Inhibitory Effects of Various Beverages on the Sulfonoconjugation of 17β-Estradiol in Human Colon Carcinoma Caco-2 Cells

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To investigate the possible effects of different beverages in the gastrointestinal tract on the sulfation of estrogen, which is a major hormone and prototype substrate for the human sulfotransferases (SULT), we analyzed the effects of these substances upon the sulfate conjugation of 17β-estradiol (E2) in the human colon carcinoma cell line Caco-2. Sulfonoconjugation activity toward E2 was measured by incubating 20 nM E2 with Caco-2 cells in the presence (5% (v/v)) of each beverage. Among the 35 beverages analyzed, four (aronia, blueberry, coffee, and peppermint) exhibited strong inhibitory effects on E2 sulfation within Caco-2 cells (IC50 values ranging from 1.9 to 4.4% (v/v)). These active beverages also strongly inhibited the cytosolic estrogen SULT activity of Caco-2 cells in vitro (IC50 values ranging from 0.18 to 0.3% (v/v)). These inhibitory activities were extractable with ethyl acetate but not hexane or n-butanol, indicating that the molecules responsible are moderately lipophilic. Coffee was found to be the most potent inhibitor but the major constituents of this beverage, caffeic acid, caffeine, and chlorogenic acid, did not show any effects on estrogen SULT activity. Kinetic analyses further indicated that the mode of inhibition by coffee is competitive. A possible relationship between the inhibition of estrogen SULT activity by coffee in the gastrointestinal tract and the reported reduction of colon cancer incidence in women who consume coffee is discussed.

Key words Caco-2; coffee; estrogen; sulfotransferase

Estrogens are a group of steroid compounds that function as the primary female sex hormones and are used in hormone replacement therapies for postmenopausal women. The role of estrogens in promoting the growth and development of the normal mammary gland but also of most estrogen receptor (ER)-positive mammary carcinomas is well established.1,2) In addition, it has been suggested that estrogen is an important physiologic regulator of intestinal calcium and phosphate absorption.3,4) Furthermore, recent epidemiologic evidence has consistently demonstrated that estrogen exposure in women reduces colon cancer risk.5–7) The cumulative data thus suggest that estrogens in the gastrointestinal tract play an important role in the normal physiologic functioning of this organ.

Sulfation is a major pathway for the inactivation of steroids including estrogens.5–8,9,10) The conjugation of estrogens with sulfate is catalyzed by a family of enzymes, the sulfotransferases (SULTs), and the resulting sulfated estrogens are unable to bind ERs.9,10) Among the four forms of human cytosolic SULTs which have been identified, estrogen SULT (termed SULT1E1) is the key enzyme that sulfates estrogens at the nanomolar concentrations at which they occur physiologically and at which they interact with the ERs.11,12) Hence, the level of SULT1E1 activity may play a key role in the maintenance of the cellular estrogen levels. Recent studies have reported that dietary flavonoids such as quercetin and resveratrol inhibit the estrogen SULT activity of human mammary epithelial, human hepatic, and jejunal S9 cellular fractions.13,14) These effects of flavonoids might affect the intracellular availability of estrogen hormones to bind their cognate receptors and influence the progression of breast cancer or colon cancer. In our current study, we investigated the possible interactions of a range of beverages known to contain numerous flavonoids and phytoestrogens with estrogen sulfation in the gastrointestinal tract. We examined sulfonoconjugation reactions in the human adenocarcinoma Caco-2 cell line by analyzing the effects of different beverages on the sulfonoconjugation of 17β-estradiol (E2).

MATERIALS AND METHODS

Materials [3H]E2 (48 Ci/mmole) and [35S]-phospho-adenosine-5’-phosphosulfate (PAPS) were purchased from PerkinElmer Life Sciences (Shelton, CT, U.S.A.). Blended coffee powder was obtained from Brooks Co., Japan. Green tea (product of Shizuoka Pref.) was purchased from the Japan Green Tea Center (Tokyo, Japan). The leaves and dried flower heads used to extract the herbal teas were purchased from Kataoka and Co., Ltd. (Tokyo, Japan). Beverages were purchased from Kirin Beverages (Tokyo, Japan). Caffeic acid, caffeine, chlorogenic acid, and reagents for the MTT assay were purchased from Sigma (St. Louis, MO, U.S.A.). All organic solvents used were of HPLC grade and purchased from Wako Chemicals (Tokyo, Japan).

