aliquots of 100 μl were sampled from the medium at various timepoints, from which 10 μl was filtered and injected onto a HPLC column. The conjugation analysis was performed on an Octa Decyl Silyl (ODS) column (Chromolith Performance RP-18e, 100 × 4.6 mmφ, Merck, Darmstadt, Germany) as described previously.⁸⁾ The mobile phase comprised 10 mM tetrabutylammonium hydrogen sulfate in water and acetonitrile (79.5:20.5). The flow rate was 1 ml/min and the temperature of the column was 40°C. Fluorescence intensity was monitored at 313 nm with excitation wavelength of 273 nm. Almost all of the signals that were derived from the culture medium constituents were eluted before 2 min. The retention times for standards, 1-naphthol, 1-naphthyl sulfate and 1-naphthyl glu-

curonide were 19.0, 17.6 and 4.6 min, respectively. Linearity of the standard curves for 1-naphthyl sulfate and glucuronide was observed up to 200 μM. The effect of coffee on the conjugation reactions was measured by adding coffee at various concentrations (0–5 %) with 25 μM 1-naphthol to the culture medium of Caco-2 cells. IC₅₀ values for the concentration-activity curves were calculated using a curve-fit program for Windows.

Microsome Preparations from TR-TBT Cells—Cells (1–2 × 10⁷) were removed from the culture dishes, washed with phosphate buffered saline, and then homogenized in 1 ml buffer A [50 mM Tris-HCl (pH 7.5), 250 mM sucrose, 0.1 mM EDTA, 3 mM 2-mercaptoethanol, 0.1 mM phenylmethylsulfonyl fluoride, 5 μg/ml antipain, and 5 μg/ml pepstatin]. The debris was removed by centrifugation at 3000 g for 15 min, after which the supernatant was centrifuged at 105000 g for 60 min. The precipitate was suspended in 100 μl buffer A and used as the microsomal fraction in the subsequent experiments.

Assay of UDP-glucuronosyl transferase (UGT) Activity—UGT activity was measured as follows; the reaction mixture (150 μl) contained 100 mM Tris-HCl (pH 7.4), 10 mM MgCl₂, 0.02 % Triton X-100, 1 mM 1-naphthol and the microsome fraction (2 μg of protein). The reactions were initiated by the addition of 0.2 mM UDP-[U-¹⁴C]glucuronic acid (0.1 μCi) and continued for 2 hr at 37°C. The reactions were stopped by the addition of 300 μl ethanol (95 %), and the mixtures were then centrifuged at 1000 g for 5 min. Aliquots (50 μl) were applied to silica-gel TLC plates (Merck 60F₂₅₄) and developed in *n*-butanol-acetone-glacial acetic acid-ammonia (30 %)-water (70:50:18:1.5:60, v/v). Radioactive compounds were analyzed by a radioimage analyzer (FLA7000, FUJIFILM, Yokohama, Tokyo, Japan).

Reverse Transcription (RT)-PCR—Total RNA was isolated from cultured cells using the guanidinium thiocyanate phenol-chloroform extraction method.¹²⁾ First strand cDNAs were synthesized from 10 μg of total RNA using 1 unit of murine moloney leukemia virus (M-MLV) reverse transcriptase with oligo(dT) primers, according to the manufacturer's protocol. PCR was then performed using this cDNA as a template with AmpliTaq Gold polymerase (Perkin-Elmer). The PCR primers used to amplify human UGT and breast cancer resistant protein (BCRP) cDNAs were designed from published sequences as follows; UGT1A1, forward

5'-GATCCCCATAGATGGCAG-3' and reverse 5'-GTATGTTTTAACACACGCAGCA-3'; UGT1A6, forward 5'-TTGCCTTCTTCCTGCTGC-3' and reverse 5'-TCTGAAGAGGTAGACGGAAAGGC-3'; BCRP, forward 5'-CTGACCCTTCCATCCTCTTC-3' and reverse 5'-CGAGGCTGGTGAATGGAGAA-3'. The amplification conditions (35 cycles) were as follows: 1 min at 94°C, 1.5 min at 54–58°C and 2 min at 72°C.

Statistics— Data were analyzed by the Student's *t*-test. *P*-values below 0.05 were considered as to be statistically significant.

