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ORIGINAL CONTRIBUTION

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Effect of 17β-oestradiol on transepithelial calcium transport in human intestinal-like Caco-2 cells and its interactions with 1,25-dihydroxycholecalciferol and 9-*cis* retinoic acid

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Abstract *Background* Oestrogen therapy helps prevent bone loss in postmenopausal women and corrects a decline in Ca absorption efficiency at the onset of menopause. However, the mechanism by which 17β-oestradiol (17β-E_2) stimulates Ca absorption is unclear. Oestrogen may exert its effect indirectly *via* increasing 1,25-dihydroxycholecalciferol (1,25 $(OH)_2D_3$) or its receptor, or act more directly on the intestines *via* the oestrogen receptor (OR). Since oestrogen also increases retinol levels, this may influence Ca absorption. *Aim* To investigate the effect of 17β-E_2 alone and in combination with 1,25 $(OH)_2D_3$ on intestinal Ca uptake and absorption in Caco-2 cells cultured under deplete- and replete-9-*cis* retinoic acid (9-*cis* RA) conditions. *Methods* Twenty-one day-old Caco-2 cell monolayers (*n* 9 wells per treatment) were exposed to 9-*cis* RA-deplete and -replete media containing dimethyl sulfoxide (control), 10 nM-1,25 $(OH)_2D_3$, 10 nM-17β-E_2, or 10 nM-1,25 $(OH)_2D_3$ plus 10 nM-17β-E_2, for 48 h. *Results* 1,25 $(OH)_2D_3$ stimulated Ca uptake, total Ca transport, calbindin D_{9K} and CaT1 mRNA levels, while 17β-E_2 and 9-*cis* RA had no effect on Ca absorption or uptake. Nor did they augment the stimulatory effect of 1,25 $(OH)_2D_3$. *Conclusion* These *in vitro* findings suggest that oestrogen does not have a direct effect on intestinal Ca absorption.

Key words oestrogen – 1,25-dihydroxycholecalciferol – retinoic acid – calcium absorption – caco-2 cells

Introduction

The onset of menopause is associated with a decline in Ca absorption efficiency, which seems undoubtedly related to ovarian hormone deficiency [1]. The impaired Ca absorption is believed to contribute to a more negative Ca balance and subsequent bone loss in postmenopausal osteoporotic women [2–5]. The cause of this Ca malabsorption has been attributed to decreased 1,25 $(OH)_2D_3$ levels [2–4], the active metabolite of vitamin D_3 and a potent regulator of Ca absorption, and/or to intestinal resistance to its action [6–8]. However, there is evidence that intestinal Ca malabsorption results directly from the low oestrogen level that is characteristic of menopause. Decreased Ca absorption due to ovarian hormone deficiency is corrected by hormone replacement therapy in some women [2, 4–5]. However, this specific intestinal effect is still controversial [9–12]. Numerous studies on animals have also produced supportive evidence for the role of oestrogen in Ca absorption [13–20]. The mechanism, however, by which this enhancement in intestinal Ca absorption occurs is unclear.

EJN 590

It has been proposed that oestrogen exerts its effect on Ca absorption by its interaction with 1,25 $(OH)_2D_3$, or its receptor (the vitamin D receptor (VDR)) [2–4]. Deficient production of 1,25 $(OH)_2D_3$ due to ovarian hormone deficiency is in line with observations that oestrogen treatment increased serum 1,25 $(OH)_2D_3$ in postmenopausal osteoporotic women [21–22]. Oestrogen treatment has been shown to increase the intestinal concentration of VDR in some studies of experimental animals [23], but not others [16].

It is, however, possible that oestrogen, itself, acts directly on the intestine to stimulate Ca absorption rather than through 1,25 $(OH)_2D_3$ or the VDR. Arjmandi et al. [13], for example, demonstrated that mucosal cells of rat intestinal tissue contain oestrogen receptor (OR) immunoreactivity, express mRNA for the OR, and respond directly to 17β-oestradiol with enhanced in vitro Ca uptake in duodenal cells that is suppressed by gene transcription and protein synthesis inhibitors, suggesting that the action of oestrogen is mediated through nuclear mechanisms. Recently, Campbell-Thompson et al. [24] found that normal human colonic mucosa expressed mRNA for ORα and -β, with mRNA for ORα much lower than for ORβ. Picotto et al. [25] also reported that 17β-oestradiol increased Ca uptake (Ca influx) into rat duodenal cells in vitro, an effect which was mediated by a rapid activation of the cAMP pathway and Ca channels. Interestingly, the stimulatory effect of 17β-oestradiol on Ca influx has also been reported for mammalian OR-positive RUCA-I (rat endometrial carcinoma) cells, but not for COS (monkey kidney) cells lacking OR [26]. The direct effect of 17β-oestradiol on Ca uptake, and more importantly, on transepithelial Ca transport (i.e., Ca absorption) in human intestinal cells, has not been fully investigated.

The objective of the present study was to investigate the possible stimulatory effect of 17β-oestradiol alone and in combination with 1,25 $(OH)_2D_3$ on intestinal Ca uptake and absorption and its mediators, calbindin D_{9K} (an important intestinal ferry protein; [27]) and the recently described epithelial Ca channel (a putative gatekeeper) [28] in Caco-2 cells cultured under conditions of deplete- and replete-1,25 $(OH)_2D_3$ and 9-*cis* retinoic acid (9-*cis* RA). 1,25 $(OH)_2D_3$ and 9-*cis* RA are known to be reduced during the menopause and to be increased by hormone replacement therapy.

