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Short communication

Lack of dose-responsive effect of dietary phyto-oestrogens on transepithelial calcium transport in human intestinal-like Caco-2 cells

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Ca absorption has been shown to be unaffected by high luminal concentrations of two commonly consumed soyabean phyto-oestrogens (PO) (genistein and daidzein) in Caco-2 cells grown under oestrogen-depleted conditions. However, these compounds exhibit dose-dependent biphasic effects in some tissues, such as reproductive tissue and bone. Thus, in light of this biphasic activity, the effect of lower concentrations of genistein and daidzein on Ca absorption requires further investigation. Therefore, the aim of the present study was to investigate the effect of a range of concentrations of 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* 12 per treatment), grown in oestrogen-deplete media, were then exposed to 10 nM-1,25-dihydroxycholecalciferol (1,25 $(OH)_2D_3$), or 1, 10 and 50 μM-genistein or -daidzein for 24 h. After exposure, transepithelial and transcellular transport of ^{45}Ca and fluorescein transport were measured. As expected, 1,25 $(OH)_2D_3$ stimulated Ca absorption in Caco-2 cells, by up regulating transcellular transport. Ca absorption was unaffected by either PO at luminal concentrations of 1, 10 or 50 μM, typical of intakes by Western and Asian populations as well as supplemental levels, respectively. The results of this model suggest that the proposed beneficial effects of supplemental levels of these PO compounds on bone mass in postmenopausal women more probably arise from direct effects on bone cells, and not by an indirect effect of these compounds on Ca absorption.

Phyto-oestrogens: Dose-response: Calcium absorption: Caco-2 cells

Dietary phyto-oestrogens (PO) are plant-derived, non-steroidal compounds which exert oestradiol-like effects in some tissues (Anderson *et al.* 1999). Their similar structure to that of mammalian oestrogens enables the binding to and activation of nuclear oestrogen receptors (OR), and furthermore, they can compete effectively with mammalian oestrogens for binding OR (Cassidy, 1996). Consequently, these compounds have received considerable research attention as possible alternatives to hormone-replacement therapy as an osteoprotective therapy. There are several lines of evidence to support a bone-conserving effect of these dietary PO. These include data from cell-culture and animal-model studies (for recent extensive reviews, see Coxam, 2003; Lieberherr *et al.* 2003), as well as a limited number of relatively short-term (3–6 month) human intervention studies, which have produced inconclusive results (for reviews, see Arjmandi, 2001; Arjmandi & Smith, 2002; Valtueña *et al.* 2003). Interestingly, Morabito *et al.* (2002) recently reported that, in a randomised 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 post-menopausal 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, similar to oestrogen (Heaney *et al.* 1978; Gallagher *et al.* 1980) may have the ability to enhance intestinal Ca absorption. For example, Arjmandi *et al.* (2002) recently reported that the rate of *in vitro* Ca transport by the intestinal cells of ovariectomised rats fed soya protein with normal isoflavone content was significantly ($P < 0.05$) higher than that from ovariectomised control animals. More recently, Cotter *et al.* (2003) used

Abbreviations: MTT, 3-(4,5-dimethylthiozol-2-yl)-2,5-diphenyltetrazolium bromide; Neutral Red, 3-amino-7-dimethylamino-2-methylphenazine hydrochloride; 1,25 $(OH)_2D_3$, 1,25-dihydroxycholecalciferol; OR, oestrogen receptor; PO, phyto-oestrogen.

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Caco-2 cells in culture (a suitable model for predicting Ca absorption in human subjects; Fleet & Wood, 1999) to investigate the effects of the isolated PO compounds, genistein and daidzein, at high luminal concentrations (50 μM; a level only achievable by dietary supplementation), on Ca absorption. There was either no effect or reduced Ca absorption in Caco-2 cells grown in oestrogen-deplete and -replete conditions, respectively. However, these compounds exhibit biphasic effects in some tissues. For example, genistein at high concentrations (about 10 to 50 μM) has been shown to inhibit the growth of OR-positive MCF-7 breast cells in culture, whereas, at concentrations below 10 μM, cell growth is stimulated by genistein (Zava & Duwe, 1997). Bone tissue in ovariectomised rats has also been shown to exhibit a biphasic response to genistein, with low doses of genistein appearing to act as an agonist at the OR, acting to maintain bone mass, whereas at higher doses the genistein is less effective and may even have adverse effects on bone cells (Anderson *et al.* 1998). Thus in light of this biphasic activity, the effect of lower concentrations of genistein and daidzein on intestinal Ca absorption requires further investigation. Such research is necessary before the concept of these compounds having an indirect effect on bone mass in postmenopausal women, through the enhancement of Ca absorption, can be discounted.

