JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

J. Agric. Food Chem. 2009, 57, 8297–8302 8297
DOI:10.1021/jf9011906

Transport and Metabolism of Equol by Caco-2 Human Intestinal Cells

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Equol is a metabolite of daidzein with greater estrogenic activity and antioxidant capacity than its precursor. Although it is known that equol is produced by the gut microflora, information regarding its transport and metabolism in the intestine is lacking. This study investigated transepithelial transport, bioconversion, and efflux of equol using differentiated cultures of Caco-2 cells to characterize its bioavailability. Uptake was directly proportional to the initial concentration in the apical compartment with maximal intracellular concentrations being reached and 20% of the total added to the apical compartment present in the basolateral compartment as free equol after 1 h. By 4 h, 73% of equol was present as β-glucuronidase/sulfatase sensitive conjugates with approximately 47 and 26% of initial equol distributed in apical and basolateral compartments, respectively. Free equol in the basolateral compartment appeared to be retrotransported, largely conjugated, and effluxed across the apical membrane. These results suggest that differences in the synthesis and efflux of equol conjugates may contribute to the marked variance in the bioavailability of equol in "producer" phenotype.

KEYWORDS: Equol; bioavailability; metabolism; glucuronide; absorption; Caco-2 cells

INTRODUCTION

Equol is a microbial metabolite of the isoflavonoid daidzein (**Figure 1**) that has received increasing attention as a result of its estrogenic and antioxidant activities. This metabolite induces estrogen-dependent transcription more effectively than other isoflavonoids (*1*) and exhibits greater estrogenic activity than genistein in rats as assessed by uterine weights of immature animals (*2*). Microflora exclusively produce the *S*-diastereoisomer of equol (*3*, *4*), which binds with higher affinity than the *R*-diastereoisomer to estrogen receptor β in vitro (*5*). Lund et al. (*6*) also reported that the estrogen-like activity of equol may be related to its ability to complex with 5α-dihydrotestosterone, but not with the androgen receptor. Indeed, subcutaneous administration of equol for 7 days decreased ventral prostate weight in rats, suggesting that equol may be efficacious in prostate cancer prevention (*6*).

Equol is a reduced metabolite of daidzein and as such has greater antioxidant capacity than its parent isoflavonoid (*2*, *7*, *8*) and suppresses superoxide formation, oxidation of LDL, and AAPH or Fe(II)-induced peroxidation in vitro (*9*−*12*). Topical application of a lotion containing 10 μM equol was shown to protect albino mice against UVA-induced lipid peroxidation (*13*), although dietary administration of equol failed to reduce plasma malonyldialdehyde in rats (*14*).

To mediate its proposed activities, equol must first be produced from daidzein in the gut and subsequently absorbed and delivered to target tissues. Although reports indicate that strict anaerobes residing in the large intestine are responsible for conversion of daidzein to equol, it is likely that conversion of daidzein to equol is mediated through the action of multiple species of bacteria (*15*, *16*). Decroos et al. (*17*) recently isolated a mixed culture of equol-producing bacteria consisting of *L. mucosae*, *E. faecium*, *F. magna*, and one unidentified species, whereas pure cultures of these individual microorganisms failed to produce equol from daidzein.

The standard marker for equol synthesis in the gastrointestinal lumen is the appearance of this metabolite in plasma and urine. Because the concentration of equol in these fluids is much lower than that of daidzein, microbial synthesis of equol is assumed to be limited. Only 30−50% of humans appear to possess microflora capable of converting daidzein to equol (*2*, *3*, *18*−*20*). However, it is possible that equol production may not be directly coupled with its absorption due to differences among individuals in the intestinal transport and metabolism of this metabolite. In this study, confluent monolayers of differentiated Caco-2 cells were used as a model for the small intestinal mucosal epithelium to characterize the uptake, metabolism, and transepithelial transport of equol.

MATERIALS AND METHODS

Supplies. A preparation containing a mixture of β-glucuronidase and sulfatase from *Helix pomatia* (G-1512), DMEM, and phenolsulfonaphthalein (phenol red) were purchased from Sigma Chemical Co. (St. Louis, MO). (*R*,*S*)-Equol (7-hydroxy-3[4′-hydroxyphenyl]chroman) was purchased from LC Laboratories (Woburn, MA). 2′,4′-Dihydroxy-2-phenylacetophenone was purchased from Indofine Chemical Co., Inc.