Caco-2 cells have been suggested to be a suitable model for predicting Ca absorption in humans [29–30]. 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 [31–32]. In particular, these cells have a functional VDR [33] and have Ca transport kinetics that suggest the presence of both saturable and non-saturable Ca transport pathway, similar to what has been observed in human and animal intestine [29]. 1,25 $(OH)_2D_3$ treatment induces the saturable, but not diffusional, component of Ca transport [30] and induces accumulation of calbindin D_{9K} and 24-hydroxylase mRNA in these cells [34–35]. In addition, the Caco-2 cells possess an OR [24, 36]. Therefore, this relatively simple in vitro method appears to be a good model for predicting Ca bioavailability in humans.

Materials and methods

Materials

Tissue culture materials, including phenol red-free Dulbecco's modified Eagle's medium with L-glutamine and sodium bicarbonate, foetal bovine serum (FBS), non-essential amino acids and phosphate buffered saline (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™, 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 $(OH)_2D_3$, 9-*cis* RA, and 17β-oestradiol were purchased from Sigma-Aldrich Ireland Ltd. Trypsin (Difco Laboratories) was supplied by Unitech, Dublin.

Conditions of cell culture

The human colon adenocarcinoma cell line, Caco-2, was purchased from the European Collection of Animal Cell Cultures (Salisbury, Wiltshire, UK). The cells were routinely grown in 75-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°C in CO_2–air (5:95, v/v). Cells were seeded at $3\times10^4/cm^2$ and passaged when reaching 90% confluency with trypsin-EDTA (prepared in PBS using EDTA (0.5 g/l) and trypsin (0.2 g/l)). Cells used in transepithelial Ca transport experiments were seeded at a density of $3\times10^4/cm^2$ onto permeable Transwell® filter inserts (24-mm diameter, 0.4-μm pore size, Costar). Cell culture media (containing FBS) were changed on alternate days for 14 days after which the cell culture media (containing charcoal-stripped, heat-inactivated FBS) was used for the last 7 days before the Ca transport study. These conditions created a low oestrogen status for the Caco-2 cells immediately preceding their exposure to treat-

ments. For mRNA studies, cells ($3\times10^4/cm^2$) were seeded into six-well culture plates (35-mm diameter, Costar). For viability studies, cells ($3\times10^4/cm^2$) were seeded into 6-well and 48-well culture plates (Costar).

Cell viability and cytotoxicity assays

The effect of 1,25 $(OH)_2D_3$, 17β-oestradiol and 9-*cis* RA on Caco-2 cell viability was investigated using the MTT [37–38] and Neutral Red [39] cell viability assays in 48-well culture plates (Costar). Lactate dehydrogenase (LDH) release from the cells into the surrounding medium was determined as an index of cytotoxicity [40].

Transepithelial electrical resistance (TEER)

For all transport experiments, the transepithelial electrical resistance (TEER) was checked prior to the experiment by a Millicell® ERS meter (Millipore Corp., Bedford, MA, USA) connected to a pair of thin side-by-side electrodes. TEER readings were taken at 37°C. A TEER value $\geq$1000 Ω cm^2 was used as an indicator that the epithelial layer was intact and ready to use for Ca transport studies.

Cell treatments

For one series of Ca transport experiments, cells grown in the Transwell® inserts (Costar), and cultured in cell culture media (containing charcoal-stripped, heat-inactivated FBS), were treated with vehicle only (for control), 10 nM-1,25 $(OH)_2D_3$ (positive control), 10 nM-17β-oestradiol or a combination of 10 nM-1,25 $(OH)_2D_3$ and 10 nM-17 β-oestradiol for 48 h. In a second series of Ca transport experiments, cells grown in the Transwell® inserts (Costar), and cultured in cell culture media (containing charcoal-stripped, heat-inactivated FBS supplemented with 10 nM-9-*cis* RA), were treated with vehicle only (control), 10 nM-1,25 $(OH)_2D_3$ (positive control), 10 nM-17β-oestradiol or a combination of 10 nM-1,25 $(OH)_2D_3$ and 10 nM-17β-oestradiol for 48 h. All compounds were added to 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 48 h after treatment.

Transepithelial calcium transport studies

The method used for determining Ca transport across the Caco-2 membrane in the present study has been described previously in detail [36]. In all studies, at least three wells were examined per treatment and experiments were repeated thrice (i.e., $n\geq9$ wells per treatment).

Reverse transcription polymerase chain reaction (RT-PCR) analysis for mRNA levels

After experimental treatments, cells were harvested and RNA was isolated and analyzed for calbindin D_{9K}, CaT1, and glyceraldehyde-3-phosphate dehydrogenase as described previously [36]. Primer sequences were (1) calbindin D_{9k} (GenBank X65869), forward 5′-ATGAGTACTAAAAAGTCTCCT-3′, reverse 5′-CTGGGATATCTTTTTTACTAA-3′; 2) CaT1 (GenBank AF365927), forward 5′-TGATGCGGCTCATCAGTGCCAGC-3′, reverse 5′-GTAGAAGTGGCCTAGCTCCTCG-3′; 3) glyderaldehyde-3-phosphate dehydrogenase (GAPDH) (GenBank AJ005371), forward 5′-CCATACAGGCAGCTTCGG-3′, reverse 5′-AGTCATCCACGAGCGATTTG-3′.

