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ORIGINAL CONTRIBUTION

Alice A. Cotter
Kevin D. Cashman

The effect of two dietary and a synthetic phytoestrogen on transepithelial calcium transport in human intestinal-like Caco-2 cells

Summary *Background* Recently, dietary phytoestrogens (PEs) have been suggested as possible alternatives to estrogen therapy, as a means of preventing bone loss associated with ovarian hormone deficiency. PEs are non-steroidal, plant-derived compounds that exhibit some estrogen-like activity in some tissues, and which appear to prevent postmenopausal bone loss. While PEs act directly on bone cells, their protective effect on bone may be partly due to their ability to enhance Ca absorption. *Aim of the study* Therefore, the aim of this study was to investigate the effect of two dietary PEs (coumestrol and apigenin) as well as a synthetic PE, ipriflavone, on Ca absorption in human Caco-2 intestinal-like cells. *Methods* Caco-2 cells were seeded onto permeable filter supports and allowed to differentiate into monolayers. On d 21, the Caco-2 monolayers (*n* 10–16 per treatment), grown in estrogen-free or low-estrogen media, were then exposed to 10 nM-1,25 $(OH)_2$ D_3, or 50 μM-ipriflavone, -coumestrol or -apigenin for 48 hours. After exposure, transepithelial and transcellular transport of ^{45}Ca and fluorescein transport (a marker of paracellular diffusion) were measured. *Results* As expected, 1,25 $(OH)_2$ D_3 stimulated Ca absorption. Treatment with coumestrol or apigenin had no effect on Ca transport. On the other hand, ipriflavone increased total Ca transport (by about 1.5-fold, $P < 0.05$) under low-estrogen conditions, but not under estrogen-free conditions. This increase in total Ca transport by ipriflavone was via an increased transcellular Ca transport (by about 2-fold, $P < 0.05$) relative to control. *Conclusion* In conclusion, the protective effect of dietary PE on bone mass would appear to be due to their direct effect(s) on bone cells, as opposed to an indirect effect on bone by stimulation of intestinal Ca absorption.

Key words phytoestrogens – ipriflavone – calcium absorption – Caco-2 cells

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A. A. Cotter · Prof. K. D. Cashman (✉)
Dept. of Food and Nutritional Sciences
University College
Cork, Ireland
Tel.: +353-21/4901317
Fax: +353-21/4270244
E-Mail: k.cashman@ucc.ie

K. D. Cashman
Dept. of Medicine
University College
Cork, Ireland

Introduction

In recent years osteoporosis has become a global health issue and is currently the major underlying cause of bone fractures in an aging population [1]. Women are at particular risk of this disease, with about 3.7-fold more women than men suffering hip fractures in the EU member states each year [2]. Estrogen deficiency is the dominating pathogenic factor for 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 [3, 4]. While there are various pharmacological interventions aimed at inhibiting bone turnover and thus preventing osteoporosis [2], estrogen therapy (or hormone replacement therapy) remains the mainstay for prevention of bone loss in postmenopausal women [5]. Decreased Ca absorption due to ovarian hormone deficiency is also corrected by hormone replacement therapy [6, 7]. However, fewer than one in four postmenopausal women decide to use hormone replacement therapy, and within six months, > 60 % of them withdraw due to concerns over an increased risk of malignancy and other side effects [8].

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Recently, attention has been focused on the so-called phytoestrogens (PE) as possible alternatives, or at least adjuncts, to hormone replacement therapy. Dietary PEs are non-steroidal compounds naturally occurring in foods of plant origin (especially soy foods). In addition, there is now also a synthetic PE-like compound, ipriflavone, which is similar in structure to the soy isoflavone, daidzein [9]. These compounds are able to compete with the principle estrogens of most mammals (17 β-estradiol and estrone) for binding estrogen receptors [ER, 10]. They have been shown in some studies, but not all [11, 12], to prevent bone loss associated with ovarian hormone deficiency (and appear to be free of the significant side effects attributed to endogenous estrogens) [13–18].