RESULTS

Metabolism of 1-Naphthol in TR-TBT 18d-1 Cells

Previously, we have analyzed the expression of drug metabolizing enzymes in TR-TBT cells. We found significant expression of CYPs and phase II conjugation enzymes and observed glucuronidation of bisphenol A, an endocrine disruptor, in these rat syncytiotrophoblast cells.^{7,8)} In our present study, we used 1-naphthol as our test compound to evaluate conjugation reactions in the TR-TBT cell line. After supplementing the TR-TBT cell cultures with 1-naphthol (0–100 μ M), the accumulation of metabolites in the growth medium was monitored via analytical HPLC over a 48 hr period. No 1-naphthyl sulfate was detected, even after 48 hr of incubation, as in the case also for bisphenol A.⁸⁾ The accumulation of 1-naphthyl glucuronide was found to be almost directly proportional to the incubation

time and the substrate concentration (Fig. 1). After 24 hr incubation, almost 50 % of the 1-naphthol supplement was found to be metabolized to a glucuronide conjugate.

Effects of Coffee upon the Conjugation of 1-Naphthol in TR-TBT Cells

Recently, many studies have shown the induction or inhibition of drug metabolizing enzymes by a variety of clinical agents, environmental chemicals and food constituents.^{13–16)} If these events occurred in the placenta, they would likely interfere with normal fetal growth and development by disturbing the metabolism of nutrients or essential endogenous molecules such as hormones. Since coffee is consumed daily worldwide and several studies have reported that it has adverse effects on fetal growth and development,^{9–11)} we examined the effects of coffee on the conjugation of 1-naphthol in TR-TBT cells in our current study. As shown in Fig. 2A, the addition of coffee to the culture medium significantly inhibited the accumulation of 1-naphthyl glucuronides at a low concentration (IC₅₀, 4.5 %). To identify the components responsible for this inhibition, we first analyzed the effect of caffeine, a major bioactive component of coffee but found that it had no inhibitory effects (Fig. 2B).

To elucidate the underlying mechanism by which coffee affects conjugation activities in TR-TBT cells, we next measured its effect on the enzymatic activity of UGT towards 1-naphthol *in vitro*. As shown in Fig. 3, exposure to coffee strongly inhibits *in vitro* UGT activity. The IC₅₀ was calculated

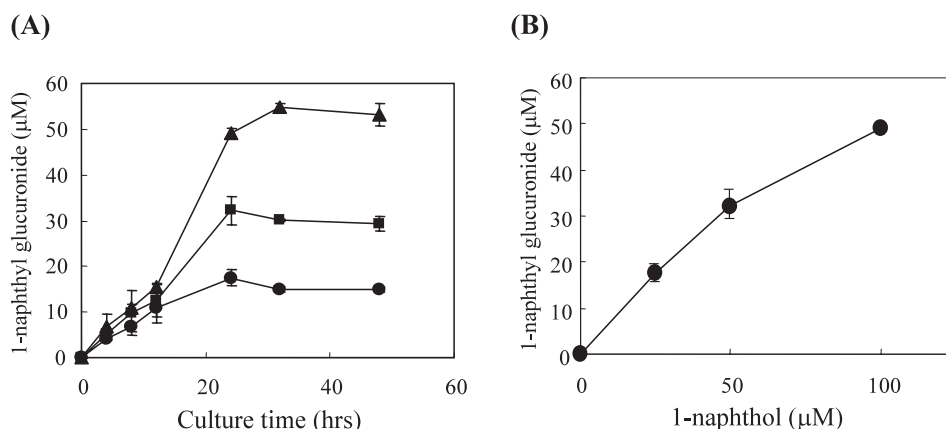


Fig. 1. 1-Naphthyl Glucuronidation in the TR-TBT 18d-1 Cell Line

1-Naphthol (0–100 μ M) was added to the growth medium of TR-TBT 18d-1 cells and aliquots were removed at the indicated times, and then analyzed by HPLC. (A) The accumulation of 1-naphthyl glucuronide was then measured for different 1-naphthol exposure levels (circle, 25 μ M; square, 50 μ M; triangle, 100 μ M). (B) 1-Naphthyl glucuronide was monitored after a 24 hr incubation. Each data point represents the mean \pm S.D. of three independent measurements.