Statistical methods

Data were normally distributed and allowed for parametric tests of significance. Data are presented as means and their pooled standard errors. Data for all toxicity variables were subjected to one-way ANOVA, with variation attributed to concentration of treatment compound [41]. To follow up the ANOVA, all pairs of means were compared by the method of least significant difference [41]. All data for Ca uptake and transport and TEER from the first series of studies (cells cultured in charcoal-stripped, heat-inactivated FBS-containing media) were subjected to two-way ANOVA, with variation attributed to 1,25 $(OH)_2D_3$ and 17β-oestradiol [41]. Ca uptake and transport and TEER in cell monolayers treated with 9-*cis* RA were compared to control cell monolayers by Student's *t* tests. Data for Ca uptake and transport and TEER from the second series of studies (cells cultured in charcoal-stripped, heat-inactivated FBS-containing media supplemented with 9-*cis* RA) were subjected to two-way ANOVA, with variation attributed to 1,25 $(OH)_2D_3$ and 17β-oestradiol [41].

Results

The effect of incremental concentrations of 1,25 $(OH)_2D_3$, 17β-oestradiol and 9-*cis* RA on Caco-2 cell survival and viability was assessed using the MTT assay, which is based on mitochondrial dehydrogenase activity, and the Neutral Red assay, where cellular uptake of the dye is indicative of cell viability. Caco-2 cell viability, as determined by either assay

Table 1 Independent and combined effect of 17β-oestradiol (17β-E_2) and 1,25 $(OH)_2D_3$ (1,25 D_3) on calcium uptake and transport in Caco-2 cell monolayers cultured in oestrogen-deplete media (Mean values and pooled standard errors of the mean)

Treatment[a]	−1,25 D_3		+1,25 D_3		Pooled SEM	Statistical significance of variance ratio (*P*), effects of:		
	−17β-E_2 (*n* 9)	+17β-E_2 (*n* 9)	−17β-E_2 (*n* 9)	+17β-E_2 (*n* 9)		1,25 D_3	17β-E_2	1,25 D_3×17β-E_2
Ca uptake (nmol/well)[b]	2.36	2.42	3.12	3.36	0.10	<0.0001	0.182	0.387
Ca Transport								
Total transepithelial:								
(nmol/well per min)	0.15	0.14	0.26	0.28	0.02	<0.0001	0.803	0.734
(%/h)	0.70	0.69	1.19	1.28	0.08	<0.0001	0.673	0.534
Transcellular[c]:								
(nmol/well per min)	0.12	0.11	0.23	0.25	0.07	<0.0001	0.903	0.212
Paracellular:								
(%/h)	0.13	0.15	0.13	0.12	0.01	0.315	0.878	0.286
TEER (Ω cm^2)	2531	2758	2368	2243	108	0.004	0.638	0.115

TEER, transepithelial electrical resistance (after 48-h exposure to the different treatments).
[a]Treatments were given for 48 h before measurement of calcium transport.
[b]Ca uptake evaluated after 60 min.
[c]Transcellular transport is total calcium transport corrected for paracellular (fluorescein) transport (for details of procedures, see *Materials and Methods* section).

was unaffected by exposure to either 1,25 $(OH)_2D_3$ or 17β-oestradiol in the range of 10^{-10} to 10^{-6} M or by exposure to 9-*cis* RA in the range of 10^{-9} to 10^{-7} M for 24 h, relative to control (data not shown). In addition, treatment with 10 nM-1,25 $(OH)_2D_3$, 10 nM-17β-oestradiol and 10 nM-9-*cis* RA for 24 h had no cytotoxic effects in Caco-2 cells, relative to controls, as assessed by the LDH release assay (data not shown).

Ca uptake into fully differentiated Caco-2 cell monolayers (cultured in charcoal-stripped, heat-inactivated FBS-containing media) was significantly (P<0.0001) increased upon exposure to 10 nM-1,25 $(OH)_2D_3$ for 48 h, irrespective of 17β-oestradiol, but was unaffected by 17β-oestradiol (Table 1).

TEER across fully differentiated Caco-2 cell monolayers (cultured in charcoal-stripped, heat-inactivated FBS-containing media) was significantly (P<0.01) reduced upon exposure to 10 nM-1,25 $(OH)_2D_3$ for 48 h, irrespective of 17β-oestradiol, but was unaffected by 17β-oestradiol (Table 1).