While the PEs act directly on bone cells, either through ER- or non-ER-mediated mechanisms [19], it is conceivable that their protective effect on bone may be partly due to their ability to enhance Ca absorption. Some of the PE compounds structurally resemble estrogen [20], and thus, similar to estrogen [19] may have the ability to enhance intestinal Ca absorption. In support of this contention, Omi et al. (1994) [21] reported that intestinal Ca absorption was significantly ($P < 0.05$) higher in ovariectomized rats given soybean milk-containing diets (rich in phyto-estrogenic compounds such as genistein and daidzein) than in rats given a control diet (containing no soybean 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 soybean milk-containing diet relative to control animals. In addition, ipriflavone, which has a bone-sparing effect in rats [22] and humans, at least in some [14, 16] but not all studies [23], has also been shown to enhance *in vitro* Ca uptake by duodenal cells of ovariectomized rats nearly as efficiently as estrogen [22]. We have recently shown that two commonly consumed soy PEs (genistein and daidzein) either have no effect or reduced Ca absorption in human intestinal-like, Caco-2, cells grown in estrogen-free or low-estrogen conditions, respectively [24]. However, there are several other PE compounds present in the diet and some of these may be even more estrogenic that genistein and daidzein, e. g., coumestrol [25]. Coumesterol has been shown to prevent postmenopausal bone loss, at least in rats [26, 27].

Therefore, the aim of the present study was to investigate the effect of two other dietary PEs (coumestrol and apigenin) and a synthetic PE, ipriflavone, on Ca absorption in Caco-2 cells, grown under estrogen-free or low-estrogen conditions (typical of that in postmenopausal women not receiving HRT).

Materials and methods

Materials

Tissue culture materials, including phenol red-free Dulbecco's modified Eagle's medium with L-glutamine and sodium bicarbonate, fetal bovine serum (FBS), minimum essential medium, non-essential amino acids, lactate dehydrogenase (LDH) colorimetric kit assay (product no. DK1340-K) and PBS were purchased from Sigma-Aldrich Ireland Ltd, Dublin, Ireland. Charcoal-stripped, heat-inactivated FBS was purchased from Valley Biomedical Inc., Winchester, VA, USA. ^{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^{TM}, Boston, MA, USA. Fluorescein sodium salt, EDTA, 3-(4,5-dimethylthiozol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 3-amino-7-dimethylamino-2-methylphenazine hydrochloride (Neutral Red), 1,25 $(OH)_2$ D_3, coumestrol and apigenin were purchased from Sigma-Aldrich Ireland Ltd. Trypsin (Difco Laboratories) was supplied by Unitech, Dublin. Ipriflavone (7, isopropoxy-3-phenyl-4H-1-benzopyran-4-one) was given to us by Sabinsa Corp, Piscataway, NJ, USA.

Conditions of cell culture and assessment of cell cytotoxicity

The conditions of Caco-2 cell culture were similar to those previously reported [24]. However, the cell culture media (phenol red-free with FBS) was changed on alternate days for 14 d after which the cell-culture media (phenol red-free containing charcoal-stripped, heat-inactivated FBS) was used for the last 7 d before the Ca transport study. These conditions created an estrogen-free environment for the Caco-2 cells preceding their exposure to treatments.

The effect of increasing concentrations of 1,25 $(OH)_2$ D_3, ipriflavone, coumestrol and apigenin on Caco-2 cell cytotoxicity was investigated using the lactate dehydrogenase (LDH) release, MTT and Neutral Red cell cytotoxicity assays as previously reported [24].