Preparation of Coffee and Herbal Tea Extracts Coffee extracts were prepared by extracting 8 g of powder with 140 ml of water at 95 °C. The dry weight of 1 ml of extract (100%) was 8.4 mg. Teas and herbal tea solutions were prepared using standard tea brewing methods as follows. Leaves or dried flower heads of different herbs (2 g) were extracted with 100 ml of water at 95 °C for 2 min. The extracts were then filtered, divided into small aliquots, and stored at −80 °C until use. Undiluted extracts were assigned a concentration of 100% (v/v).

Cell Culture Caco-2 cells were grown in 12-well plates (Iwaki, Japan) in 1 ml of MEM supplemented with 10% fetal bovine serum, 2 mM glutamine, 10 U/ml penicillin, 10 U/ml streptomycin and additional non-essential amino acids. The

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cells were kept at 37 °C in a humidified atmosphere, containing 5% CO₂. The cells were seeded in 12-well plates at a concentration of 5×10⁵ cells/ml and cultivated for up to 3 weeks with media changes every 4 or 5 d.

**Cytosolic Extract Preparation from Caco-2 Cells**

Caco-2 cells (1—2×10⁶) were removed from their culture dishes (75 mm²), washed with phosphate-buffered saline (PBS), and then homogenized in 1 ml of 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). Debris was removed by centrifugation at 3000 g for 15 min, after which the supernatant was centrifuged at 105000 g for 60 min. The clear lysates were used as cytosolic extracts in subsequent experiments.

**Estrogen SULT Assays**

For studies of E₂ sulfation in intact Caco-2 cells, the cells were grown as described above and then briefly washed with PBS. This was followed by incubation with [³H]E₂ at various concentrations (0—100 μM) in MEM supplemented with 5% charcoal-treated fetal bovine serum with or without beverage supplements at a range of concentrations (0—10% v/v). Aliquots (50 μl) of the medium were sampled at various times, and E₂ sulfate formation was assayed using the alkaline-chloroform extraction procedure. Briefly, 150 μl of 0.25 M Tris–HCl (pH 8.7) and 1 ml chloroform were added to the sampled aliquots. After mixing and centrifugation, aqueous-phase samples were subjected to liquid scintillation counting. Cytosolic estrogen SULT activity was determined using [³⁵S]PAPS as the sulfate donor and various concentrations of E₂ as the sulfate acceptor, according to a slight modification of the procedure described previously by Foldes and Meek. In this method, the reaction mixture (250 μl) contained 10 mM phosphate buffer (pH 7.4), E₂ 0—1 μM, 5.0 μM [³⁵S]PAPS (0.4 μCi) and cytosolic extract (15 μg of proteins). The mixture was incubated at 37 °C for 30 min, and the reaction was stopped by the addition of 50 μl cold 0.1 M barium acetate. Excess [³⁵S]PAPS was precipitated by the addition of 50 μl of both 0.1 M Ba(OH)₂ and 0.1 M ZnSO₄ and removed by centrifugation at 12000 g for 5 min. This precipitation procedure was then repeated, and the remaining supernatant (300 μl) was transferred to a 3 ml-liquid scintillator to determine radioactivity levels. Control reactions were established by omitting the acceptor substrate from the mixture.

**Thin-Layer Chromatography of the Labeled Products Produced by Estrogen SULT Assays**

Mixtures (20 μl) from the estrogen SULT assays were separated on silica-gel 60 F₂₅₄₄ plates (Merck, Darmstadt, Germany) in chloroform: methanol: acetic acid [60:20:3 (v/v)]. E₂-3-sulfate was used as a standard. The plates were exposed to X-ray film for 10 d at −80 °C, after which the spots were visualized with phosphomolybdic acid.

**Beverage Extraction Procedure**

Each beverage (500 μl) was extracted sequentially with an equal volume of the solvents hexane, ethyl acetate, and n-butanol. Organic and aqueous phases were then concentrated by evaporation and dissolved in 16 μl DMSO. The effects of each beverage on E₂ sulfation in Caco-2 cells were monitored using 1 μl aliquots of these fractions.

**MTT Assay**

Cytotoxic effects upon cell metabolism were investigated using the MTT assay. Cells (1×10⁴) were seeded in 96-well plates and grown for 3 weeks at 37 °C. After incubation with each test beverage for 2 h, the cells were washed with PBS and subjected to the MTT assay according to the manufacturer’s protocol (Dojin Chemicals, Tokyo, Japan).

**Statistics**

Data were analyzed using the Student’s t-test. p-values of less than 0.05 were considered to represent a statistically significant difference.

