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The effect of conjugated linoleic acid and medium-chain fatty acids on transepithelial calcium transport in human intestinal-like Caco-2 cells

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Capric (10 : 0) and lauric (12 : 0) acid and conjugated linoleic acid (CLA) have been shown to increase paracellular permeability across human intestinal-like Caco-2 cell monolayers. While this has generated interest in terms of improved drug absorption and delivery, little has been done in terms of their potential effect on nutrient transport across the intestinal epithelium. Therefore, the objective of the present study was to investigate the effect of these fatty acids on transepithelial Ca transport in Caco-2 cells. Cells were seeded onto permeable transport membranes and allowed to differentiate, over 21 d, into intestinal-like cell monolayers. Monolayers (*n* 9 per treatment) were exposed to 0 (control) or 80 μM-10 : 0, 80 μM-12 : 0, 80 μM-18 : 2, 80 μM-CLA (mixed isomers), 80 μM-*cis*-9,*trans*-11 CLA or 80 μM-*trans*-10,*cis*-12 CLA for 22 d after seeding (chronic effect) or for 24 h before Ca transport studies (acute effect) on day 22. After exposure, transepithelial and transcellular transport of ^{45}Ca, fluorescein transport (a marker of paracellular Ca transport) and transepithelial electrical resistance (TEER, an indicator of permeability) were measured. Overall Ca transport and TEER in Caco-2 cells were unaffected by exposure to any of the fatty acids for 24 h, or to 18 : 2, CLA blend or *cis*-9,*trans*-11 CLA for 22 d. Paracellular (but not total transepithelial and transcellular) Ca transport across Caco-2 cells was significantly increased ($P < 0{\cdot}01$, by about 1·5-fold relative to the control value) by exposure to 10 : 0, 12 : 0 and *trans*-10,*cis*-12 CLA for 22 d, suggesting that these non-esterified fatty acids could potentially enhance Ca absorption *in vivo*.

Conjugated linoleic acid: Calcium absorption: Caco-2 cells

Adequate Ca intake is critical to achieving optimal peak bone mass and modifies the rate of bone loss associated with ageing (National Institutes of Health, 1994). Besides the amount of Ca in the diet, the absorption of dietary Ca in foods is also a critical factor in determining the availability of Ca for bone development and maintenance (Cashman, 2002). Furthermore, the effect of dietary factors on Ca absorption is poorly understood and there is a need for detailed studies to define the ways in which food components influence Ca absorption, in order to determine how Ca bioavailability from foods can be optimised (Kennefick & Cashman, 2000).

While some dietary factors, such as phytate, casein phosphopeptides, lactose and non-digestible oligosaccharides have attracted considerable attention (Cashman, 2002), the influence of dietary lipids on Ca absorption has received much less research emphasis (Kruger & Horrobin, 1997). There have been several reports that have suggested that certain medium-chain fatty acids, such as palmitoylcarnitine (Hochman *et al.* 1994), capric (10:0) and lauric (12:0) acid (Lindmark *et al.* 1995, 1998) can increase the paracellular epithelial permeability of Caco-2 cell monolayers in culture. The Caco-2 cell line is derived from human colonic carcinoma, and can be grown in culture to differentiate into intestinal-like absorptive cells. It is a well-established model of the human intestinal epithelium (Pinto *et al.* 1983). The potential of such fatty acids for modulation of intestinal permeability has been viewed as a possible approach for enhancing the absorption and delivery of drugs (Ward *et al.* 2000). Modulation of paracellular epithelial permeability could also have a significant impact on the intestinal absorption of many nutrients, in particular ions, such as Ca^{2+}. A problem in the case of Ca, however, is that the enhanced paracellular epithelial permeability has

Abbreviations: CLA, conjugated linoleic acid; MTT, 3-(4,5-dimethylthiozol-2-yl)-2,5-diphenyltetrazolium bromide; Neutral Red, 3-amino-7-dimethylamino-2-methylphenazine hydrochloride; TEER, transepithelial electrical resistance; $1,25(OH)_2D_3$, 1,25-dihydroxycholecalciferol.