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Published on Web 08/28/2009

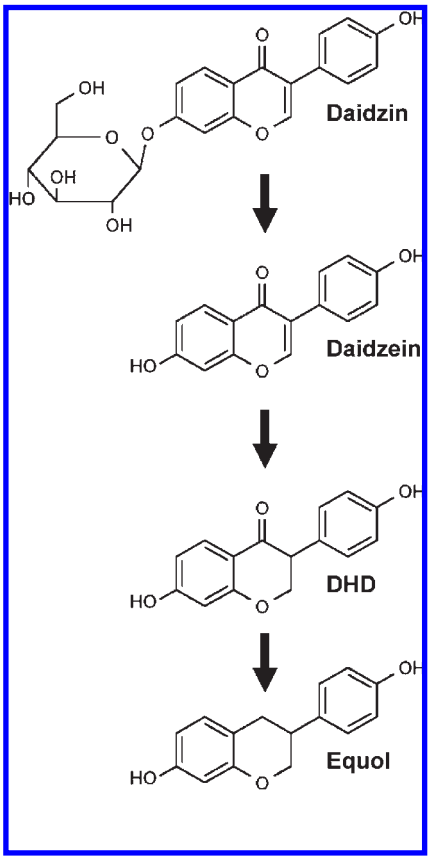

Figure 1. Biochemical conversion of dietary daidzin and daidzein to equol. Dietary daidzin is deglycosylated by mammalian lactase−phlorizin hydrolase or microbial β-glucosidase to daidzein. Dietary daidzein, or daidzein formed during intestinal deglycosylation of daidzin, is reduced to dihydrodaidzein and then to equol by intestinal microbes.

(Hillsborough, NJ). Nylon syringe filters (0.2 μm) were purchased from Alltech Associates, Inc. (Deerfield, IL). Caco-2 human colon adenocarcinoma cells were purchased from American Type Culture Collection (Rockville, MD). Pierce bicinchoninic acid protein assay kits were purchased from Thermo Fisher Scientific, Inc. (Rockford, IL). Glutamine, nonessential amino acid solution, penicillin G sodium and streptomycin sulfate, and fungizone amphotericin B were purchased from Gibco (Invitrogen Corp., Carlsbad, CA). Fetal bovine serum was purchased from Atlanta Biologicals (Lawrenceville, GA). All other reagents were purchased from Fisher Scientific Co. (Fair Lawn, NJ). Reagents used for HPLC analysis were of HPLC grade.

Cell Culture. Caco-2 cells derived from human colon adenocarcinoma were used as a model for the small intestinal mucosal epithelium to investigate the uptake, transport, and metabolism of equol. Details for growth and maintenance of these cells have been previously described (*21*, *22*). Experiments with Caco-2 cells (passages 21−35) were conducted when monolayers were 12−15 days postconfluent (18−21 days in culture). For initial experiments addressing the uptake of equol, 2.5×10^5 cells/well were seeded in six-well plates (Falcon 353046, Becton Dickinson and Co., Franklin Lakes, NJ). Caco-2 cells also were seeded at a density of 2.5×10^5 cells/well on transwell inserts to investigate transepithelial transport and metabolism (Falcon 353493, Becton Dickinson and Co.). Cellular protein concentrations were determined by Pierce BCA protein assay kit (Rockford, IL).

Preparation of Test Medium. As equol was not readily soluble in DMEM, the metabolite was complexed with Tween 20 to yield a water-soluble delivery vehicle for cell experiments. Preparation of the Tween 20 complex was as reported by O'Sullivan et al. (*23*) with slight modifications. A stock solution of equol in ethanol was prepared and filtered (0.2 μm), and its concentration was determined spectrophotometrically (ε = 5955 L mol^{-1} cm^{-1}, λ = 282 nm). Aliquots of the stock solution containing the desired mass of equol were dried under a stream of nitrogen and resolubilized in ethanol containing 1% (v/v) Tween 20. The final concentration of the vehicle in medium did not exceed 0.01%. The ethanol was evaporated under a stream of nitrogen before the addition of 100 μL of serum-free DMEM. The mixture was vortexed for 30 s followed by sonication in a water bath for 30 s. The addition of 100 μL of serum-free DMEM, vortexing, and sonication were repeated four times before final dilution with 4.5 mL of serum-free DMEM. The gradual dilution of the equol−Tween 20 mixture with serum-free DMEM facilitated optimal solubility in the aqueous medium. The final solution was filter sterilized (0.22 μm) and diluted in sterile serum-free DMEM to prepare medium with concentrations of equol ranging from 2.5 to 20 μmol/L.

