Cellular uptake of soy-derived phytoestrogens \textit{in vitro} and in human whole blood

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Abstract: Epidemiological studies comparing typical Western and traditional Eastern lifestyles indicate that dietary intake of soy-derived phytoestrogens, including genistein, daidzein, and equol, may have significant health protective effects on hormone-dependent cancers, osteoporosis and cardiovascular diseases. Phytoestrogens have been demonstrated to exert varying effects depending on tissue, endogenous hormone concentrations, and receptor types. Thus, a detailed understanding of the biodistribution and bioavailability of specific phytoestrogens is required in order to predict the subsequent biologic activities. In this study we aimed to investigate the cellular uptake of these soy-derived phytoestrogens in different cell types, including the mammary MCF-7/6 and MDAB-MB 231 cell lines, the ovarian Ishikawa Var-I cell lines and in murine adipocyte clusters. Furthermore, the biodistribution between serum and cell fraction was also investigated in human whole blood. Equol generally shows a higher cellular uptake when compared with genistein and daidzein. Therefore, equol may be more potent with respect to its relative bioactivity, which is corroborated by the observations of specific health effects associated with the equol-producer phenotype.

Key words: soy, phytoestrogens, genistein, daidzein, equol, biodistribution, cellular uptake

INTRODUCTION

Phytoestrogens are mostly non-steroidal polyphenolic plant-derived compounds that functionally mimic the activity of the human estrogen, estradiol-17β [1]. The most important phytoestrogens in the human diet are the soy-derived isoflavones, daidzein and genistein. In addition, the gastrointestinal microflora can further metabolize daidzein into the more potent estrogen equol, but this biotransformation is subject to a high inter-individual variability [2]. An increasing number of epidemiological and experimental studies suggest that the consumption of phytoestrogen-rich diets may have protective effects on estrogen-related conditions, such as menopausal symptoms [3], and estrogen-related diseases, such as prostate and breast cancers [4, 5], osteoporosis [6], and cardiovascular diseases [7]. However, consumption of too high levels of these compounds may not be without risk.

Phytoestrogens have been demonstrated to exert varying effects depending on tissue, endogenous hormone concentrations, and receptor type(s). Thus, a detailed understanding of the biodistribution and bioavailability of specific phytoestrogens is required in order to predict the subsequent biologic activities. In this study, we aimed to investigate the cellular uptakes of genistein, daidzein, and equol in different cell types. By adding the compounds individually or combined, competition effects for the estrogen receptors were investigated. Furthermore, the role of the estrogen receptor was probed by adding a pure estrogen receptor antagonist (ICI 182,780) together with the soy-derived phytoestrogens, in addition to studying the cellular uptake of the compounds in an estrogen receptor-negative cell line, MDA-MB 231. As isoflavones and derivatives are partly hydrophobic in nature, and as estrogen receptors are also present in adipose tissue [8], we speculated whether the cellular uptake of isoflavones in adipocytes would differ from that found in the human mammary and uterine carcinoma cell lines. It has, indeed, been reported that environmental estrogens such as polychlorinated bisphenols (PCBs) can accumulate in human adipose tissue [9].

MATERIALS AND METHODS

MCF-7/6 cells. MCF-7/6 cells were obtained from Dr. H. Rochefort (Unite d’Endocrinologie Cellulaire et Moléculaire, Montpellier, France) and are variants of the originally derived MCF-7 cells (from a pleural effusion of a metastasised mammary adenocarcinoma [10]). These cells possess fully characterised estrogen receptors, and several cellular activities, such as proliferation and migration \textit{in vitro}, are modulated by 17β-estradiol and other ligands of the steroid/thyroid receptor superfamily [11]. The cells were grown and cultured in a (50:50, v:v) mixture of DMEM-Ham-12, supplemented with 100 IU/ml penicillin (Gibco, Merelbeke, Belgium), 100 µg/ml streptomycin (Gibco), 2.5 µg/ml Fungizone® (Bristol-Myers Squibb, Brussels, Belgium), and 10% foetal bovine serum (FBS) in 6-well plates (Nunc, Roskilde, Denmark) in a water-
saturated atmosphere of 10% CO2, in air at 37°C. On the day of assay (near confluence), the cell medium was aspirated, the wells were washed twice with phosphate-buffered saline (PBS), and the test compounds were added to 4 ml of fresh medium containing serum (in duplicate). 0.1 M stock solutions of genistein, daidzein, and equol in DMSO were diluted in DMEM/Ham F12 to yield a final concentration of 10 μM before being added to the 6-well plates containing the cells. The compounds were either added individually or combined. The final dilution of DMSO in the cell medium did not exceed 1/1000. ICI 182,780 was added at a final concentration of 10 nM in the assay medium 10 min prior to test compounds (near confluency), the cell medium was aspirated off, the wells were washed twice with PBS, and the test compounds were added to 4 ml of fresh medium containing serum in air at 37°C. The cell medium was aspirated off, the cells were washed twice with PBS, and the test compounds added to 4 ml of fresh FBS. The cells were plated at 1.5 x 10^5 cells/75 cm² flask and passaged twice weekly. Twenty-four hours before the start of an experiment, near-confluent cells were harvested with 0.25% trypsin and plated in 6-well plates in an estrogen-free basal medium (EFBM), containing phenol red-free Ham F12 and 5% charcoal-stripped FBS. The cells were plated in 6-well plates (Nunc, Roskilde, Denmark) in a 95% humidified atmosphere at 37°C.