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only been shown with the sodium salts of these fatty acids (Lindmark *et al.* 1995, 1998), and there is a tendency for these to form soaps with Ca^{2+} (Anderberg *et al.* 1993), making the Ca unavailable for absorption. In addition, in studies of the effects of 10:0 on the intestinal epithelium, Ca^{2+} (presumably, by formation of biologically inert Ca^{2+} soaps) has been shown to dramatically reduce the absorption-enhancing effect of 10:0 to a minimum (Yata *et al.* 1983; Anderberg *et al.* 1993). The effect of the non-esterified form of these fatty acids on intestinal epithelial permeability and, in particular, on Ca absorption has not been investigated.

Recently, Roche *et al.* (2001) reported that a non-esterified isomer of conjugated linoleic acid (CLA) increased paracellular epithelial transport in Caco-2 cells. They found that Caco-2 cell monolayers exposed to the *trans*-10,*cis*-12 isomer of CLA for 14 d had a significantly increased transepithelial transfer of [^{14}C]mannitol (a marker of paracellular flux) relative to control values. The effect appeared to be related to an altered distribution within the cells of occludin and zona occludens 1, which are important protein components of tight junction complex between neighbouring intestinal cells. Interestingly, another isomer of CLA, the *cis*-9,*trans*-11 isomer, which has been suggested to be the active form, because only this isomer is incorporated into the phospholipid fraction of tissues of animals fed a mixture of CLA isomers (Ha *et al.* 1990), had no effect on paracellular permeability (Roche *et al.* 2001). Therefore, the enhanced paracellular permeability in intestinal cells by the *trans*-10, *cis*-12 isomer of CLA may represent a potential mode by which to enhance Ca absorption. However, to our knowledge, there has been no study that has investigated this.

Therefore, the objective of the present study was to investigate the effects of 10 : 0, 12 : 0, CLA (as a blend of isomers) and two specific isomers of CLA (*cis*-9,*trans*-11 and *trans*-10,*cis*-12 isomers of CLA) (all as non-esterified fatty acids) on Ca transport across polarised intestinal epithelial (Caco-2) cell monolayers.

Materials and methods

Materials

Tissue culture materials, included Dulbecco's modified Eagle's medium with L-glutamine and sodium bicarbonate, fetal bovine serum, minimum essential medium. Non-essential amino acids and PBS were purchased from Sigma-Aldrich Ireland Ltd, Dublin, 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 ($1,25(OH)_2D_3$), CLA (mixed isomers) and linoleic acid (18 : 2), 10 : 0 and 12 : 0 were purchased from Sigma-Aldrich Ireland Ltd. Isomers of CLA (*cis*-9,*trans*-11 CLA, *trans*-10,*cis*-12 CLA) were purchased from Cayman Chemicals, Ann Arbor, MI, USA.

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 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 fetal bovine serum (100 ml/l). Caco-2 cells were maintained at 37°C in a CO_2–air (5:95, v/v) atmosphere. Cells were seeded at 3×10^4/cm^2 and passaged when reaching 90 % confluency. Cells used in transepithelial Ca transport experiments were seeded at a density of 6×10^4/cm^2 onto permeable Transwell® filter inserts (24 mm diameter, 0·4 μm pore size; Costar). Cell culture media were changed on alternate days for 21 d. For viability studies, cells (6×10^4/cm^2) were seeded into forty-eight-well culture plates (Costar).

Cell viability assays

The effect of $1,25(OH)_2D_3$, and the various non-esterified fatty acids on Caco-2 cell viability was investigated using the MTT and Neutral Red cell viability assays in forty-eight-well culture plates (Costar) as described by Mossman (1983) and Edmonson *et al.* (1988) (MTT assay) and Hunt *et al.* (1987) (Neutral Red assay). 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.

