

## The effect of oestrogen and dietary phyto-oestrogens on transepithelial calcium transport in human intestinal-like Caco-2 cells

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Recently, dietary phyto-oestrogens (PO) have been suggested as possible alternatives to oestrogen therapy (hormone replacement therapy) as a means of preventing bone loss associated with ovarian hormone deficiency. While PO, which exhibit oestrogen-like activity, act directly on bone cells, their protective effect on bone may be partly due to their ability to enhance Ca absorption. Therefore, the aim of the present study was to investigate the effect of 17 $\beta$ -oestradiol and two commonly consumed soyabean PO (genistein and daidzein) on Ca absorption in the human Caco-2 intestinal-like cell model. Caco-2 cells were seeded onto permeable filter supports and allowed to differentiate into monolayers. On day 21, the Caco-2 monolayers (*n* 8–18 per treatment), grown in oestrogen-replete or -deplete media, were then exposed to 10 nM-17 $\beta$ -oestradiol, 1 nM-1,25-dihydroxycholecalciferol, or 50  $\mu$ M-genistein or -daidzein for 24 h. After exposure, transepithelial and transcellular transport of <sup>45</sup>Ca and fluorescein transport (a marker of paracellular diffusion) were measured. As expected, 1,25-dihydroxycholecalciferol stimulated Ca absorption in Caco-2 cells, by up-regulating transcellular transport, whereas 17 $\beta$ -oestradiol had no effect on Ca absorption. Unexpectedly, both PO decreased Ca absorption (by about 17–19% compared with control, *P* < 0.05), by reducing transcellular Ca transport in Caco-2 cells grown in oestrogen-replete media. This inhibitory effect disappeared when monolayers were grown in oestrogen-deplete media. In conclusion, PO at high luminal concentrations either had no effect or reduced Ca absorption in Caco-2 cells, dependent on oestrogen status. The effect of lower concentrations of these compounds needs to be investigated.

### Oestrogen: Phyto-oestrogens: Calcium absorption: Caco-2 cells

Oestrogen deficiency is a major contributory factor to the development of osteoporosis in women. In postmenopausal women it is associated with increased bone turnover and acceleration of bone loss, leading to increased susceptibility to bone fractures (Riggs & Melton, 1986; Nguyen *et al.* 1995). Oestrogen therapy (or hormone replacement therapy) remains the mainstay for prevention of bone loss in postmenopausal women (Gallagher, 2001). Decreased Ca absorption due to ovarian hormone deficiency is also corrected by hormone replacement therapy (Heaney *et al.* 1978; Gallagher *et al.* 1980; for review, see Gallagher, 1990). However, fewer than one in four postmenopausal women decide to use hormone replacement therapy, and within 6 months, >60% of

them withdraw due to concerns over an increased risk of malignancy and other side effects (Taylor, 1997).

Recently, attention has been focused on the so-called dietary phyto-oestrogens (PO) as possible alternatives, or at least adjuncts, to hormone replacement therapy. The PO are non-steroidal compounds naturally occurring in foods of plant origin (especially soyabean-based foods), and they are able to compete with the principle oestrogens of most mammals (17 $\beta$ -oestradiol and oestrone) for binding oestrogen receptors (OR; Cassidy, 1996). Several studies have shown that when ovariectomized rats were treated with either soyabean with its naturally-occurring isoflavones, a genistein-rich soyabean isoflavone preparation or purified genistein or daidzein, they retained

**Abbreviations:** FBS, fetal bovine serum; LDH, lactate dehydrogenase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Neutral Red, 3-amino-7-dimethylamino-2-methylphenazine hydrochloride; OR, oestrogen receptor; PO, phyto-oestrogen; TEER, transepithelial electrical resistance; 1,25(OH)<sub>2</sub>D<sub>3</sub>, 1,25-dihydroxycholecalciferol.

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significantly more bone mass than untreated, control animals (Blair *et al.* 1996; Arjmandi *et al.* 1996, 1998*a,b*; Picherit *et al.* 2000, 2001). To date, a limited number of relatively short-term (3–6 months) clinical studies with small subject numbers ( $n$  37–69) have examined the effect of soyabean protein or soyabean protein isolate with normal or reduced isoflavone levels on bone mineral density and/or bone mineral content (Dalais *et al.* 1998; Potter *et al.* 1998; Alekel *et al.* 2000; Hsu *et al.* 2001) and these have produced inconclusive results (for reviews, see Arjmandi, 2001; Arjmandi & Smith, 2002). Interestingly, Morabito *et al.* (2002) recently reported that in a randomized double-blind placebo controlled study, genistein treatment (56 mg/d) for 12 months was as effective as hormone replacement therapy in preventing bone loss in early postmenopausal women.

While PO act directly on bone cells (Gao & Yamaguchi, 2000; Yamaguchi & Sugimoto, 2000), it is also conceivable that their protective effect on bone may be partly due to their ability to enhance Ca absorption. Some of the PO compounds structurally resemble oestrogen (Anderson *et al.* 1999), and thus, similarly to oestrogen (Heaney *et al.* 1978; Gallagher *et al.* 1980; for review, see Gallagher, 1990) may have the ability to enhance intestinal Ca absorption. In support of this contention, Omi *et al.* (1994) reported that intestinal Ca absorption was significantly ( $P < 0.05$ ) greater in ovariectomized rats given soyabean milk-containing diets (rich in phyto-oestrogenic compounds such as genistein and daidzein) than in rats given a control diet (containing no soyabean milk) for 28 d. The authors suggest that it is possible that the enhancement in intestinal Ca absorption was the mechanism by which bone mineral density and mechanical strength of bone was significantly increased ( $P < 0.05$ ) in the rats fed the soyabean milk-containing diet relative to control animals. In addition, Arjmandi *et al.* (2002) recently reported that the rate of *in vitro* Ca transport by intestinal cells of ovariectomized rats fed soyabean protein with normal isoflavone content was significantly ( $P < 0.05$ ) greater than that from ovariectomized control animals. The effect, however, of isolated phyto-oestrogenic compounds, such as genistein and daidzein, on Ca absorption in rats has not been reported. Ipriflavone, a synthetic PO that has a bone-sparing effect in rats (Arjmandi *et al.* 2000) and human subjects (Agnusdei *et al.* 1997; Gennari *et al.* 1998; Ohta *et al.* 1999), has also been shown to enhance *in vitro* Ca uptake by rat duodenal cells (Arjmandi *et al.* 2000).

Whether PO mediate a reduction in bone loss via a direct positive effect on bone metabolism, or because of a positive effect on Ca absorption, is not known. Therefore, the aim of the present study was to investigate the effect of  $17\beta$ -oestradiol, two commonly consumed soyabean PO (genistein and daidzein) and the parent compounds of the latter (genistin and daidzin, which do not have oestrogenic activity (Miksicek, 1995)), on Ca absorption in human Caco-2 intestinal-like cells.