Therefore, the aim of the present study was to investigate the effect of a range of luminal concentrations of genistein and daidzein on Ca absorption in human Caco-2 intestinal-like cells in order to determine whether a dose-dependent effect occurs. The concentrations of genistein and daidzein used in the study are reflective of typical intakes by Western and Asian populations (low and high PO consumers, respectively), as well as of levels achieved only by supplementation.

Materials and methods

Materials

Tissue culture materials, including Dulbecco's modified Eagle's medium with L-glutamine and sodium bicarbonate, fetal bovine serum, charcoal-stripped, heat-inactivated fetal bovine serum, minimum essential medium, non-essential amino acids and PBS were purchased from Sigma-Aldrich Ireland Ltd (Dublin, Republic of Ireland). ^{45}Ca (as ^{45}Ca in an aqueous solution of $CaCl_2$, with a specific activity of 1·85 MBq/mg Ca) was purchased from Nensure™ (Boston, MA, USA). Fluorescein sodium salt, 3-(4,5-dimethylthiozol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 3-amino-7-dimethylamino-2-methylphenazine hydrochloride (Neutral Red), 1,25-dihydroxycholecalciferol (1,25 $(OH)_2D_3$), genistein and daidzein were purchased from Sigma-Aldrich Ireland Ltd (Dublin, Republic of Ireland).

Conditions of cell culture and assessment of cell viability

The conditions of Caco-2 cell culture were similar to those previously reported (Cotter *et al.* 2003). However, the cell-culture media (phenol red-free with fetal bovine serum) was changed on alternate days for 14 d after which the cell-culture media (phenol red-free containing charcoal-stripped, heat-inactivated fetal bovine serum) was used for the last 7 d before the Ca transport study. These conditions created a low-oestrogen status for the Caco-2 cells preceding their exposure to treatments.

The effect of increasing concentrations of 1,25 $(OH)_2D_3$, genistein and daidzein on Caco-2 cell viability was investigated using the MTT and Neutral Red cell viability assays as described previously (Cotter *et al.* 2003).

Cell treatments

For the Ca transport experiments, the cells grown in Transwell® inserts (Costar, Cambridge, MA, USA) were treated with the vehicle only (control), 10 nM-1,25 $(OH)_2D_3$ (positive control), or 1, 10 or 50 μM-genistein or -daidzein for 24 h. All compounds were added to the culture medium before their addition to the cells. The vehicle never exceeded 2 ml/l. Transepithelial electrical resistance measurements were taken immediately before treatment with the test compounds and 24 h after treatment as described previously (Cotter *et al.* 2003).

Transepithelial calcium transport studies

Total transepithelial, transcellular and paracellular Ca transport across the Caco-2 membrane over 60 min was determined in the present study using ^{45}Ca and fluorescein (a marker of permeability) as described in detail previously (Cotter *et al.* 2003). In all studies, at least three wells were examined per treatment. Experiments were repeated three times.

Statistical methods

The data for all variables were normally distributed and allowed for parametric tests of significance. The data are presented as means with their standard errors. The treatment effects were compared by one-way ANOVA, with variation attributed to the concentration of treatment compound (Snedecor & Cochran, 1967). To follow up the ANOVA, all pairs of means were compared by the method of least significant difference (Snedecor & Cochran, 1967).

Results

There was no effect of incremental concentrations (0–50 μM) of genistein and daidzein 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 (data not shown). In addition, the exposure of Caco-2 cells to 1,25 $(OH)_2D_3$ (the positive control for the Ca transport experiments) (in the range of 10^{-10} to 10^{-6} M) for 24 h had no effect on cell viability, using the MTT and Neutral Red assays (data not shown).

The treatment of Caco-2 cell monolayers with 10 nM-1,25 $(OH)_2D_3$ for 24 h significantly ($P<0·001$) increased the total transepithelial Ca transport compared with the

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control (Table 1). Furthermore, while paracellular Ca transport (as indicated by fluorescein transport) was unaffected, transcellular Ca transport was significantly increased ($P<0·001$) by the 1,25 $(OH)_2D_3$ treatment (Table 1).