Cellular Uptake of Equol. Monolayers were washed once with serum-free DMEM (37 °C) prior to initiating uptake experiments. Monolayers were incubated (37 °C, 5% CO_2) for 4 h in serum-free DMEM with equol (2.5−20 μmol/L) to characterize the effect of dose on cellular uptake. Separate cultures were treated with equol (10 μmol/L) for 0.5−8 h to characterize the effect of time of exposure on cellular uptake. Replicate cultures treated with the vehicle (0.01% Tween 20) alone served as controls. Aliquots of starting medium were incubated (37 °C, 5% CO_2) without cells to assess the stability of equol for the indicated incubation times. To provide initial insight as to whether cellular uptake of equol occurred by passive diffusion or facilitated transport, replicate cultures were incubated in serum-free DMEM with equol (10 μmol/L) for 1 h at either 4 or 37 °C. Samples were collected and analyzed by HPLC.

Transport and Metabolism of Equol. Caco-2 cells were cultured on transwell inserts to examine the transport and metabolism of equol. Monolayers were washed as above before the addition of 1.5 mL of serum-free DMEM with equol (10 μmol/L) to the apical compartment and 2.5 mL of serum-free DMEM to the basolateral compartment. Cultures were incubated (37 °C, 5% CO_2) for 0.5−8 h before collection of apical and basolateral media and harvest of cells to characterize the distribution and speciation (free vs conjugated) of equol as described below.

Sample Collection. Aliquots of media were collected at indicated times and centrifuged at 210*g* at 4 °C for 5 min, and the supernatants were stored under nitrogen at −20 °C until analysis by HPLC. Monolayers were washed twice with cold (4 °C) PBS to remove adsorbed material and gently scraped with a rubber policeman, and cells were collected in cold (4 °C) PBS. Cell suspensions were transferred to 15 mL conical tubes, and culture plates were scraped again to collect residual cells in cold (4 °C) PBS. The cell suspensions were centrifuged at 210*g* at 4 °C for 5 min. Following centrifugation, the supernatants were discarded and the tubes were dried with cotton swabs without disturbing the cell pellets. Pelleted cells were stored at −20 °C under nitrogen until HPLC analysis.

Barrier Integrity of Cell Monolayers. Barrier integrity of Caco-2 monolayers grown on semipermeable inserts was determined by quantitating the transfer of phenolsulfonaphthalein (phenol red), a marker of paracellular flux, from the apical compartment to the basolateral compartment as described previously (*24*). The rate of apical to basolateral flux of phenol red did not exceed 0.02%/cm^2/h for all Caco-2 cell monolayers used in these experiments.

Extraction and Analysis of Equol. Samples from cell experiments were prepared for analysis as follows. 2′,4′-Dihydroxy-2-phenylacetophenone (final concentration = 200 μmol/L) in chloroform was added as an internal standard to the samples, and cell pellets were sonicated in 0.5 mL of sodium acetate buffer (0.2 mol/L, pH 5) on ice for 10 s. A preparation containing both β-glucuronidase and sulfatase from the snail *H. pomatia* (400 units/mL) in 0.5 mL of sodium acetate buffer (0.2 mol/L, pH 5) was added to sonicates. Samples were incubated with shaking for 18−20 h at 37 °C before the addition of 4 mL of diethyl ether, and samples were vortexed at 2500 rpm for 1 min. Samples were centrifuged at 450*g* for 5 min, and the organic phase was transferred to glass vials. The extraction was repeated once with the organic phase pooled with the initial extract. Extracts were dried under nitrogen at 37 °C, redissolved in 80% methanol (0.4 mL) in water (v/v), and filtered (0.2 μm syringe filter, Alltech Associates, Inc.) prior to analysis. Replicate aliquots of cell sonicates were extracted without enzyme pretreatment to determine the amount of free equol in cells. Cellular levels of conjugated equol were calculated by subtracting the amount of free equol (no enzyme treatment) from the amount of total equol equivalents (+ β-glucuronidase/sulfatase). Similarly, aliquots

(0.5 mL) of medium from the apical and basolateral compartments were combined with the mixture of β-glucuronidase/sulfatase (400 units/mL) in 0.5 mL of sodium acetate buffer (0.2 mol/L, pH 5), incubated, and extracted as above to determine equol equivalents. Replicate aliquots also were extracted without enzyme pretreatment to determine the amount of free equol in order to calculate the amount of conjugated equol.