On the day of assay (near confluence), the cell medium was aspirated off, the wells were washed twice with PBS, and the test compounds added in 4 ml of fresh EFBM (in duplicate). 0.1 mM stock solutions of genistein, daidzein, and equol in ethanol were diluted to concentrations of 10 μM in medium before being added to the 6-well plates containing the cells. Compounds were added either individually or combined. Stock solutions provided a final dilution in the cell medium of 1/1000. Incubation times ranged from 1 h to 24 h.

After the end of incubation, the medium of each well was removed and transferred into falcon tubes. The wells were washed twice with DMEM/Ham F12 and each wash was added to the respective falcon tube. 90 μl internal standard solution (50/50 v:v) containing 0.05% formic acid. The samples were analysed using HPLC. The results are expressed as percentage uptake. Total lysates were prepared by washing the cells 3 times with PBS, and the test compounds were added to 4 ml of fresh medium containing serum (in duplicate). 0.1 M stock solutions of genistein, daidzein, and equol in ethanol were diluted to concentrations of 10 μM in medium before being added to the 6-well plates containing the cells. Compounds were added either individually or combined. Stock solutions provided a final dilution in the cell medium of 1/1000. Incubation times ranged from 1 h to 4.5 h.

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fragments. The tissue was placed directly in PBS containing collagenase (10 µg/ml) and then minced into fragments that were placed in 6-well plates. Five PBS tablets, 20 g bovine serum albumin (BSA), and 10 mg collagenase were added to 1 l of water into a sterile conical flask and the flask placed on a magnetic stirrer for ca.1 h. The pH was adjusted to 7.4 by adding hydrochloric acid or NaOH. Adipocytes were grown and cultured in (50/50, v:v) mixture of DMEM/Ham F12 supplemented with 100 IU/ml penicillin (Gibco, Merelbeke, Belgium), 100 µg/ml streptomycin (Gibco), 10% foetal calf serum (FCS) and 1.65% 1 mM HEPES buffer.

To test the experimental design and conditions, eppendorf tubes with murine adipocyte clusters were incubated with 50 µl tritiated 17β-estradiol (H-E2) at a concentration of 600 nM in 5 ml charcoal-stripped DMEM, with an incubation time of 30 min at 37°C in a 95% humidified atmosphere. An eppendorf tube containing H-E2, in medium was included as a control. After incubation, the clusters were washed with PBS and the medium, and together with PBS, was analysed using a beta counter (Beckman LS 6000IC Liquid Scintillation Counter, Beckman, Fullerton, California, USA) (10 min counts). The difference in the total amount of H-E2 added to the adipocyte clusters and the amount recovered was used as the adipocyte cellular uptake.

The same experiment was repeated, but with a combined addition of genistein, daidzein, and equol at final concentrations of 10 µM in DMEM (40 µl were added to the wells, respectively, and left to evaporate before 4 ml of medium was added). In addition, one 6-well plate was incubated with a combined solution of genistein, daidzein, and equol at a final concentration of 10 µM in order to investigate the reproducibility in uptake across the wells. The incubation time for all these plates was set to 30 min, after which the medium was removed and added to small falcon tubes. The clusters were washed with PBS, which was also added to the corresponding falcon tubes. The medium, together with PBS from each well, was extracted with 90 µl internal standard (8 mg 4-HBPH in ethyl acetate) and 7 ml ethyl acetate. The solvent was evaporated under a gentle stream of nitrogen and the residues redissolved in 150 µl water:methanol (50/50, v:v) containing 0.05% formic acid. Concentrations of genistein, daidzein, and equol were analysed using HPLC.