The treatment of Caco-2 cell monolayers with 1, 10 or 50 μM-genistein or -daidzein for 24 h had no effect on total transepithelial, paracellular or transcellular Ca transport (Table 1). Similarly, the treatment of fully differentiated Caco-2 cell monolayers with 10 nM-1,25 $(OH)_2D_3$, or 1, 10 or 50 μM-genistein or -daidzein for 24 h had no effect on transepithelial electrical resistance in the present study (Table 1).

Discussion

As expected, in the present study, the exposure of Caco-2 cell monolayers in culture to 10 nM-1,25 $(OH)_2D_3$ for 24 h stimulated total transepithelial Ca transport. This finding is in agreement with the findings of other studies that have found that 1,25 $(OH)_2D_3$ enhances Ca transport across Caco-2 cell monolayers (Fleet & Wood, 1999; Cotter *et al.* 2003). The significant enhancement of total transepithelial Ca transport by 1,25 $(OH)_2D_3$ in the present study acted as a positive control for our experiments to investigate the dose-responsiveness of dietary PO on Ca transport in the Caco-2 model.

The findings of the present study suggest that genistein and daidzein (in the range of 1, 10 or 50 μM) have no effect on total transepithelial, transcellular, or paracellular Ca transport in Caco-2 cells grown under conditions of low oestrogen status. Previously it has been reported that 50 μM-genistein and 50 μM-daidzein have no effect on the intestinal absorption of Ca when Caco-2 cells are grown under oestrogen-deplete conditions (Cotter *et al.* 2003), suggesting a lack of stimulatory effect of these compounds on Ca absorption, at least at this high luminal concentration. The present *in vitro* findings were unexpected because soyabean milks (rich sources of genistein and daidzein) have been shown to enhance Ca absorption and balance in ovariectomised animals (Omi *et al.* 1992, 1994). For example, Omi *et al.* (1994) reported that intestinal Ca absorption, determined using the metabolic balance approach at three separate occasions throughout a 28 d feeding period, was significantly ($P<0·05$) greater in ovariectomised 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 ovariectomised rats fed soya protein with normal isoflavone content were significantly ($P<0·05$) greater than those from ovariectomised control animals. Interestingly, in that study an isoflavone-deplete soya 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 soya 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 ovariectomised rat model. In their model system, the consumption of ipriflavone approximately doubled ($P<0·05$) the *in vitro* Ca uptake by intestinal cells from ovariectomised rats compared with that in cells from animals fed the control diet. Interestingly, 50 μM-ipriflavone significantly ($P<0·05$) increased total transepithelial Ca transport in our Caco-2 cell model (, unpublished results), in agreement with the findings of the animal study by Arjmandi *et al.* (2000).

The highest concentration of isoflavones used in the present and previous study (Cotter *et al.* 2003) was 50 μM. This concentration was chosen to reflect the probable

Table 1. Effect of genistein, daidzein and 1,25-dihydroxycholecalciferol (1,25 $(OH)_2D_3$) on calcium transport in Caco-2 cell monolayers cultured in oestrogen-deplete media*

(Mean values with their standard errors)

		Ca transport									
		Total transepithelial				Transcellular (nmol/well per min)‡		Paracellular (%/h)		TEER (Ω.cm^2)	
		nmol/well per min		%/h							
Treatment†	*n*	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Control	12	0·28[a]	0·01	1·48[a]	0·05	0·23[a]	0·02	0·26[a]	0·01	2207[a]	70
10 nM-1,25 $(OH)_2D_3$	12	0·41[b]	0·02	2·15[b]	0·07	0·37[b]	0·02	0·24[a]	0·01	2181[a]	92
1 μM-Genistein	12	0·26[a]	0·03	1·55[a]	0·09	0·22[a]	0·02	0·27[a]	0·02	1916[a]	118
10 μM-Genistein	12	0·24[a]	0·02	1·50[a]	0·09	0·20[a]	0·02	0·25[a]	0·01	1987[a]	81
50 μM-Genistein	12	0·26[a]	0·01	1·55[a]	0·05	0·22[a]	0·02	0·26[a]	0·01	2096[a]	77
1 μM-Daidzein	12	0·28[a]	0·02	1·55[a]	0·09	0·23[a]	0·02	0·27[a]	0·01	2008[a]	119
10 μM-Daidzein	12	0·23[a]	0·02	1·41[a]	0·06	0·19[a]	0·01	0·25[a]	0·01	2223[a]	80
50 μM-Daidzein	12	0·24[a]	0·01	1·46[a]	0·06	0·20[a]	0·01	0·24[a]	0·01	2351[a]	94
Statistical significance of effect (one-way ANOVA): *P*		<0·0001		<0·0001		<0·0001		0·380		0·114	