Caco-2 cells have been suggested to be a suitable model for predicting Ca absorption in human subjects (Giuliano & Wood, 1991; Fleet & Wood, 1999). In culture, Caco-2 cells spontaneously differentiate and form a polarized epithelial

monolayer with tight junctions and express a differentiated cell phenotype consistent with absorptive small intestine-like enterocytes (Pinto *et al.* 1983; Yee, 1997). In particular, these cells have a functional vitamin D receptor (Giuliano *et al.* 1991) and have Ca transport kinetics that suggest the presence of both a saturable and non-saturable Ca transport pathway, similar to that which has been observed in the intestine of human subjects and animals (Fleet & Wood, 1999).  $1,25$ -Dihydroxycholecalciferol ( $1,25(\text{OH})_2\text{D}_3$ ) treatment induces the saturable, but not diffusional, component of Ca transport (Giuliano & Wood, 1991) and induces accumulation of calbindin  $\text{D}_{9\text{K}}$  and 24-hydroxylase mRNA in these cells (Fleet & Wood, 1994; Fleet *et al.* 1996). In addition, the Caco-2 cells possess an OR (Campbell-Thompson *et al.* 2001). Therefore, this relatively simple *in vitro* method appears to be a good model for predicting Ca bioavailability in human subjects.

## Materials and methods

### Materials

Tissue culture materials, including Dulbecco's modified Eagle's medium with L-glutamine and sodium bicarbonate, fetal bovine serum (FBS), minimum essential medium, non-essential amino acids and PBS were purchased from Sigma-Aldrich Ireland Ltd, Dublin, Ireland.  $^{45}\text{Ca}$  (as  $^{45}\text{Ca}$  in an aqueous solution of  $\text{CaCl}_2$ , with a specific activity of  $1.85 \text{ MBq/mg Ca}$ ) was purchased from Nensure<sup>TM</sup>, Boston, MA, USA. Fluorescein sodium salt, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 3-amino-7-dimethylamino-2-methylphenazine hydrochloride (Neutral Red),  $17\beta$ -oestradiol,  $1,25(\text{OH})_2\text{D}_3$ , genistein, daidzein, genistin and daidzin were purchased from Sigma-Aldrich Ireland Ltd.

### Conditions of cell culture

The human colon adenocarcinoma cell line, Caco-2, was purchased from the European Collection of Animal Cell Cultures (Salisbury, Wilts., UK). Cells were routinely grown in  $75 \text{ cm}^2$  plastic culture flasks (Costar, Cambridge, MA, USA) in Dulbecco's modified Eagle's medium supplemented with non-essential amino acids (10 ml/l) and FBS (100 ml/l). Caco-2 cells were maintained at  $37^\circ\text{C}$  in  $\text{CO}_2$ -air (5:95, v/v). Cells were seeded at  $3 \times 10^4/\text{cm}^2$  and passaged when reaching 90% confluency. Cells used in transepithelial Ca transport experiments were seeded at a density of  $3 \times 10^4/\text{cm}^2$  onto permeable Transwell<sup>®</sup> filter inserts (24 mm diameter,  $0.4 \mu\text{m}$  pore size; Costar). Cell culture media (with FBS and containing Phenol Red) was changed on alternate days for 21 d. In one series of experiments, cell culture media (with FBS and containing Phenol Red) was changed on alternate days for 18 d after which cell culture media (without FBS and Phenol Red-free) was used for the last 3 d before the Ca transport study. These conditions created a low-oestrogen status for the Caco-2 cells immediately preceding their exposure to treatments. For mRNA studies, cells ( $3 \times 10^4/\text{cm}^2$ ) were seeded into six-well culture plates

(35 mm diameter; Costar). For viability studies, cells ( $3 \times 10^4/\text{cm}^2$ ) were seeded into six-well and forty-eight-well culture plates (Costar).

#### *Cell viability and cytotoxicity assays*

The effect of  $17\beta$ -oestradiol,  $1,25(\text{OH})_2\text{D}_3$ , genistein, genistin, daidzein and daidzin on Caco-2 cell viability was investigated using the MTT (Mossmann, 1983; Edmonson *et al.* 1988) and Neutral Red (Hunt *et al.* 1987) cell viability assays in forty-eight-well culture plates (Costar). Briefly, cells were seeded into forty-eight-well plates at  $3 \times 10^4/\text{cm}^2$  and allowed to grow for 21 d. Cells were then treated for 24 h with test compounds. Stock solutions of all test compounds were prepared in dimethyl sulfoxide, except for  $1,25(\text{OH})_2\text{D}_3$ , which was prepared in absolute ethanol. Test compounds were diluted in tissue culture medium with a final concentration of the carrier solvent not exceeding 2 ml/l. For the MTT assay, following the 24 h exposure to the test compounds, 100  $\mu\text{l}$  stock solution of MTT (3 mg/ml PBS, pH 7.4) was added to each well and the culture plate was incubated for a further 3 h at  $37^\circ\text{C}$ . The medium was then removed and the forty-eight-well culture plate was frozen at  $-20^\circ\text{C}$  overnight. After thawing, isopropanol (1 ml) was added to each well to solubilize the formazan dye. The absorbance was read at 570 nm on a plate reader. Results were expressed as a percentage of control representing the surviving fraction relative to control samples. A value  $< 85\%$  of the control value was taken as a benchmark for toxicity. For the Neutral Red assay, following the 24 h exposure to the test compounds, 1 ml media containing Neutral Red (50 mg Neutral Red (dissolved in 500  $\mu\text{l}$  dimethyl sulfoxide)/l media) was added to each well and the forty-eight-well culture plate was incubated for a further 3 h at  $37^\circ\text{C}$ . The medium was then removed and PBS added to each well. Following the removal of PBS, glacial acetic acid (10 g/l ethanol (500 ml/l) was added to solubilize the dye. The absorbance was read at 570 nm on a plate reader. Results were expressed as a percentage of the control value representing the surviving fraction relative to control samples. A value  $< 85\%$  of the control value was taken as a benchmark for toxicity.

Lactate dehydrogenase (LDH) release from cells into the surrounding medium was determined as an index of cytotoxicity (Vassault, 1983). Cells were seeded into six-well plates at  $3 \times 10^4/\text{cm}^2$  and grown for 21 d. Cells were exposed to the test compounds for 24 h as described for the MTT and Neutral Red assays. A portion (40  $\mu\text{l}$ ) of medium was taken from each well for LDH analysis and placed in a forty-eight-well plate. The remaining medium was then removed from the cells and the cells washed with PBS. The cells were then scraped and suspended in a solution of Triton X-100 (10 ml/l distilled water). The suspended cells were placed in Eppendorf tubes and sonicated on ice for 10 s. Samples were left on ice for 30 min and then centrifuged for 10 min at 1500 g. LDH analysis was carried out using a commercially available colorimetric kit assay (product no. DK1340-K; Sigma-Aldrich Ireland Ltd). The decrease in rate of absorbance (measured at 340 nm) for each sample was determined

(due to formation of NAD, as LDH reduces pyruvate to lactate). LDH release into the medium was expressed as a percentage of total LDH released from cells treated with Triton X-100.