To determine whether the cellular uptake of soy-phytoestrogens was time-dependent, and whether co-incubation of the 3 compounds together would cause competition effects for the ERs, genistein, daidzein, and equol were added separately to the wells and the solvent was evaporated before the medium was added. The final concentration of each compound in each well was 10 µM. All experiments were carried out in duplicate in 6-well plates. Incubation times were 10, 30, 90, and 270 min, respectively, at 37°C in a 95% humidified atmosphere. Next, the medium was removed, the cells were washed with PBS, and the medium and PBS for each well extracted with 90 µl internal standard (8 mg 4-HBPH in ethyl acetate) and 7 ml ethyl acetate. The solvent was evaporated under a gentle stream of nitrogen and the residues redissolved in 150 µl water:methanol (50/50, v:v) containing 0.05% formic acid.

The samples were analysed using HPLC. The HPLC-analyses were carried out using a Waters 2690 Separations HPLC module equipped with a 96% photodiode array detector (Waters, Brussels, Belgium). Chromatographic processing was carried out using Waters Millennium version 3.2 software. The reversed-phase silica column was an X Terra™ MS C18 5 µm, 4.6 x 250 mm (Waters, Brussels, Belgium). Analyses were carried out using the following gradient: 0-20 min: 90% A in B, 20-27 min: 70% A in B, 27-30 min: 90%, with solvent A being water and solvent B being acetonitrile both acidified with 0.05% formic acid. The flow rate was 1.5 ml/min. The injection volume was 20 µl. Ultraviolet detection was typically conducted at 254 nm.

Statistical methodology. All experiments were carried out in triplicate; means and SD were calculated. The statistical package SPSS for Windows version 15.0 (Chicago, IL, USA) was used and results were considered significant when P<0.05. The Kolmogorov-Smirnov and Levene's test showed that all data were normally distributed and had equal variances, respectively. Therefore, all comparisons were carried using the 1-way ANOVA with Bonferroni error protection and the paired Student’s t-test.

RESULTS

Genistein, daidzein, and equol were added at a concentration of 10 µM to the medium of MCF-7/6 cells. Cellular uptake was estimated by measuring the remaining concentrations of each compound in the medium after an incubation period of 1 h (Figure 1). For genistein and daidzein, 50-60% of the original level was found, whereas for equol only 15% could be retrieved. On the other hand, the concentrations of the respective soy-derived phytoestrogens in the corresponding cell lysates were below the level of detection; therefore, the levels lost in the medium could not be accounted for.
To further elucidate the cellular uptake of soy-derived phytoestrogens and the role of the estrogen receptors, genistein, daidzein, and equol, at a concentration of 10 μM, were added to MDA MB 231 cells. This cell line exhibits no functional estrogen receptors, and therefore provided a valuable tool for this investigation. Highly analogous results were found for the remaining levels of all phytoestrogens in the medium after an incubation period of 1h (results not shown). However, in the corresponding cell lysates, a small amount of equol equaling 0.3% of the administered dosage could be retrieved. The addition of anti-estrogen ICI 182,780 (10 nM) in either type of cell did not show any impact on the results.

The cellular uptake of genistein, daidzein, and equol was also investigated in the Ishikawa Var-1 cell line (Figure 2), both in serum-containing and in serum-free medium. After incubation for 1h all phytoestrogens were present in the medium at levels of 40-50% of the original concentration. In contrast to the results found with the mammary carcinoma cell lines, equol showed a similar response to genistein and daidzein. No significant differences were noticed between the presence or absence of serum for both genistein and daidzein. However, significantly less equol could be retrieved in the medium after incubation in the presence of serum (*p<0.05).

Next, the cellular uptake of phytoestrogens in murine adipocyte clusters was investigated. First, as a control, the uptake of 17β-estradiol was investigated using a tritium labeled probe. The mean number of counts of 3H-E₂ in medium from murine adipocyte clusters was found to be lower than the counts in medium only, indicating cellular uptake of estrogens in adipocytes (Table 1).

After dosing genistein, daidzein, and equol to murine adipocyte clusters in medium, increased cellular concentrations of genistein, daidzein, and equol in murine adipocytes were determined with increased incubation times (Figure 3). These observations are in line with a general decrease in concentrations in the medium. Cellular uptake of equol was not evident until 90 min, but increased further up to 270 min.