**RESULTS**

**Detection of Estrogen SULT Activity in Caco-2 Cells**

We previously reported that the SULT1E1 gene is expressed in differentiated Caco-2 cells. However, the activity of SULTs toward estrogens has yet to be determined. We therefore measured the estrogen SULT activity levels in differentiated Caco-2 cells using E₂ as a substrate. Figure 1 shows estrogen SULT activities in intact cells (A) and in cytosolic extracts (B). Two types of estrogen SULT activity were observed in both assays. TLC analysis revealed that the mobilities of the two labeled products were identical to that of E₂-3-sulfate (Fig. 2). Based on the concentration range observed in these analyses, the activity seen in the nanomolar range in both assays is likely to be due to SULT1E1 activity. The activity at higher E₂ concentrations may be due to phenol SULTs (mainly SULT1A1 and SULT1A3 activities). These data prompted us to investigate the effects of beverages on...
estrogen SULT activity using E2 as a substrate at a nanomolar concentration (20 nM).

**Effects of Different Beverages upon E2 Sulfation in Intact Caco-2 Cells** We measured E2 sulfation levels by incubating intact Caco-2 cells with 20 nM E2 in the presence or absence of different beverages at 5% concentrations (v/v) for 2 h. To compare the effects of beverages, we used percentage (v/v) concentrations taking the original concentrations as 100% (v/v), because each beverage contains different amounts of ingredients. Figure 3 shows the results of these measurements. Among the 35 beverages tested in this experiment, four were found to reduce E2 sulfation activity to less than 50% of the control levels: aronia, 28%, blueberry, 50%, coffee, 18.6%, and peppermint, 42%. IC50 measurements (the concentration at which 50% inhibition was achieved) were then performed for these four beverages (Fig. 4). Coffee was found to be the most potent of the four (IC50, 1.9% v/v). It was also noteworthy that none of these four active beverages showed any cytotoxicity up to a 10% (v/v) concentration, as judged by the MTT assay (data not shown).

**Effects of Inhibitory Beverages on the Cytosolic Estrogen SULT Activity of Caco-2 Cells** To elucidate further the inhibitory activities of the four beverages identified in the initial screening, we next measured the effects of these active substances on estrogen SULT activity in vitro using cytosolic extracts of Caco-2 cells. As shown in Fig. 5, strong inhibition was observed for each of these beverages in this experiment, and the IC50 values were almost an order of magnitude lower than those obtained with intact cells.

**Characterization of the Inhibitory Activities of Beverages against E2 Sulfation** To analyze the inhibitory activities of aronia, blueberry, coffee, and peppermint extracts upon E2 sulfation in more detail, we extracted each of these beverages using different solvents (hexane, ethyl acetate, and n-butanol). The results of the inhibiting fractions upon the E2 sulfation levels in intact Caco-2 cells were then determined. As shown in Fig. 6, almost all of the inhibitory activities of these beverages could be extracted by ethyl acetate, except in the case of blueberry. However, only weak inhibition was obtained using the n-butanol-extracted fractions in each case.

**Further Characterization of the Inhibitory Activity of Coffee against E2 Sulfation** Since coffee is a commonly consumed beverage worldwide, we analyzed its inhibitory activities against E2 sulfation in more detail. For this purpose, we examined the effect of coffee fractions against estrogen SULT activity in vitro. As shown in Fig. 7A, these inhibitory activities were mainly contained in the ethyl acetate fraction. A Lineweaver–Burk plot of the inhibition also revealed that the mode of inhibition is competitive (Fig. 7B). Since coffee is known to contain several bioactive substances that can be extracted with ethyl acetate such as caffeic acid, caffeine, and chlorogenic acid,18) we also measured the effects of these constituents on estrogen SULT activity in intact Caco-2 cells. No inhibition by any of these compounds was observed even at concentrations up to 100 μM (data not shown).

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**Fig. 2. Identification of Labeled Products Using TLC**

The metabolites labeled with [3H]E2 (lane 3) and [35S]PAPS (lane 4) in the estrogen SULT assays shown in Fig. 1 were separated on silica TLC plates. The radioactive spots obtained were visualized with autoradiography. Standard E2 (lane 1) and E2-3-sulfate (lane 2) were visualized with phosphomolybdic acid. The large spot at the origin is free [35S]PAPS.