#### *$17\beta$ -Oestradiol stimulation of Caco-2 cell growth*

The responsiveness of Caco-2 cells to  $17\beta$ -oestradiol-stimulated cell growth was assessed by the method of Di Domenico *et al.* (1996). In brief, Caco-2 cells were seeded at  $3 \times 10^4/\text{cm}^2$  in  $25 \text{ cm}^2$  plastic culture flasks (Costar) in Dulbecco's modified Eagle's medium, supplemented with non-essential amino acids (10 ml/l) and charcoal stripped heat-inactivated FBS (50 ml/l). Following 24 h adherence to the flask, media was removed and cells were fed media without  $17\beta$ -oestradiol (control) or media containing  $17\beta$ -oestradiol (10 nM) with or without 1  $\mu\text{M}$ -4-hydroxytamoxifen (Sigma-Aldrich Ireland Ltd). In order to achieve maximum effect, 1  $\mu\text{M}$ -4-hydroxytamoxifen was added 1 h prior to addition of  $17\beta$ -oestradiol. Media were replaced every second day for 6 d. On days 2, 3, 5 and 6 cells were passaged using Phenol Red-free trypsin-EDTA and cells counted in a haemocytometer in quadruplicate. Each experiment was carried out in triplicate.

#### *Transepithelial electrical resistance*

For all transport experiments, the transepithelial electrical resistance (TEER) was checked prior to the experiment by a Millicell<sup>®</sup> ERS meter (Millipore Corp., Bedford, MA, USA) connected to a pair of thin side-by-side electrodes. TEER readings were taken at  $37^\circ\text{C}$ . A TEER value  $\geq 400 \Omega \cdot \text{cm}^2$  was used as an indicator that the epithelial layer was intact and ready to use for Ca transport studies.

#### *Cell treatments*

For Ca transport experiments, cells grown in the Transwell<sup>®</sup> inserts (Costar) were treated with vehicle only (for control), 1 nM- $1,25(\text{OH})_2\text{D}_3$  (positive control), 10 nM- $17\beta$ -oestradiol or 50  $\mu\text{M}$ -genistein, -genistin, -daidzein or -daidzin for 24 h. All compounds were added to complete culture medium prior to their addition to the cells. The vehicle never exceeded 2 ml/l. TEER measurements were taken immediately prior to treatment with test compounds and 24 h after treatment.

#### *Transepithelial calcium transport studies*

The method used for determining Ca transport across the Caco-2 membrane in the present study is a modification of the methods of Giuliano & Wood (1991) and Fleet & Wood (1994). Transepithelial transport of Ca was studied with Caco-2 cells grown on permeable membrane supports for 21 d, by which time the cells are fully differentiated. On the day of an experiment, after cells were treated for 24 h with the test compounds, the medium was removed and the inserts rinsed with buffer. The buffer was prepared fresh before use and consisted of 140 mM-NaCl, 5.8 mM-KCl, 1.2 mM- $\text{CaCl}_2$ , 0.8 mM- $\text{MgSO}_4$ , 0.44 mM- $\text{KH}_2\text{PO}_4$ ,

0.34 mM- $\text{Na}_2\text{HPO}_4$ , 4 mM-glutamine, 25 mM-glucose and 20 mM-4-(2-hydroxy-ethyl)-1-piperazine-ethanesulfonic acid, pH 7.4. After rinsing, 2.6 ml buffer was added to the lower chamber of the Transwell<sup>®</sup> inserts (Costar). At time 0, 1.5 ml transport buffer was added to the upper chamber of the Transwell<sup>®</sup> inserts. This consisted of the same buffer as the lower chamber except it also contained  $^{45}\text{Ca}$  (with an activity of 148 kBq/ml) and 5.3 mM-fluorescein (as the sodium salt). Fluorescein was included in the transport buffer as a means of measuring paracellular (diffusional) transport across the Caco-2 monolayer (Lindmark *et al.* 1998). Following the addition of the transport buffer to the Transwell<sup>®</sup> inserts (Costar) the plates were covered and incubated at 37°C in a shaking water-bath (set to forty-eight oscillations per min) for 60 min. At 30 and 60 min after addition of  $^{45}\text{Ca}$ -labelled transport buffer, duplicate portions (10  $\mu\text{l}$ ) of the buffer from the lower chamber (basolateral buffer) were taken from each well and placed in wells of a blackened ninety-six-well plate (Costar). When samples had been taken at both time points, 200  $\mu\text{l}$  50 mM-*n*-ethylmorpholine buffer (pH 8.0) was added to each of the ninety-six wells and fluorescence (excitation 485 nm, emission 535 nm) was measured in a Spectrafluor+ Tecan fluorescence plate reader (Hombrechtikon, Switzerland). In addition, at the same time points duplicate portions (50  $\mu\text{l}$ ) of the basolateral buffer were taken for determination of  $^{45}\text{Ca}$  content. Samples of the basolateral buffer were placed in scintillation vials and 5 ml liquid scintillation cocktail (biodegradable counting scintillant; Amersham International Plc., Amersham, Bucks., UK) was added to each vial. Counts were measured on a Beckman LS 6500 multipurpose liquid scintillation counter (Beckman Instruments Inc., Fullerton, CA, USA). An equal volume of fresh basolateral buffer was added back to the lower chamber following each sampling point. The concentration of fluorescein appearing in the lower buffer after 60 min was determined using a standard curve of fluorescein and this value was expressed as a percentage of the total fluorescein added to the upper chamber of the Transwell<sup>®</sup> inserts (Costar). This represented the paracellular route of Ca transport. The amount of  $^{45}\text{Ca}$  appearing in the basolateral buffer was expressed as a percentage of the total  $^{45}\text{Ca}$  applied to the upper chamber. This represented total transepithelial  $^{45}\text{Ca}$  transport (i.e. by both the paracellular and transcellular transport routes) and was expressed both as %/h and nmol transported/min per well during the 30–60 min time interval. By subtracting the percentage of fluorescein transport per h from the total  $^{45}\text{Ca}$  transport (%/h), the amount of  $^{45}\text{Ca}$  crossing the Caco-2 cell monolayer by the transcellular (active) route was calculated and expressed as nmol/well per min. In all studies, at least three wells were examined per treatment. Experiments were repeated three times.

#### *Reverse transcription–polymerase chain reaction analysis for mRNA levels*

After experimental treatments, cells (grown in FBS–Phenol Red-containing media) were harvested and RNA was isolated and analysed for calbindin  $\text{D}_{9\text{K}}$  and glyceraldehyde-3-phosphate dehydrogenase. RNA was also isolated

from post-confluent, fully differentiated Caco-2 cells (grown in FBS–Phenol Red-containing media) and analysed for OR $\alpha$  and - $\beta$ . mRNA levels were determined by reverse transcription–polymerase chain reaction analysis (Wood *et al.* 2001). The glyceraldehyde-3-phosphate dehydrogenase mRNA level was used as a constitutively expressed control gene. Primer sets for glyceraldehyde-3-phosphate dehydrogenase, calbindin  $\text{D}_{9\text{K}}$ , OR $\alpha$  and - $\beta$  were derived from previously published sequences (Fleet & Wood, 1994; Arai *et al.* 2000; Wood *et al.* 2001). To minimize the potential for variability in the reverse transcriptase reaction, cDNA was prepared from total cellular RNA for all samples at the same time, using the same reagents. Polymerase chain reaction products were subjected to electrophoresis (agarose (2%) gel). Relative amounts of amplified polymerase chain reaction product from each experimental condition were visualized under u.v. light and digitized with the Kodak Digital Science DC 120 Zoom Digital Camera and Electrophoresis Documentation System (Eastman Kodak Company, Rochester, NY, USA). Relative amounts of the product were estimated by digital densitometry using Kodak Digital Science Analysis System 120 quantification software (Eastman Kodak Company, Scientific Imaging Systems, Rochester, NY, USA). Calbindin  $\text{D}_{9\text{K}}$  expression was normalized relative to the expression of glyceraldehyde-3-phosphate dehydrogenase mRNA.