Equol concentrations were determined using a Waters 2695 separations module (Milford, MA), a Waters 2996 photodiode array detector, and a Nova-Pak C-18 reversed-phase column (150 mm × 3.9 mm i.d., 4 μm, 60 Å pore size) with an inline Nova-Pak C-18 guard column (20 mm × 3.9 mm i.d., 4 μm). Sample injection volume was ≤20 μL, and data were collected from 210 to 400 nm. HPLC separation was achieved using a mobile phase consisting of 1.0% acetic acid in water (v/v; solvent A), acetonitrile (solvent B), and methanol (solvent C) with the following linear solvent gradient at 0.55 mL/min (25 ± 5 °C): 0−1 min, 75% A, 12% B, 13% C; 1−14 min, from 75 to 49% A, from 12 to 25% B, from 13 to 26% C; 14−15 min, from 49 to 10% A, from 25 to 45% B, from 26 to 45% C; 15−19 min, no change; 19−20 min, from 10 to 75% A, from 45 to 12% B, from 45 to 13% C; and, 20−25 min, no change. Equol was identified by retention times and UV absorption spectra of pure (≥98%) equol standard. Samples were quantitated using standard curves with HPLC peak areas as a function of concentrations that were determined spectrophotometrically.

Statistics. SPSS release 14.0 for Windows (SPSS, Inc., Chicago, IL) was used for all statistical analyses. All experiments had a minimum of three independent observations for each test group. Each experiment was replicated at least once such that $N \geq 6$ unless indicated otherwise. Data were expressed as means ± SEM when applicable. Comparison of means between two groups was performed using independent Student's t tests. Comparison of means between more than two groups was performed using one-way analysis of variance (ANOVA) followed by Bonferroni post hoc comparison of statistical significance. Linear regression was performed when correlational analysis was warranted. Results were considered to be statistically significant when $P < 0.05$.

RESULTS

A preliminary study showed that equol was stable (>95% recovery) in test medium added to wells without cells and incubated for as long as 8 h. Initial experiments were conducted with Caco-2 cells grown on plastic to characterize the apparent uptake of equol from the apical compartment. Because all samples were treated with the β-glucuronidase/sulfatase mixture before extraction (i.e., the extent of phase II metabolism of equol was not determined in these experiments), results are presented as equol equivalents. Cellular accumulation of equol equivalents during 4 h of incubation was directly proportional ($r^2 = 0.99$) to the initial concentration of equol in the medium (**Figure 2**). Intracellular quantities of equol equivalents represented 4.6−6.4% of the initial amount of equol in the medium. No gross morphological signs of toxicity were observed after incubation in medium containing as much as 20 μmol/L equol. When Caco-2 cells were incubated in medium containing 10 μmol/L equol for 0.5−8 h, apparent uptake was rapid with maximum intracellular amounts accumulated within 1 h (**Figure 3**). By 8 h, intracellular equol equivalents decreased ($P < 0.05$) to 60% of the amount that had accumulated at 1 h. This decrease in cellular equol equivalents suggested efflux or conversion of equol to metabolites. The rapid accumulation of equol equivalents by Caco-2 cells also suggested that equol passively diffused into the cells. This possibility was supported by the observation that cellular levels of equol equivalents were 30% greater ($P < 0.05$) in Caco-2 cells incubated in medium with equol at 4 °C compared to 37 °C.

To examine equol metabolism and transport, Caco-2 cells were cultured on transwell inserts. The maximum intracellular accumulation of equol equivalents occurred within the first hour of incubation from medium containing 10 μmol/L free equol