TEER, transepithelial electrical resistance (after 24 h exposure to the different treatments).
[a,b] Mean values within a column with unlike superscript letters were significantly different (ANOVA followed by least significant difference test, $P<0·001$).
* For details of procedures, see Cotter *et al.* (2003).
† Treatments were given for 24 h before measurement of Ca transport.
‡ Transcellular transport is total Ca transport corrected for paracellular (fluorescein) transport (for details, see Cotter *et al.* (2003)).

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maximal luminal (small intestine) concentrations in the subjects participating in the various dietary intervention trials which have investigated the effect of PO on bone (for reviews, see Arjmandi, 2001; Arjmandi & Smith, 2002; Valtueña *et al.* 2003). Although PO primarily mimic the actions of oestrogen (Setchell, 1998), anti-oestrogenic effects have also been observed *in vivo*, whereby the effect of synthetic or natural oestrogens is counteracted by administered isoflavones or their presence in the diet (Mazur *et al.* 1998). Oestrogens exhibit biphasic responses that are highly dose-dependent (Setchell, 1998) and similar actions have been suggested for PO in various tissues (Kaplanski *et al.* 1981; Molteni *et al.* 1995; Anderson *et al.* 1998; Picherit *et al.* 2001). In rat bone tissue, low doses of genistein function as an oestrogen agonist, helping to preserve bone mass post-ovariectomy, while higher doses are less effective and potentially could have adverse effects on bone cells (Anderson *et al.* 1998). Cell growth and cell proliferation are also influenced by the PO concentration. Elattar & Virji (2000) found that genistein and another PO-like compound, quercetin, exert a biphasic effect on the growth and proliferation of the human oral squamous carcinoma cell line SCC-25, while the growth of MCF-7 cells is also dependent on PO concentration (Zava & Duwe, 1997; Hsieh *et al.* 1998). For example, genistein at high concentrations (about 10 to 50 μM) has been shown to inhibit the growth of OR-positive MCF-7 breast cells in culture, whereas, at concentrations below 10 μM, cell growth is stimulated by genistein (Zava & Duwe, 1997). In isolated bovine granulosa cells and MCF-7 cells, PO exhibit a dose-related biphasic effect on steroidogenesis and aromatase inhibition, respectively (Kaplanski *et al.* 1981; Almstrup *et al.* 2002). Therefore, in light of the mounting evidence that PO can influence biological processes in a dose-dependent biphasic manner, it was felt important to investigate the effect of a concentration range of PO on Ca transport in the present study. The lower concentrations of isoflavones (1 and 10 μM) used in the present study were chosen to reflect the luminal (small intestine) concentrations typical of European and Asian adult populations who have on average daily total isoflavone intakes of less than 1 mg and between 20 and 50 mg, respectively (Chen *et al.* 1999; van Erp-Baart *et al.* 2003). However, there was no evidence of a dose-dependent biphasic effect of PO on Ca absorption in the Caco-2 cells in the present study.

In conclusion, the present study of Caco-2 cells, which possess an ORβ (to which PO bind almost as well as oestrogens; Kuiper *et al.* 1997) and which are capable of a functional response to oestrogen (Cotter *et al.* 2003), confirms previous findings. The study found that Ca absorption is not enhanced by two commonly consumed soyabean PO (genistein and daidzein) at high luminal concentrations. However, the study also importantly expanded on these previous findings to clearly demonstrate a lack of effect of these PO at lower luminal concentrations, which are more typical of habitual dietary levels in European and Asian populations. Thus, the results of this model suggest that the proposed beneficial effects of supplemental levels of PO compounds, such as genistein and daidzein, on bone mass in postmenopausal women more probably arise from direct effects on bone cells, and not by an indirect effect of these compounds on Ca absorption. While Caco-2 cells are considered a suitable model for predicting Ca absorption in human subjects (Fleet & Wood, 1999), the findings of the present and previous studies in Caco-2 cells in culture, which suggest that PO do not improve Ca absorption, would need to be confirmed in human studies.

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