Cell treatments

For Ca transport experiments, the cells grown in Transwell® inserts (Costar, Cambridge, MA, USA) were treated with vehicle only (for control), 10 nM-1,25 $(OH)_2$ D_3 (positive control), or 50 μM-ipriflavone, -coumestrol or -apigenin for 48 h. All compounds were added to culture medium before their addition to the cells. The vehicle never exceeded 2 ml/l. Transepithelial electrical resistance (TEER) measurements were taken immediately before treatment with test compounds and 48 h after

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treatment as described previously [24]. A TEER value $\geq 1000\ \Omega \cdot cm^2$ was used as an indicator that the epithelial layer was intact and ready to use for Ca transport studies [28].

Transepithelial calcium transport studies

Total transepithelial, transcellular and paracellular Ca transport across the Caco-2 cell membrane over 60 min was determined in the present study using ^{45}Ca and fluorescein (a marker of permeability) as described in detail previously [24]. In all studies, at least three wells were examined per treatment. Experiments were repeated three times.

Statistical method

Data for all toxicity variables and TEER were normally distributed and allowed for parametric tests of significance. Data for all Ca transport variables (except transcellular Ca transport in studies using estrogen-free media) were not normally distributed and therefore, were log transformed prior to statistical analysis, to achieve a near-normal distribution. Treatment effects were compared by one-way ANOVA, with variation attributed to type of treatment compound [29]. To follow up the ANOVA, all pairs of means were compared by the method of least significant difference [29].

Results

LDH release from Caco-2 cells was around 5 % and was not significantly different in any of the treatments compared to controls (data not shown). Furthermore, there was no effect of incremental concentrations of ipriflavone, coumestrol and apigenin (0–50 μM) on Caco-2 cell cytotoxicity as assessed using the MTT and Neutral Red uptake assays (data not shown). In addition, exposure of Caco-2 cells to 1,25 $(OH)_2$ D_3 (positive control for the Ca transport experiments) for 24 h had no cytotoxic effect as assessed by all three assays (data not shown).

Treatment of fully differentiated Caco-2 cell monolayers (grown in media containing FBS) with 10 nM-1,25 $(OH)_2$ D_3 for 48 h led to a significant ($P < 0.05$) decrease in TEER (Table 1). On the other hand, treatment of the Caco-2 cell monolayers with 50 μM-ipriflavone for 48 h led to a significant ($P < 0.01$) increase in TEER, whereas treatment with 50 μM-coumestrol or -apigenin for 48 h had no effect on TEER (Table 1).

Treatment of Caco-2 cell monolayers (grown in media containing FBS) with 10 nM-1,25 $(OH)_2$ D_3 for 48 h significantly ($P < 0.01$) increased the total transepithelial Ca transport compared with control values (Table 1). Furthermore, while paracellular Ca transport (as indicated by fluorescein transport) was unaffected, transcellular Ca transport was significantly increased ($P < 0.01$) by the 1,25 $(OH)_2$ D_3 treatment (Table 1).

Treatment of Caco-2 cell monolayers (grown in media containing FBS) with 50 μM-coumestrol or -apigenin for 48 h had no effect on total transepithelial, paracellular or transcellular Ca transport (Table 1). Treatment of Caco-2 cell monolayers with 50 μM-ipriflavone for 48 h significantly ($P < 0.05$) increased total transepi-

Table 1 Effect of coumestrol, apigenin, ipriflavone and 1,25-dihydroxycholecalciferol (1,25 $(OH)_2$ D_3) on calcium transport in Caco-2 cell monolayers cultured in a low-estrogen environment* (Mean values with their standard errors)