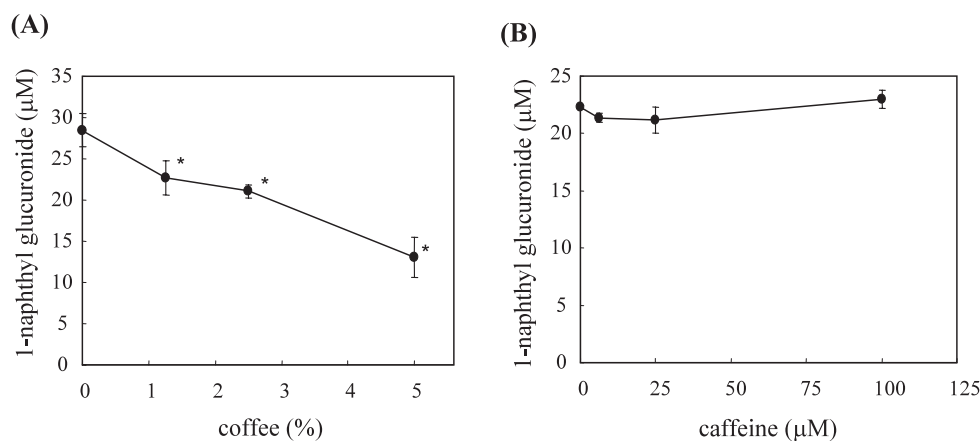


Fig. 2. Effects of Coffee and Caffeine on the Glucuronidation of 1-Naphthol in TR-TBT Cells

Coffee (A) or caffeine (B) was added to the culture medium with 25 µM 1-naphthol. The production of 1-naphthyl glucuronide in the medium was then measured by analytical HPLC after 24 hr. Each data point represents the mean \pm S.D. of three independent measurements. *, $p < 0.05$. Undiluted coffee was assigned a concentration of 100 % (v/v).

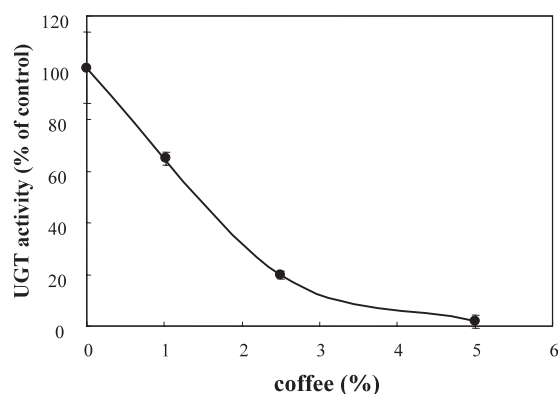


Fig. 3. Effect of Coffee on the *in vitro* UGT Activity of TR-TBT Cells

The UGT activities of 1-naphthol was measured in the presence or absence of coffee at the indicated concentrations (0–5 %). The activity levels are represented as a percentage of the control, without coffee treatment. The IC_{50} value was determined to be 1.5 % using a curve-fit program for Windows. All values represent the averages of two experiments with error bars.

to be 1.5 % (v/v).

Effects of Coffee on the Expression of UGT and BCRP Genes

Glucuronidation of 1-naphthol is catalyzed by members of the UGT1 subfamily, principally UGT1A6.¹⁷⁾ Recent studies have demonstrated the induction or reduction of different isoforms of the UGT1A subfamily in Caco-2 cells by dietary flavonoids, such as chrysin and quercetin.^{14, 18, 19)} Coffee also contains several phenolic compounds with antioxidant properties and its total polyphenol content has been reported to range from 200 to 550 mg per standard serving.^{20, 21)} To examine the possibility that the reduction of glucuronidation of 1-naphthol by coffee is due to its effects upon the

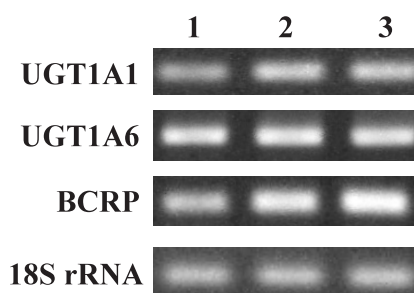


Fig. 4. Effects of Coffee on the Expression of the UGT1A1, UGT1A6 and BCRP Genes

Total RNA was isolated from TR-TBT 18d-1 cells treated with coffee for 24 hr and RT-PCR analysis of the UGT1A1, UGT1A6 and BCRP genes was then performed. These expression levels were normalized to those of 18S rRNA. Lane 1, without coffee; lane 2, 2.5 % coffee; lane 3, 5 % coffee.

expression of UGT1 genes, we analyzed UGT1A1 and UGT1A6 genes by RT-PCR after coffee treatment of TR-TBT cells for 24 hr. As shown in Fig. 4, a slight induction of UGT1A1 expression but no obvious change in UGT1A6 expression was observed in this analysis.