Finally, the distribution of genistein, daidzein, and equol was investigated in cellular and serum fractions of human blood 24 h after intake of 0.5 l soymilk, and 8 h after intake of 1 soy tablet EB 605. At 24 h after soymilk intake, only equol could be detected with an approximate 2:1 ratio in serum and cell fraction, respectively, while, 8 h after soy tablet intake, all compounds could be detected in both the serum and cellular fractions in blood (Figure 4). Equol was present in very small concentrations, while genistein and daidzein were equally

<table>
<thead>
<tr>
<th>Sample</th>
<th>Mean counts per min (cpm) of β-radiation</th>
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<tr>
<td>Medium (control)</td>
<td>188,457</td>
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<tr>
<td>Medium from murine adipocyte clusters (n = 2)</td>
<td>94,393</td>
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* Significantly different at p<0.05.

Figure 1 Recovery (%) of genistein, daidzein, and equol (10 μM) in medium of MCF-7/6 cells after 1 h of incubation (n = 6, mean ± SD).

Figure 2 Recovery (%) of genistein, daidzein, and equol (10 μM) in medium of Ishikawa Var-1 cells. Cells were kept in respective media for 24 h before the start of the experiment to establish possible effects of serum proteins on cellular uptake of these compounds. The incubation period was 0.5 h (n = 6, mean ± SD).

Figure 3 Recovery (%) of genistein, daidzein, and equol (10 μM) in a) murine adipocyte clusters, b) in medium of murine adipocytes over time (n = 4, mean ± SD).

Figure 4 Recovery (%) of genistein, daidzein, and equol (10 μM) in serum-free DMEM and DMEM from murine adipocytes.
distributed both in serum and in the cellular fractions with a serum/cell fraction ratio of 10.

**DISCUSSION**

Cellular uptakes of genistein, daidzein, and equol in MCF-7/6 cells, Ishikawa Var-1 cells, MDA-MB 231 cells, and murine adipocyte cells were examined by measuring the respective concentrations in the medium and, if possible, in the cells. Furthermore, the distribution of these soy-derived phytoestrogens in the serum and cell fraction of whole human blood was also investigated. After incubation with MCF-7/6 cells, genistein and daidzein were retrieved from the medium in significantly higher concentrations than equol. This suggests a high and preferential uptake of equol by the MCF-7/6 cells. The discrepancy between the phytoestrogen concentrations in the medium and those in the cell fractions (below detection level) may suggest either extensive loss during the washing step or rapid biotransformation. Considering the relatively large wash volume, it is likely that some or all of the phytoestrogens originally present in the cells were removed during washing. When all compounds were co-incubated at 10 μM in the medium, a highly analogous pattern of uptake was observed (results not shown). Furthermore, the cellular uptake of genistein, daidzein, and equol (10 μM) in MCF-7/6 cells in the presence of ICI 182,780 (10 nM) was investigated (results not shown). Interestingly, the uptake of the soy-derived phytoestrogens in MCF-7/6 cells was largely unaffected by the addition of ICI 182,780, suggesting that estrogen receptors are not involved in the cellular uptake mechanism. This is also substantiated by the higher affinities of genistein for both ERα and ERβ, compared with that of daidzein, which, in that case, should lead to a higher uptake of genistein. Nagel et al. suggested that some compounds with low affinity for the estrogen receptor require longer incubations to reach steady-state conditions [14]. They also found that estrogen receptor down-regulation in MCF-7/6 cells occurred between 1–5 h, but the concentrations of estrogen receptors did not change further between 8–18 h. However, during initial experiments to optimize the incubation times, no significant differences in isoflavone uptake over time were observed for the tested cells. Further confirmation for the non-involvement of estrogen receptors was obtained with MDA-MB 231 cells. These cells are derived from a mammary adenocarcinoma cell line and the 231 variant lacks both estrogen receptors. The results were highly analogous to those obtained with MCF-7/6 cells. Interestingly, the results with Ishikawa Var-1 cell lines differed significantly from the mammary cell lines. For all soy-derived phytoestrogens ca 50% of the applied concentration could be retrieved from the medium, both in serum-free and serum-added conditions. Both serum albumin and sex hormone binding globulin (SHBG), that can bind the studied phytoestrogens, are present in charcoal-stripped serum; however significant amounts of SHBG are denatured during heat inactivation of the serum (the final concentration in 10% serum is < 3 nmol/l). It is indicated, therefore, that, at least under the conditions described here, the presence or absence of these proteins will not greatly influence cellular uptake. As such, only slightly less equol could be found after incubation in the presence of serum.