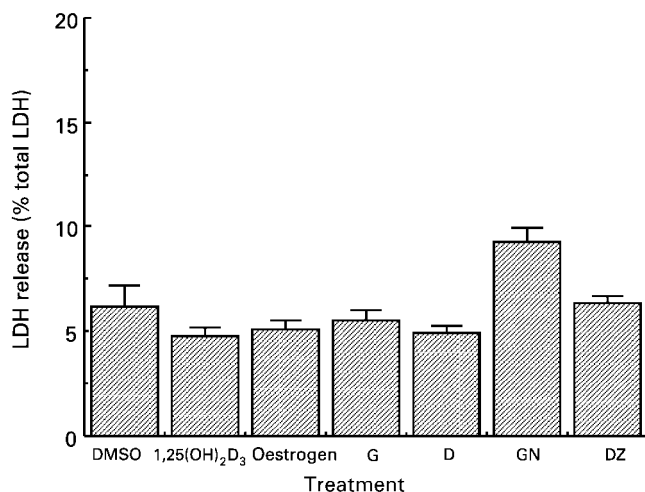
#### *Statistical methods*

Data for all variables (except the % LDH release data) were normally distributed and allowed for parametric tests of significance. The % LDH release data was cos transformed prior to statistical analysis, to achieve a near-normal distribution. Results are presented as mean values with their standard errors. Treatment effects were compared by one-way ANOVA, with variation attributed to concentration of treatment compound (cell viability and cytotoxicity data) or type of treatment compound (cell growth data and Ca transport data) (Snedecor & Cochran, 1967). To follow up the ANOVA, all pairs of mean values were compared by the method of least significant difference (Snedecor & Cochran, 1967).

#### **Results**

Treatment with 50  $\mu\text{M}$ -genistein, -genistin, -daidzein, or -daidzin for 24 h had no cytotoxic effects in Caco-2 cells, relative to controls, as assessed by the LDH release assay (Fig. 1). There was no effect of incremental concentrations (0–50  $\mu\text{M}$ ) of genistein, genistin, daidzein and daidzin on Caco-2 cell survival and viability after 24 h of exposure as determined using the MTT assay, which is based on mitochondrial dehydrogenase activity, and the Neutral Red assay, which is based on cellular uptake of the dye (results not shown). In addition, exposure of Caco-2 cells to  $1,25(\text{OH})_2\text{D}_3$  (positive control for the Ca transport experiments) and  $17\beta$ -oestradiol (in the range of  $10^{-10}$ – $10^{-6}$  M for both compounds) for 24 h had no effect on cell viability, using the MTT and Neutral Red assays, or on cell cytotoxicity (at a concentration of 1 nM



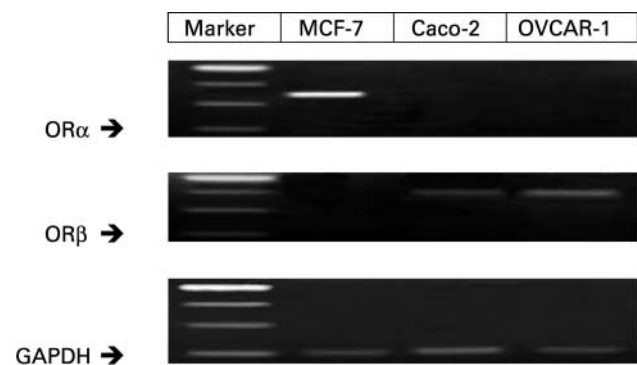


**Fig. 1.** Effect of treatment for 24 h on the percentage of lactate dehydrogenase (LDH) release from Caco-2 cells. LDH activity in the medium was measured and expressed as a percentage of total LDH released from Caco-2 cells treated with Triton X-100 (for further details, see p. 756). Treatments were: dimethyl sulfoxide (DMSO, control); 1 nM-1,25-dihydroxycholecalciferol (1,25(OH)<sub>2</sub>D<sub>3</sub>); 10 nM-17 $\beta$ -oestradiol (oestrogen); 50  $\mu$ M-genistein (G); 50  $\mu$ M-daidzein (D); 50  $\mu$ M-genistin (GN); 50  $\mu$ M-daidzin (DN). Values are means with their standard errors shown by vertical bars for at least three independent experiments. Data were not normally distributed and thus were transformed (cos) before statistical analysis. The effect of treatment on % LDH release was analysed using one-way ANOVA of the (cos) transformed data. No significant ( $P > 0.05$ ) concentration effects were found.

for 1,25(OH)<sub>2</sub>D<sub>3</sub> and 10 nM for 17 $\beta$ -oestradiol), using the LDH release assay (results not shown).

The post-confluent, fully differentiated Caco-2 cells used in the present study (grown in FBS-Phenol Red-containing media) expressed mRNA for OR $\beta$  but not - $\alpha$  (Fig. 2). cDNA from MCF-7 (breast cancer) and OVCAR-1 (ovarian cancer) cells in culture were used as positive controls for mRNA for OR $\alpha$  and - $\beta$  respectively.

Addition of 10 nM-17 $\beta$ -oestradiol to the Phenol Red-free medium containing charcoal-stripped, heat-inactivated FBS



**Fig. 2.** Expression of oestrogen receptor (OR)  $\beta$  but not OR $\alpha$  in Caco-2 cells. Reverse transcriptase-polymerase chain reaction for OR $\alpha$  or - $\beta$  and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were performed simultaneously on mRNA from MCF-7 (breast) cells or from OVCAR-1 (ovarian) cells (positive controls for OR $\alpha$  and - $\beta$  respectively) and on mRNA from Caco-2 cells. Marker, 500 (double band), 400, 300 and 200 bp. For details of procedures, see p. 756. Representative ethidium bromide staining of OR $\alpha$  (345 bp), OR $\beta$  (393 bp) and GAPDH (200 bp) transcripts.

induced a significant ( $P < 0.001$ ) increase in Caco-2 cell number (Fig. 3). Inclusion of 1  $\mu$ M-4-hydroxytamoxifen, a partial oestrogen agonist, prevented the 17 $\beta$ -oestradiol-induced proliferative effect (Fig. 3).