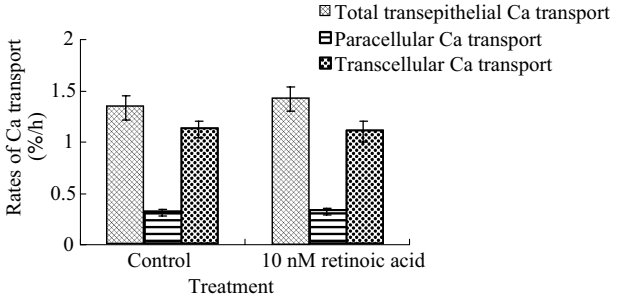

Fig. 1 Effect of 9-*cis* RA treatment for 48 h on total, paracellular and transcellular Ca transport across Caco-2 cell monolayers grown in charcoal-stripped, heat-inactivated foetal bovine serum-containing media. No significant treatment effects were found (P>0.05)

The rate of total transepithelial Ca transport across fully differentiated Caco-2 cell monolayers (cultured in charcoal-stripped, heat-inactivated FBS-containing media) was significantly (P<0.0001) increased upon exposure to 10 nM-1,25 $(OH)_2D_3$ for 48 h, irrespective of 17β-oestradiol, but was unaffected by 17β-oestradiol (Table 1). Similarly, the rate of transcellular Ca transport across fully differentiated Caco-2 cell monolayers was significantly (P<0.0001) increased upon exposure to 10 nM-1,25 $(OH)_2D_3$ for 48 h, irrespective of 17β-oestradiol, but was unaffected by 17β-oestradiol, while paracellular Ca transport (as indicated by fluorescein transport) was unaffected by 1,25 $(OH)_2D_3$ or 17β-oestradiol (Table 1).

Treatment of fully differentiated Caco-2 cell monolayers with 10 nM-9-*cis* RA for 48 h had no significant effect on total transepithelial, transcellular or paracellular calcium transport (Fig. 1). Similarly, TEER across the monolayers was unaffected by with 9-*cis* RA for 48 h (mean (SD) 1922 (501) vs. 1669 (559) Ω cm^2, for control and 10 nM-9-*cis* RA, respectively; P=0.260). TEER across fully differentiated Caco-2 cell monolayers (cells cultured in charcoal-stripped, heat-inactivated FBS-containing media supplemented with 9-*cis* RA) was unaffected by 1,25 $(OH)_2D_3$ or 17β-oestradiol (Table 2).

Ca uptake into and the rate of total transepithelial Ca transport across fully differentiated Caco-2 cell monolayers (cells cultured in charcoal-stripped, heat-inactivated FBS-containing media supplemented with 9-*cis* RA) were significantly (P<0.0001 and P<0.05,

Table 2 Independent and combined effect of 17β-oestradiol (17β-E_2) and 1,25 $(OH)_2D_3$ (1,25 D_3) on calcium uptake and transport in Caco−2 cell monolayers cultured in oestrogen-deplete media supplemented with 9-*cis* RA (Mean values and pooled standard errors of the mean)

Treatment[a]	−1,25 D_3		+1,25 D_3		Pooled SEM	Statistical significance of variance ratio (*P*), effects of:		
	−17β-E_2 (*n* 9)	+17β-E_2 (*n* 9)	−17β-E_2 (*n* 9)	+17β-E_2 (*n* 9)		1,25 D_3	17β-E_2	1,25 D_3×17β-E_2
Ca uptake (nmol/well)[b]	4.27	4.21	5.10	5.12	0.10	<0.0001	0.909	0.836
Ca Transport								
Total transepithelial:								
(nmol/well per min)	0.24	0.23	0.32	0.39	0.05	0.015	0.553	0.456
(%/h)	1.42	1.34	1.86	2.03	0.17	0.018	0.668	0.979
Transcellular[c]:								
(nmol/well per min)	0.18	0.18	0.26	0.32	0.04	0.013	0.517	0.488
Paracellular:								
(%/h)	0.32	0.27	0.34	0.36	0.04	0.198	0.689	0.446
TEER (Ω cm^2)	1669	2052	1541	1622	186	0.158	0.238	0.439

TEER, transepithelial electrical resistance (after 48-h exposure to the different treatments).
[a]Treatments were given for 48 h before measurement of calcium transport.
[b]Ca uptake evaluated after 60 min.
[c]Transcellular transport is total calcium transport corrected for paracellular (fluorescein) transport (for details of procedures, see *Materials and Methods* section).

respectively) increased upon exposure to 10 nM-1,25 $(OH)_2D_3$ for 48 h, irrespective of 17β-oestradiol, but was unaffected by 17β-oestradiol (Table 2). Similarly, the rate of transcellular Ca transport across fully differentiated Caco-2 cell monolayers was significantly ($P<0.05$) increased upon exposure to 10 nM-1,25 $(OH)_2D_3$ for 48 h, irrespective of 17β-oestradiol, but was unaffected by 17β-oestradiol, while paracellular Ca transport (as indicated by fluorescein transport) was unaffected by 1,25 $(OH)_2D_3$ or 17β-oestradiol (Table 2).

Levels of mRNA for calbindin D_{9K} and CaT1 genes were increased in Caco-2 cells exposed to 10 nM-1,25 $(OH)_2D_3$, alone or in combination with 17β-oestradiol, for 48 h compared to that in cells exposed to dimethyl sulfoxide (control) (Fig. 2). mRNA levels for calbindin D_{9K} and CaT1 genes were unaffected in Caco-2 cells which were exposed to 10 nM-17β-oestradiol, and exposure to 10 nM-17β-oestradiol did not augment the 1,25 $(OH)_2D_3$ induced increase in mRNA levels, relative to control (Fig. 2).