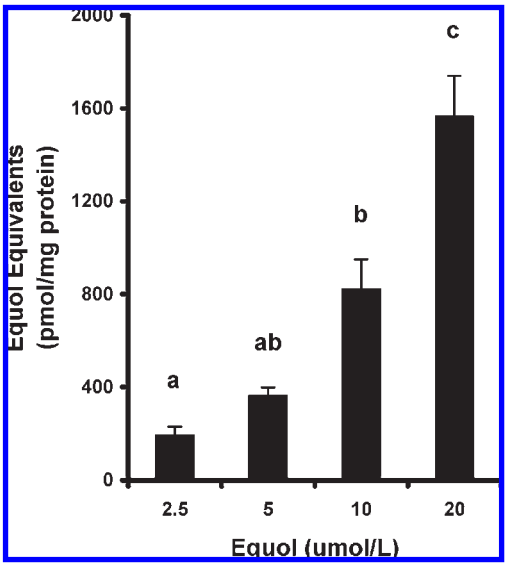

Figure 2. Apparent uptake of equol by Caco-2 cells is proportional to its concentration in medium. Caco-2 cells were incubated in DMEM containing the indicated concentrations of equol for 4 h. Washed cells were collected, incubated with β-glucuronidase, and analyzed by HPLC as described under Materials and Methods. Data are means ± SEM ($N = 6$ at each concentration). Means that do not share a common letter differ significantly ($P < 0.05$) from one another as indicated by one-way analysis of variance (ANOVA) followed by Bonferroni posthoc comparison of statistical significance.

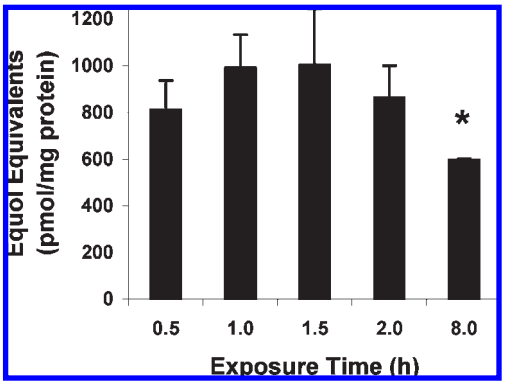

Figure 3. Maximum cellular accumulation of equol equivalents occurs by 1 h but decreases with increased incubation. Caco-2 cells were exposed to 10 μmol/L equol for the indicated times. Cellular content of equol equivalents was determined as described under Materials and Methods. Data are means ± SEM ($N = 6$ for each exposure time). The presence of an asterisk above the bar at 8 h indicates that the cellular content of equol equivalents was significantly less ($P < 0.05$) than that at 1 h as determined by an independent t test.

(**Figure 4A**). Glucuronidase/sulfatase-sensitive (phase II) conjugates of equol accounted for all equol that was detected in cells from 0.5 to 2 h. Cellular equol equivalents declined rapidly after 1 h with the concentration of conjugated equol steadily increasing in both the apical (**Figure 4B**) and basolateral (**Figure 4C**) compartments. By 4 h, 73% of equol was present as β-glucuronidase/sulfatase-sensitive conjugates with approximately 47 and 26% of initial equol distributed in apical and basolateral compartments, respectively. By 8 h, approximately 98% of the equol in cultures existed as phase II conjugates with nearly 73% of the total located in the apical compartment. Approximately 20% of total equol in the culture was present as free equol in the