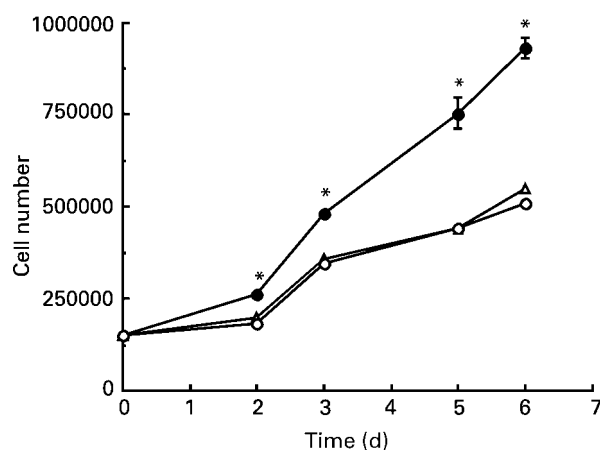
Treatment of fully differentiated Caco-2 cell monolayers (grown in media containing FBS and Phenol Red) with 1 nM-1,25(OH)<sub>2</sub>D<sub>3</sub>, 10 nM-17 $\beta$ -oestradiol, or 50  $\mu$ M-genistein, -genistin, -daidzein or -daidzin for 24 h had no effect on TEER in the present study (Tables 1 and 2).

Treatment of Caco-2 cell monolayers (grown in media containing FBS and Phenol Red) with 1 nM-1,25(OH)<sub>2</sub>D<sub>3</sub> for 24 h significantly ( $P < 0.05$ ) increased the total transepithelial Ca transport compared with control values (Tables 1 and 2). Furthermore, while paracellular Ca transport (as indicated by fluorescein transport) was unaffected, transcellular Ca transport was significantly increased ( $P < 0.05$ ) by the 1,25(OH)<sub>2</sub>D<sub>3</sub> treatment (Tables 1 and 2).

Treatment of Caco-2 cell monolayers (grown in media containing FBS and Phenol Red) with 10 nM-17 $\beta$ -oestradiol for 24 h had no effect on total transepithelial, paracellular or transcellular Ca transport (Table 1). Unexpectedly, treatment of Caco-2 cell monolayers (grown in media containing FBS and Phenol Red) with 50  $\mu$ M-genistein or -daidzein for 24 h significantly ( $P < 0.05$ ) decreased total transepithelial Ca transport, by about 19 and about 17% respectively compared with control values (Table 1). Furthermore, while paracellular transport was unaffected, transcellular Ca transport was significantly ( $P < 0.05$ ) decreased by treatment with both PO (Table 1).

Treatment of Caco-2 cell monolayers (grown in media containing FBS and Phenol Red) with 50  $\mu$ M-genistein or -daidzin (the parental compounds for genistein and daidzein respectively) for 24 h had no effect on total transepithelial, paracellular or transcellular Ca transport (Table 2).

Total transepithelial, paracellular and transcellular Ca transport were unaffected in Caco-2 cell monolayers in



**Fig. 3.** Effect of 17 $\beta$ -oestradiol and partial oestrogen agonist (hydroxytamoxifen) on Caco-2 cell growth. Cells were grown in the absence (○) (control) and in the presence of either 10 nM-17 $\beta$ -oestradiol (●) or 10 nM-17 $\beta$ -oestradiol and 1  $\mu$ M-4-hydroxytamoxifen (Δ). For details of procedures, see p. 756. Values are means with their standard errors shown by vertical bars for the cell number of each well averaged from three different experiments. Means values were significantly different from those of the controls: \* $P < 0.05$ .

**Table 1.** Effect of 17 $\beta$ -oestradiol, genistein, daidzein and 1,25-dihydroxycholecalciferol (1,25(OH) $_2$ D $_3$ ) on calcium transport in Caco-2 cell monolayers cultured in oestrogen-replete media\*  
(Mean values with their standard errors)

Treatment†	n	Calcium transport									
		Total transepithelial				Transcellular		Paracellular		TEER	
		nmol/well per min		%h		(nmol/well per min)‡		(%h)		( $\Omega \cdot \text{cm}^2$ )	
		Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Control	16	0.42 <sup>a</sup>	0.03	1.94 <sup>a</sup>	0.10	0.37 <sup>a</sup>	0.02	0.23 <sup>a</sup>	0.02	748 <sup>a</sup>	22
1 nM-1,25(OH) $_2$ D $_3$	17	0.50 <sup>b</sup>	0.02	2.26 <sup>b</sup>	0.06	0.45 <sup>b</sup>	0.02	0.19 <sup>a</sup>	0.01	710 <sup>a</sup>	24
10 nM-17 $\beta$ -Oestradiol	18	0.39 <sup>ac</sup>	0.02	1.77 <sup>ac</sup>	0.10	0.34 <sup>ac</sup>	0.02	0.23 <sup>a</sup>	0.02	747 <sup>a</sup>	21
50 $\mu$ M-Genistein	9	0.34 <sup>c</sup>	0.02	1.60 <sup>c</sup>	0.09	0.30 <sup>c</sup>	0.02	0.22 <sup>a</sup>	0.01	727 <sup>a</sup>	33
50 $\mu$ M-Daidzein	9	0.35 <sup>c</sup>	0.02	1.66 <sup>c</sup>	0.07	0.30 <sup>c</sup>	0.02	0.20 <sup>a</sup>	0.02	755 <sup>a</sup>	29
Statistical significance of effect (one-way ANOVA): P		0.0003		$\leq 0.0001$		$\leq 0.0001$		0.521		0.680	

TEER, transepithelial electrical resistance (after 24 h exposure to the different treatments).

<sup>a,b,c</sup>Mean values within a column with unlike superscript letters were significantly different (ANOVA followed by least significant difference test,  $P < 0.05$ ).

\* For details of procedures, see p. 756.

† Treatments were given for 24 h before measurement of calcium transport.

‡ Transcellular transport is total calcium transport corrected for paracellular (fluorescein) transport (for details, see p. 756).

which FBS and Phenol Red had been withdrawn for 72 h (i.e. low-oestrogen conditions) previous to their exposure to 10 nM-17 $\beta$ -oestradiol or 50  $\mu$ M-genistein or -daidzein for 24 h (Table 3). Similarly, 50  $\mu$ M-genistein or -daidzein had no effect on TEER in these monolayers. Treatment of the Caco-2 monolayers with 1 nM-1,25(OH) $_2$ D $_3$  for 24 h, after the period of low-oestrogen exposure, significantly ( $P < 0.05$ ) increased total transepithelial and transcellular Ca transport, whereas TEER and paracellular Ca transport were unaffected (Table 3). Mean TEER values (range 1545–1721  $\Omega \cdot \text{cm}^2$ ) in these Caco-2 cell monolayers were significantly greater than those in monolayers grown in oestrogen-replete media (range 402–748  $\Omega \cdot \text{cm}^2$ ).

mRNA levels for calbindin D $_{9K}$  were significantly ( $P < 0.01$ ) increased in Caco-2 cells (grown in media containing FBS and Phenol Red) exposed to 1 nM-1,25(OH) $_2$ D $_3$  for 24 h compared with that in cells exposed to dimethyl sulfoxide (control) (Fig. 4). mRNA

levels for calbindin D $_{9K}$  were unaffected in Caco-2 cells (grown in media containing FBS and Phenol Red) which were exposed to 10 nM-17 $\beta$ -oestradiol or 50  $\mu$ M-genistein or -daidzein for 24 h, relative to control values (Fig. 4).