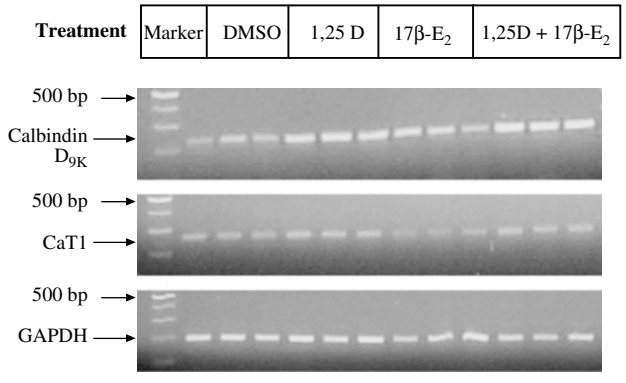

Fig. 2 Effect of treatment for 48 h on calbindin D_{9K} and CaT1 mRNA accumulation in Caco-2 cells. Treatments were: dimethyl sulfoxide (DMSO, control); 10 nM-1,25-dihydroxycholecalciferol (1,25D; 10-nM 17β-oestradiol (17β-E_2); 10 nM-1,25-dihydroxycholecalciferol and 10 nM 17β-oestradiol (1, 25+17β-E_2). Marker, 500 (double band), 400, 300 and 200 bp. Calbindin D_{9K}, CaT1 and glyceraldehydes-3-phosphate dehyrogenase (GAPDH) mRNA levels were assessed by reverse transcriptase-polymerase chain reaction. Representative gels from individual experiments

Discussion

As expected, in the present study, exposing Caco-2 cell monolayers in culture to 10 nM-1,25 $(OH)_2D_3$ for 48 h stimulated Ca uptake and total transepithelial Ca transport. This is in agreement with the findings of other studies that have found that 1,25 $(OH)_2D_3$ enhanced Ca transport in Caco-2 cells [29–30, 34–36, 42–44]. The enhancement of total transepithelial Ca transport by 1,25 $(OH)_2D_3$ was accompanied by an increased transcellular Ca transport, whereas paracellular Ca transport was unaffected. This is in agreement with the findings of several other studies [29–30, 34–35], supporting the notion that 1,25 $(OH)_2D_3$ regulates intestinal Ca absorption by increasing the transcellular flux of Ca through enterocytes [45]. The mechanism by which 1,25 $(OH)_2D_3$ increased transcellular Ca transport is likely to be related to the increase in levels of mRNA for the intestinal calcium binding protein (calbindin D_{9K}), and presumably also in the protein level, although this was not determined. Calbindin D_{9K} is proposed to function as either an intracellular Ca buffer or an

intracellular ferry protein that facilitates diffusion of Ca across the enterocyte [46]. That 1,25 $(OH)_2D_3$-induced an increase in mRNA for calbindin D_{9K} is in agreement with similar findings of several other studies [29, 34–36, 47].

The newly discovered CaT1 epithelial Ca channel, a primary gatekeeper protein which may regulate homeostatic modulation of intestinal Ca absorption efficiency, has recently been reported to be up-regulated in Caco-2 cells by 1,25 $(OH)_2D_3$ treatment for 24 h [47], suggesting another possible means by which 1,25 $(OH)_2D_3$ increases Ca uptake and transport. mRNA levels for CaT1 were relatively low in untreated Caco-2 cells but increased when cells were exposed to 1,25 $(OH)_2D_3$ for 48 h.

Treatment with 10 nM-17β-oestradiol for 48 h had no effect on Ca uptake or total transepithelial Ca transport in differentiated Caco-2 cells (grown in charcoal-stripped, heat-inactivated FBS-containing media). The concentration of 17β-oestradiol (10 nM) used in the present study, while high in terms of physiological circulating concentrations (typically around 325 and 75 pM, in HRT-treated and non-treated postmenopausal women, respectively; [48]), was chosen as it has been used in several studies of Ca uptake into intestinal cells in culture [13, 25–26]. The lack of effect of 17β-oestradiol (and its lack of augmentation of the stimulatory effect of 1,25 $(OH)_2D_3$) on transepithelial Ca transport are in agreement with preliminary findings which suggest that 100 nM-17β-oestradiol has no stimulatory effect (and lack of its augmentation of the stimulatory effect of 100 nM-1,25 $(OH)_2D_3$) on transepithelial Ca transport in Caco-2 cells grown in media containing charcoal-stripped FBS (personal communication from S Taparia and RJ Wood).

While the lack of effect of 17β-oestradiol on Ca uptake into human intestinal cells has not been reported previously, the findings of the present study are in contrast with those which show that 10 nM-17β-oestradiol significantly ($P<0.05$) increased Ca uptake into rat duodenal cells [13, 25]. In addition, the findings of these Caco-2 studies appear to be in contrast to the findings of oestrogen-depleted ovariectomized rats [15–16, 18, 49] and postmenopausal women [2–5], which suggest that oestrogen stimulates Ca absorption in vivo. However, this is not a universal finding in human trials. For example, Gallagher et al. [10] reported a lack of direct effect of oestrogen treatment on Ca absorption in postmenopausal women. It is of interest that, even though 1,25 $(OH)_2D_3$ stimulated Ca absorption in that study, there was no augmentation of the stimulatory effect of 1,25 $(OH)_2D_3$ by oestrogen. Others have also reported a lack of effect of oestrogen on Ca absorption in postmenopausal women [49–50]. Moreover, Holzherr et al. [12] reported a decrease in Ca absorption in postmenopausal women 8 months after commencing HRT.