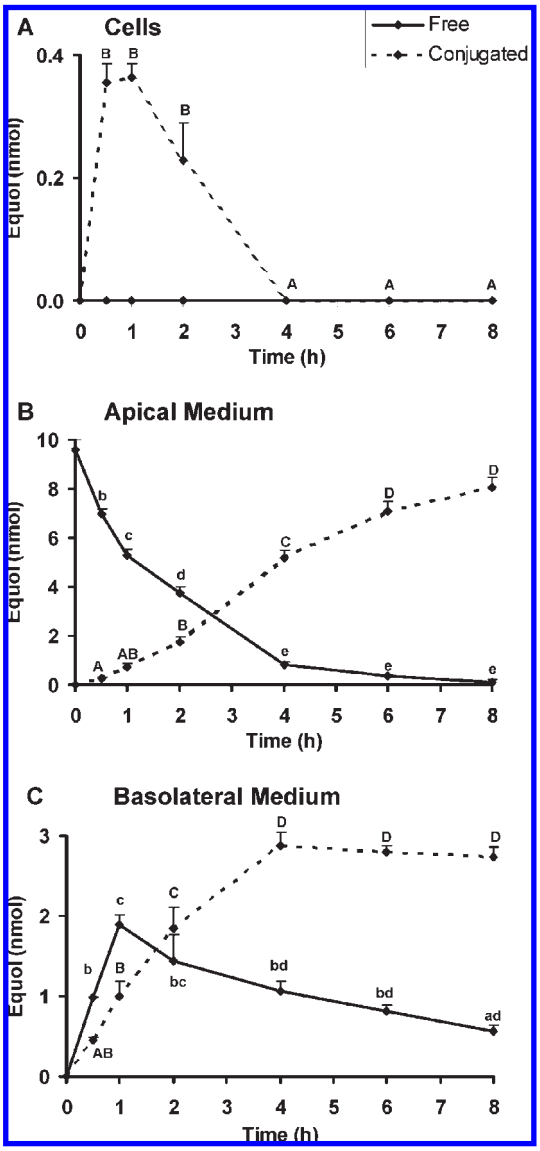

Figure 4. Uptake, metabolism, and transport of equol by Caco-2 cells. Equol (10 μmol/L) was added to the apical compartment of monolayers of Caco-2 cells on transwell inserts at 0 h. Cells and apical and basolateral media were collected from 0 to 8 h and analyzed by HPLC as described under Materials and Methods. Data are means ± SEM for four to six replicate cultures. Means within a free (lower case) or conjugated (upper case) group for the cellular (**A**), apical (**B**), or basolateral (**C**) compartment that do not share a common letter differ significantly ($P < 0.05$) from one another by one-way analysis of variance (ANOVA) followed by Bonferroni posthoc comparison of statistical significance. Note that indicators of statistical significance at 0 h are "A" for conjugated equol and "a" for free equol. Differences between means for conjugated and free equol are not depicted here.

basolateral compartment at 1 h. This reflected transepithelial transport of free equol because barrier function of the monolayers was intact as assessed by apical to basolateral flux of phenol red. The concentration of free equol in the basolateral compartment steadily decreased thereafter without a change in equol equivalents in the well. This suggested retrotransport of equol across the basolateral membrane with subsequent conjugation and efflux into the apical and basolateral compartments. In a separate experiment, cells were exposed to spent medium containing 98% equol conjugates. Concentrations of equol equivalents in apical and basolateral compartments remained unchanged after 4 h, indicating that only free equol is transported into Caco-2 cells (data not shown).

DISCUSSION

To our knowledge this is the first study to address the transport and metabolism of equol in Caco-2 human intestinal cells. Here, we show that equol rapidly reaches its maximum concentration in Caco-2 cells and is extensively metabolized to phase II conjugates that are effluxed across the apical and basolateral membranes of Caco-2 cells. As the enzyme preparation from *H. pomatia* used in our study has both glucuronidase and sulfatase activities, the speciation of the equol conjugates produced by Caco-2 cells will require further investigation. Partial conversion of genistein and daidzein to glucuronide and sulfate derivatives and the transport of the free and conjugated species of these isoflavonoids into the apical and basolateral compartments of Caco-2 cell cultures have been previously reported (*25*, *26*). Similarly, free and conjugated species of genistein and daidzein are present in the plasma of human subjects after the administration of soy isoflavonoids (*27*, *28*), and genistein glucuronides were the predominant conjugates in the plasma of rats administered genistein (*29*, *30*).

Equol transported from the apical to the basolateral compartment in the Caco-2 transport model represents the bioavailable fraction of the metabolite. We found that nearly 20% of the starting amount of equol in the apical compartment was transported as free equol to the basolateral chamber within 1 h. In vivo, this fraction of free equol would be transported to peripheral tissues, where it would be taken up, metabolized, and eventually excreted in urine or bile. As the duration of incubation in the closed Caco-2 culture system increased, the concentration of free and conjugated equol in the basolateral compartment decreased and increased, respectively, with the conjugates representing 77% of the total equol equivalents by 6 h. Glucuronides are the predominant conjugate (>90%) of equol in humans (*2*, *31*) and rats (*32*), although free equol and sulfate conjugates also have been identified. Our results suggest that at least a portion of the equol conjugates present in human plasma are synthesized during the absorption process. Efflux of intracellular equol across the apical membrane of Caco-2 cells is representative of excretion into the intestinal lumen in vivo. Efflux of conjugated equol into the apical compartment was evident within 0.5 h and increased to approximately 73% of the initial concentration by 8 h. This suggests that the efflux of conjugates across the apical membrane is more efficient than the transfer of equol conjugates across the basolateral membrane.