### Discussion

As expected, in the present study exposing Caco-2 cell monolayers in culture to 1 nM-1,25(OH) $_2$ D $_3$  for 24 h stimulated total transepithelial Ca transport. This finding is in agreement with the findings of other studies that have found that physiological concentrations of 1,25(OH) $_2$ D $_3$  enhanced Ca transport in Caco-2 cells (Giuliano & Wood, 1991; Fleet & Wood, 1994, 1999; Fleet *et al.* 1996; Chirayath *et al.* 1998; Shao *et al.* 2001). In the present study, the enhancement of total transepithelial Ca transport by 1,25(OH) $_2$ D $_3$  was accompanied

**Table 2.** Effect of genistin, diadzin and 1,25-dihydroxycholecalciferol (1,25(OH) $_2$ D $_3$ ) on calcium transport in Caco-2 cell monolayers cultured in oestrogen-replete media\*  
(Mean values with their standard errors)

Treatment†	n	Calcium transport									
		Total transepithelial				Transcellular		Paracellular		TEER	
		nmol/well per min		%h		(nmol/well per min)‡		(%h)		( $\Omega \cdot \text{cm}^2$ )	
		Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Control	10	0.69 <sup>a</sup>	0.02	3.16 <sup>a</sup>	0.05	0.65 <sup>a</sup>	0.02	0.23 <sup>a</sup>	0.01	409 <sup>a</sup>	10
1 nM-1,25(OH) $_2$ D $_3$	11	0.78 <sup>b</sup>	0.02	3.56 <sup>b</sup>	0.06	0.72 <sup>b</sup>	0.02	0.28 <sup>a</sup>	0.02	415 <sup>a</sup>	10
50 $\mu$ M-Genistin	10	0.64 <sup>a</sup>	0.02	3.04 <sup>a</sup>	0.08	0.59 <sup>a</sup>	0.02	0.27 <sup>a</sup>	0.03	411 <sup>a</sup>	11
50 $\mu$ M-Diadzin	11	0.65 <sup>a</sup>	0.03	3.03 <sup>a</sup>	0.07	0.60 <sup>a</sup>	0.03	0.25 <sup>a</sup>	0.02	402 <sup>a</sup>	7
Statistical significance of effect (one-way ANOVA): P		0.0007		$\leq 0.0001$		0.0009		0.277		0.800	

TEER, transepithelial electrical resistance (after 24 h exposure to the different treatments).

<sup>a,b</sup>Mean values within a column with unlike superscript letters were significantly different (ANOVA followed by least significant difference test,  $P < 0.05$ ).

\* For details of procedures, see p. 756.

† Treatments were given for 24 h before measurement of calcium transport.

‡ Transcellular transport is total calcium transport corrected for paracellular (fluorescein) transport (for details, see p. 756).

**Table 3.** Effect of 17 $\beta$ -oestradiol, genistein, diadzein and 1,25-dihydroxycholecalciferol (1,25(OH) $_2$ D $_3$ ) on calcium transport in Caco-2 cell monolayers cultured in oestrogen-deplete media\*

(Mean values with their standard errors)

Treatment†	n	Calcium transport									
		Total transepithelial				Transcellular (nmol/well per min)‡		Paracellular (%/h)		TEER ( $\Omega \cdot \text{cm}^2$ )	
		nmol/well per min		%/h		Mean	SE	Mean	SE	Mean	SE
Control	10	0.34 <sup>a</sup>	0.05	2.29 <sup>a</sup>	0.24	0.30 <sup>a</sup>	0.04	0.38 <sup>a</sup>	0.04	1721 <sup>a</sup>	47
1 nM-1,25(OH) $_2$ D $_3$	8	0.59 <sup>b</sup>	0.06	3.45 <sup>b</sup>	0.45	0.51 <sup>b</sup>	0.07	0.40 <sup>a</sup>	0.07	1664 <sup>a</sup>	99
10 nM-17 $\beta$ -Oestradiol	8	0.32 <sup>a</sup>	0.03	2.43 <sup>a</sup>	0.20	0.27 <sup>a</sup>	0.02	0.39 <sup>a</sup>	0.02	1545 <sup>a</sup>	31
50 $\mu$ M-Genistein	10	0.41 <sup>a</sup>	0.07	2.08 <sup>a</sup>	0.35	0.30 <sup>a</sup>	0.04	0.42 <sup>a</sup>	0.03	1560 <sup>a</sup>	26
50 $\mu$ M-Diadzein	10	0.36 <sup>a</sup>	0.05	2.32 <sup>a</sup>	0.29	0.31 <sup>a</sup>	0.04	0.36 <sup>a</sup>	0.05	1656 <sup>a</sup>	63
Statistical significance of effect (one-way ANOVA): P		0.018		0.045		0.013		0.951		0.203	

TEER, transepithelial electrical resistance (after 24 h exposure to the different treatments).

<sup>a,b</sup>Mean values within a column with unlike superscript letters were significantly different (ANOVA followed by least significant difference test,  $P < 0.05$ ).

\* For details of procedures, see p. 756.

† Treatments were given for 24 h before measurement of calcium transport.

‡ Transcellular transport is total calcium transport corrected for paracellular (fluorescein) transport (for details, see p. 760).