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**Fig. 3. Effects of Various Beverages upon the Estrogen SULT Activity Levels in Intact Caco-2 Cells** Each of the indicated beverages was added to the culture medium of Caco-2 cells at a final concentration of 5% (v/v) along with 20 nM [3H]E2 and incubated for 2 h. Aliquots (50 μl) of the medium were subsequently sampled and E2 sulfate formation was assayed using the alkaline–chloroform extraction procedure. The activity was calculated as a percentage of the controls (no beverage added). The values shown represent mean with standard deviation of triplicate determinations. The results for coffee, teas, and various herbs are shown in (a) and (b) and those for fruit juices in (c) in ascending order of E2 sulfate activity. * p<0.05 (n=3).
DISCUSSION

The effects of different beverages on estrogen sulfoconjugation activity in Caco-2 cells were investigated. We initially detected two types of E2 sulfation activities in both intact cells and cytosolic extracts. We speculated that the activity detected at lower E2 concentrations would be attributable to SULT1E1, which has a $K_m$ in the nanomolar range for estrogens, and that the activity at higher E2 concentrations would be due to SULT1A1 or SULT1A3, both of which have a $K_m$ in the micromolar range for estrogenic compounds.9) Although it is difficult to estimate precisely the intracellular concentrations of estrogens, it is probable that the relevant concentration in vivo will be in the nanomolar range only. We thus chose to use E2 as a substrate to measure estrogen SULT activity at a concentration of 20 nm, which would be in the range of its physiological levels. In addition, at this concentration of E2, sulfation will be solely catalyzed by SULT1E1. Moreover, the $K_m$ for the activity measured at lower E2 concentrations was calculated to be 14 nm from kinetic analyses using cytosolic extracts of Caco-2 cells.

Among the panel of 35 beverages tested in the present analyses at a concentration of 5% (v/v), aronia, blueberry, coffee, and peppermint were found to inhibit E2 sulfation significantly in intact Caco-2 cells. Many of these beverages have been reported previously to contain inhibitors of SULT activity both in vivo and in vitro.19–23) Teas (green tea and black tea), herbs (jasmine and rosemary), coffee, and fruit...
The aronia, blueberry, coffee, and peppermint beverages also displayed inhibitory activity against in vitro estrogen SULT activity, although the IC₅₀ values in this case are markedly different from those measured for intact cells (aronia, 0.3% vs. 4.1%; blueberry, 0.20% vs. 4.4%; coffee, 0.18% vs. 1.9; peppermint, 0.21% vs. 3.8%). One possible explanation for this discrepancy is low permeability of the inhibitors into the cells. In addition, the major inhibitory activities of the beverages could be extracted by ethyl acetate, suggesting that the chemical nature of the inhibitors is slightly lipophilic. Sugar conjugates of flavonoids are thus possible candidates because most beverages contain many such conjugates and they often show low permeability across the cell membrane. In the case of coffee, among its bioactive constituents that have been well characterized are caffeic acid, caffeine, and chlorogenic acid. However, none of these compounds showed any inhibitory effects against cellular estrogen SULT activity. Further characterization of the inhibitory constituent(s) of coffee will thus be necessary in future experiments.

Sulfoconjugation is thought to play a significant role in the metabolism of estrogens and contribute to the maintenance of the circulating levels of these hormones. Under physiologic conditions, E₂ is more likely to be sulfated by estrogen SULT (SULT1E1) than by other isoforms of this enzyme. SULT1E1 is expressed in many human tissues including the liver and jejunum, mammary epithelial cells, endometrium, and testis. Recent studies have reported that dietary flavonoids such as quercetin and resveratrol inhibit the estrogen SULT activity of human mammary epithelial cells and of human hepatic and jejunal S9 fractions. These effects of flavonoids might therefore affect the intracellular availability of estrogen hormones to their receptors and influence the progression of breast and colon cancers. In this regard, our present data indicate that globally popular beverages such as coffee and peppermint tea might also influence the cellular levels of estrogen through inhibition, thereby modulating the physiologic effects of this hormone in the gastrointestinal tract such as calcium and phosphate intakes.

Ethinyl estradiol, a systemic oral contraceptive, has been reported to be inactivated predominantly in the intestine rather than in the liver by SULT1E1, a key enzyme in the metabolism of this factor. Previously, we reported that coffee inhibits the phenol SULT activity of Caco-2 cells both in vivo and in vitro. Taken together with the data in our present study, coffee consumption might therefore influence the bioavailability of orally administered drugs that are catalyzed by SULTs, and this in turn may lead to an increase in their clinical effectiveness and/or adverse reactions.

Coffee was found in our present analyses to be the most potent inhibitor of estrogen SULT activity in Caco-2 cells. Several epidemiological studies showed coffee consumption to be associated with a lower risk of certain types of cancers, particularly those of colorectal origin, and that estrogen exposure in women also results in a reduction in colon cancer risk. Recently Giroux et al. have reported that a lack of estrogen receptor β enhances tumorigenesis in the small intestine in mice. If coffee drinking reduces estrogen SULT...
activity in the gastrointestinal tracts, the level of free estrogen in turn would increase. This effect might thus be related to the preventive effects of coffee against carcinogenesis in the intestine and colon. Further studies to elucidate this possibility will be of great interest in the future.

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