While Arjmandi et al. [13] suggest that the effect of oestrogen on Ca uptake in their in vitro model is *via* a nuclear OR-mediated mechanism, Picotto et al. [25] suggest that the rapidity of the effect of 17β-oestradiol could be due to a cell surface signalling OR, and probably not the nuclear receptor, since the pure anti-oestrogen ICI 182,780 did not abolish the 17β-oestradiol-induced stimulation of Ca uptake by rat duodenal cells in vitro. Interestingly, Dopp et al. [26] reported that 10 nM-17β-oestradiol increased Ca uptake into OR-positive rat endometrial carcinoma (RUCA-1) cells but not in COS (monkey kidney) cells, which lack OR. We have previously shown that our Caco-2 cells, which are colonocytes in origin but spontaneously differentiate in culture into small intestinal-like enterocytes, express mRNA for ORβ but not the ORα [36], in agreement with the findings of Campbell-Thompson et al. [24] which also show that ORβ message and protein were expressed in Caco-2 cells, but that ORα mRNA was undetectable. Campbell-Thompson et al. [24], on the other hand, showed that normal human colonic mucosa as well as colon tumour samples expressed mRNA for ORα and ORβ, with message for ORα being much lower than for ORβ. It is unclear whether epithelial cells in the human small intestinal tissue express ORα. For example, Salih et al. [51] have shown that there is heterogeneity within intestinal OR proteins in rats, with the duodenum expressing a variant OR devoid of the classical DNA binding domain, whereas, the ileum, jejunum and colon expressed a more typical OR. While it is possible that the lack of mRNA for ORα, and presumably of the ORα protein in our Caco-2 cells, might, in part, explain the lack of stimulatory effect of 17β-oestradiol on Ca transport, we have, however, previously shown that the Caco-2 cells were capable of a functional biological response to 17β-oestradiol (i.e., a proliferative effect), which was inhibited by OH-Tamoxifen, a partial oestrogen agonist [36].

There was no effect of 10 nM-17β-oestradiol on calbindin D_{9K} or CaT1 mRNA levels. These findings are in agreement with those of Wood et al. [47] who reported that exposure of Caco-2 cells to 100 nM-17β-oestradiol had no effect on CaT1 mRNA levels.

The charcoal-stripped, heat-inactivated FBS-containing media used in the present study has dramatically reduced concentrations of various steroid hormones, including 17β-oestradiol and 1,25 $(OH)_2D_3$. It also has reduced retinoic acid levels

and this may influence the potential of 1,25 $(OH)_2D_3$ to enhance transcellular Ca absorption. For example, the reduced 9-*cis* RA levels may limit the induction of gene transcription by the 1,25 $(OH)_2D_3$-VDR-retinoic acid receptor (RXR) complex. 9-*cis* RA, the ligand for the RXR, with 1,25 $(OH)_2D_3$ has been shown to increase parathyroid hormone receptor/parathyroid hormone-related protein receptor mRNA levels 4-fold in renal distal convoluted tubule cells, whereas 1,25 $(OH)_2D_3$ on its own increase mRNA levels by only 70% [52]. However, addition of 9-*cis* RA had no effect on the stimulatory potential of 1,25 $(OH)_2D_3$ on Ca transport in the Caco-2 cells.

In addition to increasing circulating oestrogen and 1,25 $(OH)_2D_3$ levels, HRT has been shown to increase circulating retinol concentrations [53–54]. It is unclear whether increased levels of 9-*cis* RA, influences the enhancement of Ca absorption by HRT by superactivation of the 1,25 $(OH)_2D_3$-VDR-RXR complex. In the present study, Ca transport was not influenced by oestrogen in the presence of 9-*cis* RA. This is the first study, to our knowledge, of the effect of 9-*cis* RA (alone or in conjunction with other steroid hormones) on Ca transport.

In conclusion, the findings of our in vitro study would suggest that oestrogen does not have a direct effect on intestinal Ca absorption. It may be that oestrogen therapy in some individuals enhances Ca absorption indirectly by increasing the circulating levels of 1,25 $(OH)_2D_3$ in postmenopausal women or increasing the intestinal concentration of VDR. However, should the presence of the OR-α be confirmed in healthy human small intestinal tissue, especially the duodenal tissue, then these studies should be repeated with Caco-2 cell lines transfected with OR-α, before a direct effect on intestinal Ca absorption can be discounted.