Extensive metabolism of particular phytoestrogens has been observed in MCF-7 and other mammary cancer cell lines, and this metabolism seems to differ depending on the cell lines [15]. Nagel et al. found that after incubation with MCF-7 cells in serum-free medium for 18 h, genistein and equol were not metabolised, whereas only 15% coumestrol remained after incubation in the serum-free medium [14]. In serum, genistein and coumestrol were metabolised to a similar extent, 53 and 68%, respectively. Peterson et al. showed that genistein was taken up and concentrated 20-fold above the medium concentration within human mammary epithelial cells [16]. Growth inhibition of various isoflavones in ZR-75-1, BT-20, T47D and MCF-7 cells was studied and it was found that the latter 2 cell lines produced hydroxylated and methylated metabolites of genistein and biochanin A, whereas the other cell lines did not. Peterson et al. further hypothesised that the metabolism may render more active forms of genistein (and biochanin A) in human breast cancer cells [15]. At a genistein concentration of 3.7 μM, they found a substantial conversion (89–100%) of genistein into a more polar product in each of the cell lines. However, for higher concentrations of genistein, great differences between the metabolites from each of the cell lines were observed. This is

![Figure 4](image-url) Concentrations of genistein, daidzein, and equol (10 μM) in serum and cells after soy isoflavone intake. a) Blood was taken from a female volunteer 24 h after intake of 0.5 l soy milk; b) blood was taken from a female volunteer 8 h after intake of a soy tablet (300 mg genistein/genistin, 111 mg daidzein/daidzin, 45 mg glycitein/glycitin) (Mean ± SD of 3 injections).
in contrast to a non-transformed human mammary epithelial cell line that did not significantly metabolise genistein nor biochanin A [16]. This is also in contradiction to the findings by Nagel et al. (1998), who found no metabolism of genistein and equol after incubation with MCF-7 cells in serum-free medium for 18 h [14]. However, they did observe significant metabolism of coumestrol (15% recovery) under the same conditions, in addition to pronounced metabolism of genistein and coumestrol in serum-containing medium (53% and 68%, respectively). Furthermore, enterolactone has been found to be rapidly sulphated by both MCF-7 human breast cancer cells and HepG2 human liver cancer cells, and the monosulphate was the predominant metabolite in these cell cultures [17]. Therefore, it is possible that the reduced concentrations of genistein and, possibly, also of equol and daidzein, found in the medium in some of the experiments, is due not only to cellular uptake, but also to metabolism of the compounds. However, given the relatively short incubation times and the serum free conditions used, the contribution of biotransformation to the reduced recoveries is most likely limited.

The cellular uptake of genistein, daidzein, and equol was also investigated in murine adipocyte clusters. Adipose tissue is specialised connective tissue that functions as the major storage site for fat in the form of triglycerides. The adipocyte is one of the most metabolically active tissues in the human body, nearly tripling the blood circulation of any other organ. These cells are unique in comparison to any other cell type in the human body, and their hydrophobic nature renders investigation of the concentration of dietary estrogens interesting. Furthermore, in addition to xenoestrogens, it is known that tamoxifen accumulates to some extent in human breast fat tissue [18]. From the radioactive 17β-estradiol (E2) count, it is clear that E2 was taken up by the adipocyte clusters. In addition, the concentrations of soy-derived phytostrogens in the medium were monitored over time, showing increasing cellular and decreasing media concentrations of both genistein and daidzein. Equol concentrations decreased in the medium for a period up to 270 min. Although equol was only detected at very low levels initially, it was found in the highest amounts in the adipocyte clusters at later time points.

Finally, cellular uptake of soy-derived phytostrogens was also studied in human blood fractions. It is generally known that cellular concentrations are better indicators of long-term dietary intake compared to serum concentrations (e.g. for vitamin C [19]). It is known that the half-lives of both genistein and daidzein are between 6-8 h, whereas, for equol, due to its intestinal microbial production from daidzein, the half-life approaches 24 h [20]. We therefore studied human sera and cellular fractions after soy isoflavone intakes to establish the concentrations of genistein, daidzein, and equol. At 24 h after 0.5 l soymilk intake, some daidzein was still detected in serum; however, equol was found in both serum and cell fractions. This is consistent with the longer half-life of equol compared with the 2 isoflavones. At 8 h after intake of 3 soy tablets (300 mg genistein/gensitin, 111 mg daidzein/daidzin, 45 mg glycitein/glycitin), both genistein and daidzein were found in the serum and cellular fraction, whereas the level of equol was much smaller.

The combined results suggest that the uptake of soy-derived phytostrogens into several cell types may be different depending on the nature of the compound. The observed differences indicate the importance of detailed investigations of bioavailability and biodistribution in order to estimate possible effects observed both in vitro and in vivo. The cellular uptake, and therefore the ability to elicit biological activities, may be somewhat higher for equol than for either genistein and daidzein. This may contribute to the health effects due to soy intake observed in equol producers versus non-equol producers [21-26].

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