Preparation of brush border enzymes

Cells were grown for 21 d using the cell culture conditions described earlier. On day 22, cells were washed twice with PBS and scraped into 2 mM Tris–50 mM mannitol (pH 7·1) buffer. Cells were then sonicated with six 10 s bursts separated by 10 s intervals. A few grains of $CaCl_2$ were added to the sonicated cells and the cells put on ice for 10 min. The sonicates were then centrifuged for 10 min at 950 ***g*** at 4°C and the resulting supernatant fraction was further centrifuged for 30 min at 33 500 ***g***. The resulting pellet containing the brush border enzymes was re-suspended in distilled water and stored at −70°C until required for determination of brush border enzyme activities.

Brush border enzyme analysis

The activities of three marker enzymes, namely, alkaline phosphatase, aminopeptidase N and sucrase–isomaltase were used to establish the degree of differentiation of the Caco-2 cells. Alkaline phosphatase was determined using a modification of the method of Colbeau & Maroux (1978). In brief, alkaline phosphatase was measured using *p*-nitrophenyl phosphate (4·5 mM in diethanolamine–HCl buffer (pH 9·75) containing Triton-X 100 (0·5 ml/l)) as a substrate. The reaction was performed for 30 min at 37°C. The reaction was stopped by the addition of 2 M-NaOH and absorbance was read at 405 nm (molar extinction coefficient 17 000 $M^{-1}\,cm^{-1}$). Aminopeptidase

N activity was measured by the method of Svensson *et al.* (1978). Sucrase–isomaltase activity was measured using a modification of the method of Messer & Dahlqvist (1966). In brief, sucrose (56 mM) was used as substrate and the reaction was performed for 60 min at 37°C. At the end of the 60 min, *O*-dianisidine dihydrochloride was added to derivatise the glucose formed during the reaction. Absorbance was read at 452 nm and the amount of glucose formed was determined using a standard curve of glucose concentration *v.* absorbance. Enzyme activity is expressed as mU activity/min per mg brush border protein. One unit of activity (of all three enzymes) is defined as the amount of enzyme required to hydrolyse 1 μmol substrate/min under the experimental conditions.

Transepithelial electrical resistance

For all transport experiments, the transepithelial electrical resistance (TEER; a measure of the integrity of polarised epithelial cell monolayers) was checked before the experiment by a Millicell® ERS meter (Millipore Corp., Bedford, MA, USA) connected to a pair of thin side-by-side electrodes as described by Tanaka *et al.* (1995). TEER readings were taken at 37°C. A TEER value $\geq 800\,\Omega\cdot cm^2$ 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® (Costar) inserts were treated with vehicle only (for control), 10 nM-$1,25(OH)_2D_3$ (positive control) for 24 h, or 80 μM-18 : 2, 80 μM-10 : 0, 80 μM-12 : 0, 80 μM-CLA blend, 80 μM-*cis*-9,*trans*-11 CLA or 80 μM-*trans*-10, *cis*-12 CLA (all as the non-esterified forms of the fatty acids) for 24 h (for acute studies) on day 21 or for 22 d after initial seeding of the cells (for longer-term studies). All compounds were added to complete culture medium before their addition to the cells. The vehicle never exceeded 1 ml/l. TEER measurements were taken immediately before treatment with test compounds and 24 h after treatment (for acute studies) and after 22 d (for longer-term studies).

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 exposure of the cells to the non-esterified fatty acids (for 24 h or 22 d) or to $1,25(OH)_2D_3$ (for 24 h), 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-$CaCl_2$, 0·8 mM-$MgSO_4$, 0·44 mM-KH_2PO_4, 0·34 mM-Na_2HPO_4, 4 mM-glutamine, 25 mM-glucose and 20 mM-4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid, pH 7·4. After rinsing, 2·6 ml of this buffer was added to the lower chamber of the Transwell® (Costar) inserts. At time zero, 1·5 ml transport buffer was added to the upper chamber of the Transwell® inserts. This consisted of the same buffer as the lower chamber (see composition of buffer earlier) except that it also contained ^{45}Ca (with an activity of 148 kBq/ml) and 5·3 mM-fluorescein (as the sodium salt). Fluorescein was included in the transport buffer, in place of Phenol Red, as used by Fleet & Wood (1994), a means of measuring paracellular (diffusional) transport across the Caco-2 monolayer (Lindmark *et al.* 1998). 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® (Costar) inserts. This represented the paracellular route of Ca transport. The amount of ^{45}Ca appearing in the basolateral buffer was expressed as a percentage of the total ^{45}Ca applied to the upper chamber. This represented total transepithelial ^{45}Ca transport (i.e. by both the paracellular and transcellular transport routes) and was expressed as both %/h and nmol transported/min per well. By subtracting the % fluorescein transport/h from the total ^{45}Ca transport (%/h), the amount of ^{45}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.