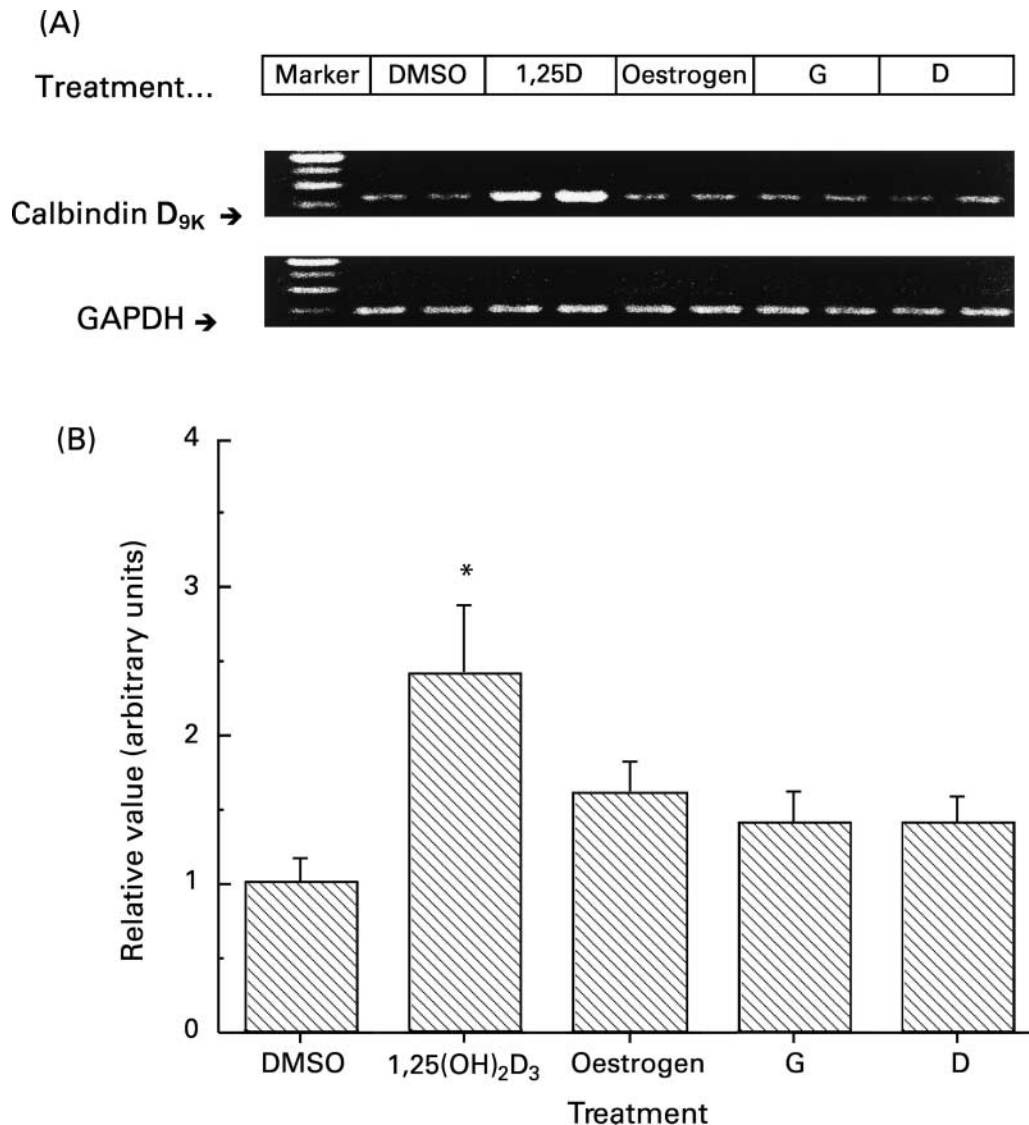
by an increased transcellular Ca transport, whereas paracellular Ca transport was unaffected. This finding is in agreement with the findings of several other studies (Giuliano & Wood, 1991; Fleet & Wood, 1994, 1999; Fleet *et al.* 1996). The significant enhancement of total transepithelial Ca transport by 1,25(OH) $_2$ D $_3$  in the present study acted as a positive control for our present experiments to investigate the influence of 17 $\beta$ -oestradiol and dietary PO on Ca transport in the Caco-2 model.

In the present study, treatment with 10 nM-17 $\beta$ -oestradiol for 24 h had no effect on total transepithelial Ca transport in differentiated Caco-2 cells (grown in FBS-Phenol Red-containing media). The concentration of 17 $\beta$ -oestradiol (10 nM) used in the present study, while high in terms of physiological circulating concentrations (typically about 325 and 75 pM in hormone replacement therapy-treated and non-treated postmenopausal women, respectively; Morabito *et al.* 2002), was chosen as it has been shown to significantly ( $P < 0.05$ ) increase Ca uptake in rat duodenal cells *in vitro* (Arjmandi *et al.* 1993). These findings agree with preliminary findings that suggest that 100 nM-17 $\beta$ -oestradiol does not influence Ca transport in Caco-2 cells grown in oestrogen-replete media (S Taparia and RJ Wood, personal communication). To test whether 17 $\beta$ -oestradiol might stimulate Ca transport in Caco-2 cells maintained in an oestrogen-depleted environment, the experiments were repeated in Caco-2 cell monolayers that were grown in media devoid of FBS and Phenol Red (which may have some oestrogenic activity; Welshons *et al.* (1988)) for 72 h before their exposure to 17 $\beta$ -oestradiol for a subsequent 24 h. However, under these conditions of low oestrogen status, 17 $\beta$ -oestradiol (10 nM) had no effect on Ca transport in Caco-2 cells. These *in vitro* findings, however, appear to be in contrast to the findings in oestrogen-depleted ovariectomized rats (O'Loughlin & Morris, 1998; Colin *et al.* 1999; Ten Bolscher *et al.* 1999; Arjmandi *et al.* 2000) and postmenopausal women (Heaney *et al.* 1978; Gallagher *et al.* 1980;

for review, see Gallagher, 1990), which suggest that oestrogen stimulates Ca absorption *in vivo*.

Ten Bolscher *et al.* (1999) suggest that oestrogen stimulates intestinal Ca absorption *in vivo* via an OR-mediated effect. The Caco-2 cells used in the present study expressed mRNA for OR $\beta$  but not  $\alpha$ . These findings are in agreement with the recent findings of Campbell-Thompson *et al.* (2001) which show that OR $\beta$  message and protein were expressed in Caco-2 cells, but that OR $\alpha$  mRNA were undetectable. Campbell-Thompson *et al.* (2001), on the other hand, showed that normal human colonic mucosa as well as colon tumour samples expressed mRNA for OR $\alpha$  and  $\beta$ , with mRNA for OR $\alpha$  being much lower than for OR $\beta$ . The lack of mRNA for OR $\alpha$ , and presumably of the OR $\alpha$  protein in the Caco-2 cells in the present study, although this was not determined, might, partly explain the lack of stimulatory effect of 17 $\beta$ -oestradiol on Ca transport. In the present study, addition of 10 nM-17 $\beta$ -oestradiol to a Phenol Red-free medium containing a charcoal-stripped, heat-inactivated FBS induced a proliferative effect in subconfluent, undifferentiated Caco-2 cells. Furthermore, hydroxytamoxifen, a partial oestrogen agonist, inhibited this proliferative effect. These findings, collectively, would suggest that the Caco-2 cells used in the present study were capable of a functional response to 17 $\beta$ -oestradiol. These findings are in agreement with those of Di Domenico *et al.* (1996), who investigated the functionality of the OR in Caco-2 cells.