Treatment[d]	n	Calcium transport									
		Total transepithelial				Transcellular (nmol/well per min)[e]		Paracellular (%/h)		TEER ($\Omega \cdot cm^2$)	
		(nmol/well per min)		(%/h)							
		Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Control	12	0.36[a]	0.04	1.95[a]	0.03	0.22[a]	0.04	0.46[a]	0.04	1633[a]	57
10 nM-1,25 $(OH)_2$ D_3	12	0.56[b]	0.04	3.04[b]	0.05	0.46[b]	0.05	0.44[a]	0.05	1381[b]	42
50 μM-Ipriflavone	14	0.54[b]	0.06	3.27[b]	0.06	0.44[b]	0.07	0.63[a]	0.04	2064[c]	60
50 μM-Apigenin	14	0.37[a]	0.04	1.87[a]	0.04	0.30[a]	0.04	0.53[a]	0.05	1672[a]	60
50 μM-Coumestrol	14	0.45[a]	0.04	2.33[a]	0.03	0.34[a]	0.04	0.46[a]	0.03	1730[a]	56
(one-way ANOVA): P		0.017		0.006		0.011		0.474		< 0.0001	

TEER transepithelial electrical resistance (after 48 h exposure to the different treatments);
[a, b, c] Mean values within a column with unlike superscript letters were significantly different (ANOVA followed by least significant difference test, $P < 0.05$);
[d] Treatments were given for 48 h before measurement of calcium transport;
[e] Transcellular transport is total calcium transport corrected for paracellular (fluorescein) transport (for details, see Materials and methods);
* For details of procedures, see Materials and methods

thelial transport and transcellular Ca transport, but had no effect on paracellular Ca transport (Table 1).

Treatment of fully differentiated Caco-2 cell monolayers (grown in media containing charcoal-stripped, heat-inactivated FBS) with 10 nM-1,25 $(OH)_2$ D_3 for 48 h led to a significant ($P < 0.05$) decrease in TEER (Table 2). On the other hand, treatment of the Caco-2 cell monolayers with 50 μM-ipriflavone for 48 h led to a significant ($P < 0.01$) increase in TEER, whereas treatment with 50 μM-coumestrol or -apigenin for 48 h had no effect on TEER (Table 2).

Treatment of Caco-2 cell monolayers (grown in media containing charcoal-stripped, heat-inactivated, FBS) with 10 nM-1,25 $(OH)_2$ D_3 for 48 h significantly ($P < 0.01$) increased the total transepithelial Ca transport compared to control (Table 2). Furthermore, while paracellular Ca transport (as indicated by fluorescein transport) was unaffected, transcellular Ca transport was significantly increased ($P < 0.01$) by the 1,25 $(OH)_2$ D_3 treatment (Table 2).

Treatment of Caco-2 cell monolayers (grown in media containing charcoal stripped heat inactivated FBS) with 50 μM-ipriflavone, -coumestrol or -apigenin for 48 h had no effect on total transepithelial, paracellular and transcellular Ca transport (Table 2).

Discussion

While ideally Ca absorption should be measured by human studies, such studies are often very time consuming and expensive to run [30]. As an alternative, experimental animal models, especially the laboratory rat, have been used quite extensively for studying Ca bioavailability, as humans and rats display similar absorption mechanisms for Ca and a number of dietary and physiological factors affect Ca absorption similarly in the two species [31]. However, while studies using laboratory animals are less expensive than studies in humans, they are somewhat limited by uncertainties with regard to differences in metabolism between animals and humans [30]. More recently, Caco-2 cells have been shown to be a good model for studying Ca transport in humans [24, 28, 32, 33]. Although derived originally from a human colon adenocarcinoma, in culture these cells undergo spontaneous structural and functional differentiation so as to yield a cell line which express characteristics of mature small intestinal enterocytes [32]. For example, once differentiated these cells form a polarised epithelial monolayer with tight intercellular junctions, and microvilli of the brush border membrane, and the cells excrete border-associated enzymes. The presence of a functional vitamin D receptor [34], Ca transport kinetics which suggest the presence of both transcellular and paracellular pathways [35], and the accumulation of calbindin D_{9K} and 24-hydroxylase mRNA following treatment with 1,25 $(OH)_2$ D_3, which is known to stimulate Ca transport [32, 35], all substantiate the use of Caco-2 cells as a model for predicting Ca transport in humans under certain conditions. Furthermore, we have recently reported that the Caco-2 cells used in the present study express mRNA for ERβ, even though mRNA for ERα is undetectable [24]. The presence of ERβ in Caco-2 cells is important in the present study because PEs bind only weakly with the ERα but complex with the ERβ almost as well as estrogen [36]. In addition, the Caco-2 cells are capable of a functional response to estrogen [24].