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References

1. Heaney RP, Recker RR, Stegman MR, Moy AJ (1989) Calcium absorption in women: relationships to calcium intake, estrogen status and age. J Bone Miner Res 4:469–475
2. Gallagher JC (1990) The pathogenesis of osteoporosis. Bone Miner 9:215–227
3. Gallagher JC, Riggs BL, Eisman J, Hamstra A, Arnaud SB, DeLuca HF (1979) Intestinal calcium absorption and serum vitamin D metabolites in normal subjects and ostroporotic patients: effects of age and dietary calcium. J Clin Invest 64:729–736
4. Gallagher JC, Riggs BL, DeLuca HF (1980) Effect of estrogen on calcium absorption and serum vitamin D metabolites in postmenopausal osteoporosis. J Clin Endocrinol Metab 51:1359–1364
5. Heaney RP, Recker RR, Saville PD (1978) Menopausal changes in calcium balance performance. J Lab Clin Med 92:953–963
6. Francis RM, Peacock M, Taylor GA, Storer JH, Nordin BE (1984) Calcium malabsorption in elderly women with vertebral fractures: evidence for resistance to the action of vitamin D metabolites on the bowel. Clin Sci 66:103–107
7. Gennari C, Agnusdei D, Nardi P, Civitelli R (1990) Estrogen preserves a normal intestinal responsiveness to 1, 25-dihyroxyvitamin D3 in oophorectomized women. J Clin Endocrinol Metab 71:1288–1293
8. Morris HA, Need AG, Horowitz M, O'Loughlin PD, Nordin BEC (1991) Calcium absorption in normal and osteoporotic postmenopausal women. Calcif Tissue Int 49: 240–243
9. Aitken JM, Armstrong E, Anderson JB (1972) Osteoporosis after oophorectomy in the mature female rat and the effect of oestrogen and-or progestogen replacement therapy in its prevention. J Endocrinol 55:79–87
10. Gallagher JC, Fowler SE, Detter JR, Sherman SS (2001) Combination treatment with estrogen and calcitriol in the prevention of age-related bone loss. J Clin Endocrinol Metab 86:3618–3628
11. Heaney RP (1976) Estrogens and postmenopausal osteoporosis. Clin Obstet Gynecol 19:791–803
12. Holzherr ML, Retallack RW, Gutteridge DH, Price RI, Faulkner DL, Wilson SG, Will RK, Stewart GO, Stuckey GA, Prince RL, Criddle RA, Kent GN, Bhagat CI, Dhaliwal SS, Jamrozik K (2000) Calcium absorption in postmenopausal osteoporosis: benefit of HRT plus calcitriol, but not HRT alone, in both malabsorbers and normal absorbers. Osteoporos Int 11:43–51
13. Arjmandi BH, Salih MA, Herbert DC, Sims SH, Kalu DN (1993) Evidence for estrogen receptor-linked calcium transport in the intestine. Bone Miner 21:63–74
14. Arjmandi BH, Hollis BW, Kalu DN (1994) In vivo effect of 17β-estradiol on intestinal calcium absorption in rats. Bone Miner 26:181–189
15. Arjmandi BH, Khalil DA, Hollis BW (2000) Ipriflavone, a synthetic phytoestrogen, enhances intestinal calcium transport *in vitro*. Calcif Tissue Int 67:225–229
16. Colin EM, Van Den Bemd GJ, Van Aken M, Christakos S, De Jonge HR, Deluca HF, Prahl JM, Birkenhager JC, Buurman CJ, Pols HA, Van Leeuwen JP (1999) Evidence for involvement of 17 beta-estradiol in intestinal calcium absorption independent of 1,25-dihydroxyvitamin D3 level in the Rat. J Bone Miner Res 14:57–64
17. Kalu DN, Liu CC, Hardin RR, Hollis BW (1989) The aged rat model of ovarian hormone deficiency bone loss. Endocrinology 124:7–16
18. O'Loughlin PD, Morris HA (1998) Oestrogen deficiency impairs intestinal calcium absorption in the rat. J Physiol 15:313–322