In addition to metabolism by drug metabolizing enzymes, the export of metabolites across the plasma membrane via transporters is also an essential pathway for the elimination of xenobiotics. The current evidence indicates that flavonoids interact with ATP-binding cassette (ABC) efflux transporters, mainly P-glycoprotein, multidrug resistance-associated protein (MRP) 1, MRP2 and BCRP.²²⁾ Since 1-naphthyl sulfate and glucuronide are possible substrates of BCRP,²³⁾ we measured the expression of the BCRP gene by RT-PCR after treatment of TR-TBT cells with 5 % coffee for 24 hr. As shown in Fig. 4, we detected a slight induction of

BCRP by coffee.

DISCUSSION

In our current study, we report the inhibitory effects of coffee on the conjugation of 1-naphthol in the rat syncytiotrophoblastic cell line, TR-TBT 18d-1, which was previously established from the Tg-rat placenta. We observed an accumulation of 1-naphthyl glucuronide in the culture medium of TR-TBT cells (Fig. 1) but no 1-naphthyl sulfate was detected (data not shown). The absence of sulfoconjugate is surprising because the significant expression of phenol sulfating sulfotransferase (SULT) 1 family genes has been previously reported in rat syncytiotrophoblast cell lines⁷⁾ and the transporter BCRP gene was found to be expressed in the TR-TBT 18d-1 cell line in our present analyses. To further elucidate this phenomenon, future functional studies of SULT and BCRP will be needed.

Coffee was found to reduce the levels of 1-naphthol glucuronidation in TR-TBT cells in both a dose and culture time-dependent manner (IC_{50} of 4.5 % at 24 hr; Fig. 2A). The inhibitory component in coffee in this regard is unlikely to be caffeine, however, since no inhibition of 1-naphthol conjugation was detected at a dose of up to 100 μ M, a concentration which corresponds to an approximately 10–20 % coffee extract solution. This result is similar to the case for bisphenol A; caffeine did not show any inhibition on the glucuronidation of bisphenol A in the same cells.⁸⁾ Other than caffeine, coffee extracts contain many bioactive components such as chlorogenic acid, cafestol, and kahweol. Further investigations will be needed to identify the component of coffee responsible for the inhibition of glucuronidation.

Our current study demonstrated that coffee strongly inhibited the *in vitro* UGT activity toward 1-naphthol (Fig. 3). The IC_{50} value was almost identical to that measured in intact cells (4.5 %), suggesting that the inhibitory effect of coffee on the glucuronyl conjugation of 1-naphthol might be partly due to direct interaction of active component(s) with the enzyme proteins inside the cell. Further analysis will need to be undertaken to clarify the molecular basis of this inhibition.

Previously we have found that coffee produces only a very weak inhibition of *in vitro* UGT activity of human colon carcinoma Caco-2 cells towards 1-naphthol at high doses (20 % inhibition by a

10 % coffee solution).²⁴⁾ However, our current study showed that coffee strongly inhibits *in vitro* UGT activity of TR-TBT cells. This discrepancy might be due to the species difference of UGT enzyme structures between human and rat, or due to the difference of assay conditions. Further investigation for the discrepancy must be carried out in the future.

We also examined the effects of coffee upon the expression of the enzymes, UGT1A1, UGT1A6 and BCRP, but no obvious reduction was detected, and a marginal induction was observed for the UGT1A1 and BCRP genes (Fig. 4). This induction was also observed following incubation with caffeine (data not shown), suggesting caffeine in coffee may be responsible in a part for the induction of gene expression.

Glucuronidation is generally considered to be a detoxification reaction that produces metabolites from both exogenous and endogenous substrates that are more polar and more readily eliminated. Since UGT activity is not observed in fetal liver as a major pathway for xenobiotic and endobiotic detoxification,²⁵⁾ the activity of this enzyme in syncytiotrophoblasts may play a major protective role during gestation through the metabolic processing and clearance of compounds. In addition, UGTs are also associated with the inactivation of some hormones such as testosterone, estrone and thyroid hormones.²⁶⁾ We speculate that the inhibition of UGT activity by constituents of coffee might interfere with normal fetal growth and development and in this regard there are several reports showing a correlation between coffee consumption and fetal death or abnormal development. A possible correlation between the inhibition by coffee of glucuronidation in the placenta and adverse effects upon fetal development should be further evaluated.

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