In the present study, the increase in total transepithe-

Table 2 Effect of coumestrol, apigenin, ipriflavone and 1,25-dihydroxycholecalciferol (1,25 $(OH)_2$ D_3) on calcium transport in Caco-2 cell monolayers cultured in an estrogen-free environment* (Mean values with their standard errors)

Treatment[d]	n	Calcium transport									
		Total transepithelial				Transcellular (nmol/well per min)[e]		Paracellular (%/h)		TEER ($\Omega \cdot cm^2$)	
		(nmol/well per min)		(%/h)							
		Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Control	11	0.27[a]	0.02	1.50[a]	0.10	0.24[a]	0.01	0.27[a]	0.06	1163[a]	41
10 nM-1,25 $(OH)_2$ D_3	10	0.45[b]	0.03	2.25[b]	0.16	0.35[b]	0.02	0.24[a]	0.07	1001[b]	25
50 μM-Ipriflavone	15	0.34[a]	0.04	1.92[a]	0.17	0.28[a]	0.02	0.34[a]	0.05	1890[c]	47
50 μM-Apigenin	13	0.32[a]	0.03	1.73[a]	0.11	0.27[a]	0.02	0.30[a]	0.05	1283[a]	29
50 μM-Coumestrol	16	0.29[a]	0.03	1.75[a]	0.13	0.28[a]	0.02	0.27[a]	0.05	1086[a]	33
(one-way ANOVA): P		0.014		0.018		0.020		0.710		< 0.0001	

TEER transepithelial electrical resistance (after 48 h exposure to the different treatments)

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

[d] Treatments were given for 48 h before measurement of calcium transport

[e] Transcellular transport is total calcium transport corrected for paracellular (fluorescein) transport (for details, see Materials and methods)

* For details of procedures, see Materials and methods

lial Ca transport across fully differentiated Caco-2 cell monolayers in culture following exposure to 10 nM-1,25 $(OH)_2$ D_3 for 48 h is in agreement with findings of other studies, which also found that 1,25 $(OH)_2$ D_3 enhanced total Ca transport in Caco-2 cells [24, 28, 32, 33, 35]. The significant enhancement of total transepithelial Ca transport by 1,25 $(OH)_2$ D_3 acted as a positive control for our experiments to investigate the influence of two dietary PEs, coumestrol and apigenin, and a synthetic PE, ipriflavone, on Ca transport in the Caco-2 cell model.

In the present study, the two dietary-based PEs, namely coumestrol and apigenin, had no effect on transepithelial Ca transport in Caco-2 cells grown in low-estrogen or estrogen-free conditions. We have recently reported that two other PE, genistein and daidzein, which are soybean-based PE, had no effect on Ca transport in Caco-2 cells grown in estrogen-free conditions, while they significantly reduced Ca transport in low-estrogen conditions [24]. Coumestrol is a coumestan found primarily in soybean sprout, clover, alfalfa and fodder crops [37], while apigenin is a flavone consumed mainly in the form of millet [38]. Both compounds structurally resemble estrogen [20] and bind to the human ER [39] and thus, similar to estrogen [6, 7] may have the ability to enhance intestinal Ca absorption. Coumestrol has been shown to have bone-conserving properties in experimental animal models. For example, coumestrol has been reported to increase the Ca content of 9-d-old chick embryonic femurs in organ culture [40] and to inhibit release ^{45}Ca from 20-d-old fetal rat femora, suggesting a role for this PE in promoting bone mineralization and inhibiting bone resorption. Interestingly, 3,9-bis-(N, N-dimethylcabamoyloxy)-5H-benzofuro-[3,2-C]-quinoli-ne-6-one, a derivative of coumestrol, is a potent inhibitor of bone resorption and a stimulator of bone formation [41]. Dodge et al. (1996) [26] showed that oral supplementation for 5 weeks (starting 1 month after ovariectomy (OVX)) with coumestrol (0.1, 1, 10 and 30 mg/kg body weight) effectively spared the OVX-induced bone loss in 6-month-old rats. In addition, Draper et al. (1997) [27] found that coumestrol (injected intramuscularly as cottonseed oil twice weekly for 6 weeks) prevented OVX-induced bone loss in skeletally mature rats. The rate of bone resorption (as determined by urinary deoxypyridinoline) was lower in rats treated with coumestrol compared to control. The authors suggested that the osteoprotective effect was likely a result of coumestrol's estrogenic effect, possibly on improving Ca balance [27]. However, an effect of coumestrol on intestinal Ca absorption was not evident in human Caco-2 cells in the present study.