The findings of the present study in the Caco-2 cell model suggest that genistein and daidzein, two commonly consumed soyabean-based PO, at relatively high concentrations reduced intestinal absorption of Ca. The concentration of isoflavones used in the present study (i.e. 50  $\mu$ M) was chosen to reflect luminal (small intestine) concentrations in subjects participating in the various dietary intervention trials which have investigated the effect of PO on bone (Dalais *et al.* 1998; Potter *et al.* 1998; Alekel *et al.* 2000; Hsu *et al.* 2001; Morabito *et al.* 2002). This is



**Fig. 4.** Effect of treatment for 24 h on calbindin D<sub>9K</sub> accumulation in Caco-2 cells. Treatments were: dimethyl sulfoxide (DMSO, control); 1 nM-1,25-dihydroxycholecalciferol (1,25(OH)<sub>2</sub>D<sub>3</sub>); 10 nM-17 $\beta$ -oestradiol (oestrogen); 50  $\mu$ M-genistein (G); 50  $\mu$ M-daidzein (D). Marker, 500 (double band), 400, 300 and 200 bp. Calbindin D<sub>9K</sub> mRNA levels were assessed by reverse transcriptase–polymerase chain reaction and normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression. For details of procedures, see p. 756. (A), a representative gel from one experiment (*n* 2); (B), relative expression of calbindin D<sub>9K</sub> mRNA levels. Values are means with their standard errors shown by vertical bars for at least six wells per treatment. The effect of treatment was analysed by one-way ANOVA followed by least significant difference test. Mean value was significantly different from that of the control: \**P*<0.05.

the first study, to our knowledge, to investigate the effect of PO on Ca absorption in a human intestinal cell line. These *in vitro* findings were unexpected because soyabean milks (rich sources of genistein and daidzein) have been shown to enhance Ca balance in experimental animals (Omi *et al.* 1992, 1994). For example, Omi *et al.* (1994) reported that intestinal Ca absorption, determined using the metabolic balance approach on three separate occasions throughout a 28 d feeding period, was significantly (*P*<0.05) greater in ovariectomized rats given a soyabean milk-containing diet than in rats given a control diet (containing no soyabean milk). Moreover, Arjmandi *et al.* (2002) recently reported that the rates of *in vitro* Ca transport by duodenal, ileal, and colonic cells of ovariectomized rats fed soyabean protein with normal isoflavone content

were significantly (*P*<0.05) greater than those from ovariectomized control animals. Interestingly, in that study an isoflavone-deplete soyabean protein also significantly (*P*<0.05) increased Ca transport by ileal cells, but not duodenal or colonic cells (Arjmandi *et al.* 2002), suggesting that perhaps the soyabean protein itself enhances the Ca transport even in the absence of isoflavones. The effect of isolated PO compounds on Ca absorption in rats has not been reported. However, Arjmandi *et al.* (2000) reported that ipriflavone, a synthetic PO, enhanced *in vitro* intestinal Ca transport in an ovariectomized rat model. In their model system, consumption of ipriflavone approximately doubled (*P*<0.05) the *in vitro* Ca uptake by intestinal cells from ovariectomized rats compared with that in cells from animals fed the control diet.



It should be noted that these studies were carried out in ovariectomized animals that would be expected to have a low endogenous oestrogen status. Therefore, to test whether the genistein and daidzein might stimulate Ca transport in Caco-2 cell monolayers in conditions of reduced oestrogen status, the Ca transport experiments in the present study were repeated in Caco-2 cell monolayers that were grown in media devoid of FBS and Phenol Red for 72 h before their exposure to the PO compounds for a subsequent 24 h. Under these conditions of low oestrogen status, the inhibitory effect of the two PO on Ca transport disappeared, but also of note, there was no evidence of a stimulatory effect on Ca transport.

It is possible that the inhibitory effect of the two PO, at high luminal concentrations, on Ca transport in Caco-2 cells grown in oestrogen-replete conditions in the present study was due to their binding to the OR $\beta$  in the intestinal cells and hence competitively inhibiting oestrogen uptake. This mechanism is believed to underpin the inhibitory effect of isoflavones on breast and colon tumour growth (Messina *et al.* 1994; Zava & Duwe, 1997). Anderson *et al.* (1998) reported biphasic effects of genistein on rat bone tissue, whereby at low doses, genistein appears to be an agonist at the OR locus, whereas at higher doses, it is less effective and may even have adverse effects on bone cells. In light of this biphasic activity, the effect of lower concentrations of these isoflavones on Ca transport in Caco-2 cells warrants investigation.

Genistein and other isoflavones bind only weakly with the OR $\alpha$  but complex with OR $\beta$  almost as well as oestrogens (Kuiper *et al.* 1997). As already mentioned earlier, the Caco-2 cells in the present study expressed mRNA for the OR $\beta$  and, presumably expressed the OR $\beta$  protein, although this was not determined. Interestingly, in the present study, genistin and daidzin, which do not have oestrogenic activity (Miksicek, 1995), did not influence transepithelial Ca transport, suggesting that the inhibition of Ca transport was only associated with the aglycoside form of these isoflavones (i.e. genistein and daidzein) and not their respective glycoside. The reduction in total transepithelial Ca transport in Caco-2 cells treated with genistein and daidzein in the present study occurred by a down-regulation of the transcellular Ca transport process. The mechanism for this down-regulation is unclear. There is some evidence of a regulatory effect of dietary PO on Ca-binding proteins. For example, Taylor *et al.* (1999) reported that calbindin D<sub>28K</sub> protein levels in the brain of female fetuses from pregnant rats fed a PO-free diet were significantly ( $P < 0.05$ ) greater than those in the brains of fetuses from pregnant rats fed a PO-containing diet. Similarly, Lephart *et al.* (2000) showed that consumption of PO, in the form of soyabean-based diets, for 5 weeks lead to a significant ( $P < 0.05$ ) decrease in calbindin levels in the brain of male rats relative to that in control animals fed a PO-free diet. In the present study, mRNA levels for the intestinal Ca-binding protein (calbindin D<sub>9K</sub>), which is proposed to function as either an intracellular Ca buffer or an intracellular ferry protein that facilitates diffusion of Ca across the enterocyte (Bronner *et al.* 1986), were unaffected by either genistein or daidzein treatment. As expected, 1,25(OH)<sub>2</sub>D<sub>3</sub> treatment significantly ( $P < 0.01$ )

increased the levels of mRNA for calbindin D<sub>9K</sub> in the Caco-2 cells used in the present study. This finding is in agreement with similar findings of several other studies (Fleet & Wood, 1994, 1999; Fleet *et al.* 1996; Wood *et al.* 2001). Transcription of calbindin D<sub>9K</sub> gene in Caco-2 cells was also unaffected by 17 $\beta$ -oestradiol, even though this gene has an oestrogen response element (Klinge, 2001).

The down-regulation of transcellular Ca transport by PO may arise by a non-genomic effect. For example, genistein has been reported to directly inhibit the activity of certain cellular regulatory proteins, including tyrosine kinases and topoisomerases (Akiyama *et al.* 1987; Markovits *et al.* 1989). Inhibition of tyrosine kinases may adversely affect Ca transport since they are involved in 1,25(OH)<sub>2</sub>D<sub>3</sub>-induced translocation of the vitamin D receptor from nucleus to the plasma membrane (Capiati *et al.* 2002). At the plasma membrane, the vitamin D receptor may mediate rapid, non-genomic responses, such as transcaltachia (the rapid hormonal stimulation of intestinal Ca transport) (Norman *et al.* 2002).

In conclusion, the findings of the present study would suggest that the beneficial effects of supplemental levels of PO compounds, such as genistein and daidzein, on bone mass in postmenopausal women (Dalais *et al.* 1998; Potter *et al.* 1998; Morabito *et al.* 2002) are more likely to arise from direct effects on bone cells, and not by an indirect effect of these compounds on Ca absorption. However, as these compounds exhibit biphasic effects in some tissues, the effect of lower concentrations of genistein and daidzein on intestinal Ca absorption requires further investigation.

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