19. Russell JE, Morimoto S, Birge SJ, Fausto A, Avioli LV (1986) Effects of age and estrogen on calcium absorption in the rat. J Bone Miner Res 1:185–189
20. Schulz SR, Morris HA (1999) Ionized calcium and bone turnover in the estrogen-deficient rat. Calcif Tissue Int 65:78–82
21. Lund B, Sorensen H, Lund B, Agner E (1982) Serum 1, 25-dihydroxyvitamin D in normal subjects and in patients with postmenopausal osteopenia. Horm Metab Res 14:271–274
22. Stock JL, Coderre JA, Mallette LE (1985) Effects of a short course of estrogen on mineral metabolism in postmenopausal women. J Clin Endocrinol Metab 61:595–600
23. Chen C, Noland KA, Kalu DN (1997) Modulation of intestinal vitamin D receptor by ovariectomy, estrogen and growth hormone. Mech Ageing Dev 99:109–122
24. Campbell-Thompson M, Lynch IJ, Bhardwaj B (2001) Expression of estrogen receptor (ER) subtypes and ER beta isoforms in colon cancer. Cancer Res 61:632–640
25. Picotto G, Massheimer V, Boland R (1996) Acute stimulation of intestinal cell calcium influx induced by 17β-estradiol via cAMP messenger system. Mol Cell Endocrinol 119:129–134
26. Dopp E, Vollmer G, Hahnel C, Grevesmühl Y, Schiffman D (1999) Modulation of the intracellular calcium level in mammalian cells caused by 17β-estradiol, different phytoestrogens and the anti-estrogen ICI 182780. J Steroid Biochem Mol Biol 68:57–64
27. Bronner F (1992) Current concepts of calcium absorption- An overview. J Nutr 122:641S–643S
28. Peng JB, Chen XZ, Berger UV, Vassilev PM, Tsukaguchi H, Brown EM, Hediger MA (1999) Molecular cloning and characterization of a chennel-like transporter mediating intestinal calcium absorption. J Biol Chem 274:22739–22746
29. Fleet JC, Wood RJ (1999) Specific $1,25(OH)_2D_3$-mediated regulation of transcellular calcium transport in Caco-2 cells. Am J Physiol 276:G958–G964
30. Giuliano AR, Wood RJ (1991) Vitamin D-regulated calcium transport in Caco-2 cells: unique in vitro model. Am J Physiol 260:G207–G212
31. Pinto M, Robine-Leon S, Appay MD, Kedinger M, Triadow N, Dussaulx E, Lacroix B, Simon-Assmann P, Haffer K, Fogh J, Zweibaum A (1983) Enterocyte-like differentiation and polarization of the human colon carcinoma cell line Caco-2 in culture. Biol Cell 47:323–330
32. Yee S (1997) In vitro permeability across Caco-2 cells (colonic) can predict in vivo (small intestinal) absorption in man-fact or myth. Pharmacol Res 14:763–766
33. Giuliano AR, Franceschi RT, Wood RJ (1991) Characterization of the vitamin D receptor from the Caco-2 human colon carcinoma cell line: effect of cellular differentiation. Arch Biochem Biophys 285:261–269
34. Fleet JC, Wood RJ (1994) Identification of calbindin D-9k mRNA and its regulation by 1,25-dihydroxyvitamin D_3 in Caco-2 cells. Arch Biochem Biophys 308:171–174
35. Fleet JC, Bradley J, Reddy GS, Ray R, Wood RJ (1996) 1 alpha,25-$(OH)_2$-vitamin D_3 analogs with minimal in vivo calcemic activity can stimulate significant transepithelial calcium transport and mRNA expression in vitro. Arch Biochem Biophys 15:228–234
36. Cotter AA, Jewel C, Cashman KD (2003) The effect of oestrogen and dietary phyto-oestrogens on transepithelial calcium transport in human intestinal-like Caco-2 cells. Br J Nutr 89:755–765
37. Edmonson JM, Armstrong LS, Martinez AO (1988) A rapid and simple MTT-based assay for determining drug sensitivity in monolayer cultures. J Tissue Cult Methods 11:15–17
38. Mossman T (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods 65:55–63
39. Hunt SM, Chrzanowski C, Barnett CR, Brand HN, Fawell JK (1987) A comparison of in vitro cytotoxicity assays and their application to water samples. Altern Lab Anim 15:20–29
40. Vassault A (1983) In: Bergmeyer HV (ed) Methods of enzymatic analysis, Vol 3, 3rd edn. Deer-field Beach, FL, VCH Weinheim, W. Germany, pp118–126
41. Snedecor GW, Cochran WG (1967) Statistical methods Iowa State University Press Ames
42. Chirayath MV, Gajdzik L, Hulla W, Graf J, Cross HS, Peterlik M (1998) Vitamin D increases tight-junction conductance and paracellular Ca^{2+} transport in Caco-2 cell cultures. Am J Physiol 274:G389–G396
43. Cotter AA, Cashman KD (2004) Lack of dose-responsive effect of dietary phyto-oestrogens on transepithelial calcium transport in human intestinal-like Caco-2 cells. Br J Nutr 91:5–9
44. Shao A, Wood RJ, Fleet JC (2001) Increased vitamin D receptor level enhances 1,25-dihydroxyvitamin D_3-mediated gene expression and calcium transport in Caco-2 cells. J Bone Miner Res 16:615–624
45. Nellans HN (1990) Intestinal calcium absorption. Interplay of paracellular and cellular pathways. Miner Electrolyte Metab 16:101–108
46. Bronner F, Pansu D, Stein WD (1986) An analysis of intestinal calcium transport across the rat intestine. Am J Physiol 250:G561–G569
47. Wood RJ, Tchack L, Taparia S (2001) 1,25-Dihydroxyvitamin D_3 increases the expression of the CaT1 epithelial calcium channel in the Caco-2 human intestinal cell line. BMC Physiol 1:11–18
48. Morabito N, Crisafulli A, Vergara C, Gaudio A, Lasco A, Frisina N, D'Anna R, Corrado F, Pizzoleo MA, Cincotta M, Altavilla D, Ientile R, Squadrito F (2002) Effects of genistein and hormone-replacement therapy on bone loss in early postmenopausal women: a randomized double-blind placebo-controlled study. J Bone Miner Res 17:1904–1912
49. Ten Bolscher M, Netelenbos JC, Barto R, Van Buuren LM, Van der vijgh WJ (1999) Estrogen regulation of intestinal calcium absorption in the intact and ovariectomized adult rat. J Bone Miner Res 14:1197–1202
50. Dijkgraaf-ten Bolscher M, Neele SJ, Barto R, van der Vijgh WJ, Netelenbos JC (2000) Effect of estrogen on intestinal strontium absorption in postmenopausal women. Maturitas 36:195–201
51. Salih MA, Sims SH, Kalu DN (1996) Putative intestinal estrogen receptor: evidence for regional differences. Mol Cell Endocrinol 121:47–55
52. Sneddon WB, Barry EL, Coutermarsh BA, Gesek FA, Liu F, Friedman PA (1998) Regulation of renal parathyroid hormone receptor expression by 1,25-dihydroxyvitamin D_3 and retinoic acid. Cell Physiol Biochem 8:261–277
53. Bergmann S, Siegert G, Lattke P, Jaross W, Drecan-Team (1995) Changes in selenium and vitamin A serum concentrations in females as a result of the menopause. Arteriosclerosis 115:S110
54. Meram I, Balat O, Tamer L, Ugur MG (2003) Trace elements and vitamin levels in menopausal women receiving hormone replacement therapy. Clin Exp Obstet Gynecol 30:32–34