In the present study, treatment with 50 μM-ipriflavone for 48 h stimulated total transepithelial Ca transport in differentiated Caco-2 cells grown under low-estrogen conditions (media contained about 50 pM, close to that reported for postmenopausal women not receiving HRT; 75 pM [42]). This enhancement of total transepithelial Ca transport was achieved by an increased transcellular transport of Ca, whereas paracellular Ca transport was unaffected. This is the first study, to our knowledge, to investigate the effect of ipriflavone on Ca absorption in a human intestinal cell line. These *in vitro* findings are in line with those of Arjmandi et al. (2000) [22] who reported that ipriflavone enhanced *in vitro* intestinal Ca transport in an OVX rat model. In their model system, consumption of ipriflavone approximately doubled ($P < 0.05$) the *in vitro* Ca uptake by duodenal cells from OVX rats compared to that in cells from animals fed the control diet [22]. On the other hand, they appear to be in contrast to the findings of a recent three-year randomized controlled study by Alexandersen et al. (2001) [23] which failed to find any effect of ipriflavone on BMD or Ca and bone metabolism in postmenopausal osteoporotic women (not on HRT).

The mechanism by which PE influence Ca absorption is unclear and warrants further investigation. There is some evidence of a regulatory effect of dietary PE on Ca binding proteins. For example, Lephart et al. [43] showed that consumption of PE, in the form of soy diets, for 5 weeks led to a significant ($P < 0.05$) decrease in calbindin D_{28K} protein levels in the brain of male rats relative to that in control animals fed a phytooestrogen-free diet. However, mRNA levels for the intestinal calcium binding protein (calbindin D_{9K}), which is proposed to function as either an intracellular Ca buffer or an intracellular ferry protein that facilitates diffusion of Ca across the enterocyte, were unaffected by either genistein or daidzein treatment in our previous study [24]. Genistein has been reported to directly inhibit the activity of certain cellular regulatory proteins, including tyrosine kinases and topoisomerases [44]. Inhibition of tyrosine kinases may influence 1,25 $(OH)_2$ D_3-induced translocation of the VDR from nucleus to the plasma membrane [45], which is important for induction of active Ca transport. However, daidzein which does not inhibit tyrosine kinases, also reduced intestinal Ca absorption [24]. Furthermore, the mechanism by which ipriflavone stimulates Ca absorption is also unclear.

In conclusion, the findings of the present study *in vitro* would suggest that the dietary PEs, coumestrol and apigenin, had no effect on intestinal Ca transport, whereas the synthetic PE, ipriflavone enhanced intestinal Ca absorption in Caco-2 cells grown under low-estrogen conditions, a model of postmenopausal Ca absorption. More research is needed to understand the mechanisms of this stimulatory effect. Thus, the proposed beneficial effects of dietary-based PE compounds on bone mass in postmenopausal women are more likely to arise from direct effects on bone cells, and not by an indirect effect of these compounds on Ca absorption.

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