We observed significantly higher intracellular quantities of equol in Caco-2 cells incubated at 4 °C compared with those incubated at 37 °C. This is presumably because equol passively diffused across the apical membrane and both the activity of phase II enzymes and efflux of conjugated equol were attenuated at the lower temperature. Several other investigators reported similar rates for the transcellular flux of genistein from the apical to basolateral and basolateral to apical compartments, suggesting passive diffusion of this compound across the plasma membrane of Caco-2 cells (*33*, *34*).

Phase II metabolism of equol may attenuate in vivo bioavailability and bioefficacy of this compound. Ju and colleagues (*35*) reported that equol significantly increased proliferation of MCF-7 estrogen-dependent human breast cancer cells in vitro. However, dietary supplementation of equol failed to increase proliferation of MCF-7 cells implanted in ovariectomized athymic mice. The authors speculated that this lack of estrogenic response in vivo is due to "deactivation" of equol by phase II metabolism. Similarly, Selvaraj et al. (*36*) found that mice administered equol

subcutaneously had significantly higher uterine masses than mice that were fed equol. We observed that phase II conjugates of equol were not taken up by Caco-2 cells incubated in conditioned medium containing only conjugated equol (data not shown). Equol conjugates effluxed into the gastrointestinal lumen may be hydrolyzed by microbial β-glucuronidases and further metabolized by the microflora. Alternatively, free equol may be taken up by intestinal epithelial cells once again for transport across the basolateral membrane as the free or conjugated compound (*37*, *38*).

In light of the potential for equol to act as a natural health-promoting compound, attempts have been made to modulate its production by manipulating the intestinal microflora. Equol production requires a microbial consortium, but may also be affected by a number of nongenomic and genomic factors such as carbohydrate and fat intake (*19*, *20*, *39*), alcohol intake (*39*), food matrix (*40*), race (*41*), and gender (*19*). Recent demonstration that the addition of an equol-producing bacterial consortium isolated from feces of equol producers converted a nonproducing flora to an equol-producing flora in vitro (*17*) provides support for continued efforts to manipulate the microbial community in the host gut.

A potential limitation of our study is that differentiated Caco-2 cells are phenotypically more similar to small intestinal enterocytes than colonocytes. Because equol production likely occurs exclusively in the colon (*42*), the relevance of exposure to small intestine-like cells may be challenged. However, an alternative strategy for obtaining the health-promoting benefits of equol is oral administration. Setchell et al. (*2*) has reported that an oral dose of 25 mg of equol was rapidly absorbed with maximum plasma concentration observed after 4−6 h. We have found that equol, much like its precursor daidzein (*43*), is stable during simulated gastric and small intestinal digestion and is readily bioaccessible (data not shown). This further supports the beneficial potential of orally administered equol to individuals classified as equol "non-producers".

In summary, our results suggest that free equol passively diffuses across the apical membrane of Caco-2 cells with approximately 20% of the total from the apical compartment transported as free equol into the basolateral compartment within 1 h. Intracellular equol not transported across the basolateral membrane was efficiently converted to phase II conjugates that were effluxed across both the apical and basolateral membranes. We speculate that differences in the extent of phase II metabolism and efflux of equol may contribute to the wide variance reported in the apparent bioavailability of equol in individuals classified as equol "producers" (*44*). Numerous polymorphisms that affect the expression and function of gene products that participate in phase I and II metabolism and the transport of xenobiotics have been identified (*45*). For example, variants of the human UGT1A3 gene coding for UDP-glucuronosyltransferase 1A3 have been shown to affect the efficiency of glucuronidation of quercetin, luteolin, kaempferol, and estrone (*46*). Moreover, the frequency distribution of alleles and single-nucleotide polymorphisms in UGT1A3 was found to significantly differ for Chinese Han and German−Caucasian populations (*46*). Elucidation of the specific enzymes and transporters responsible for equol metabolism and the affects of polymorphisms in their respective genes are likely to provide further insights about the bioavailability and bioefficacy of equol in individuals.

ABBREVIATIONS USED

AAPH, 2,2′-azobis(2-amidinopropane) dihydrochloride; BCA, bicinchoninic acid; DHD, dihydrodaidzein; DMEM, Dulbecco's modified Eagle's medium; HPLC, high-performance liquid chromatography; LDL, low-density lipoprotein; PBS, phosphate-buffered saline; UVA, ultraviolet A.

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Received April 8, 2009. Revised manuscript received July 28, 2009. Accepted July 29, 2009. Supported by USDA IFAFS Grant 2001-52102-11333 and the Ohio Agricultural and Research Development Center.