Statistical methods

Data for all variables were normally distributed and allowed for parametric tests of significance. Data are presented as mean values with their standard errors. Treatment effects were compared by ANOVA, with variation attributed to concentration of treatment compound (cell viability data) or type of treatment compound (Ca transport data) (Snedecor & Cochran, 1967). To follow up the ANOVA, all pairs of mean values were compared by Tukey's multiple comparison test. The percentage detectable difference in total Ca transport in the Caco-2 cell model used in the present study was estimated *a priori* using a power calculation as described by Dallal (1990). This calculation is based on the observed CV of the Ca transport results and the number of replicates per treatment.

Results

The effect of incremental concentrations of 18 : 2, 10 : 0, 12 : 0, CLA (blend of isomers), *cis*-9,*trans*-11 CLA and *trans*-10,*cis*-12 CLA on Caco-2 cell survival and viability was assessed using the MTT assay, which is based on mitochondrial succinic dehydrogenase activity, and the Neutral Red assay, which is based on cellular uptake of the dye as an indicator of cell viability. Caco-2 cell viability was unaffected ($P > 0{\cdot}05$) by exposure to any of the non-esterified fatty acid compounds for 24 h up to concentrations of 80 μM, relative to control (Figs 1 and 2). Increasing concentrations of 12:0, CLA (blend of isomers), *cis*-9,*trans*-11 CLA and *trans*-10,*cis*-12 CLA above 80 μM (and up to 200 μM) had no effect on cell viability, relative to control

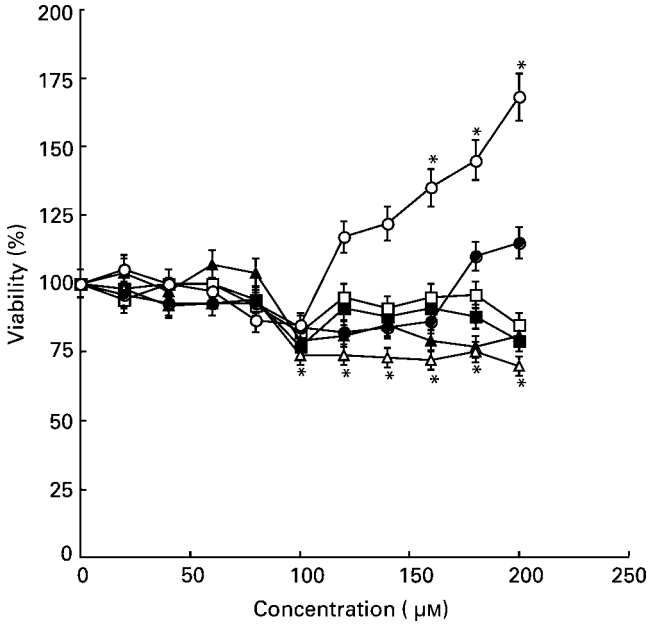

Fig. 1. Effect of 24 h incubation with fatty acids (○, 10 : 0; ●, 12 : 0; △, 18 : 2; ▲, conjugated linoleic acid (CLA) mixed blends; □, *cis*-9,*trans*-11 CLA; ■, *trans*-10,*cis*-12 CLA) on survival of Caco-2 cells, measured by 3-(4,5-dimethylthiozol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. For details of procedures, see p. 640. Results are expressed as surviving fraction relative to vehicle control (ethanol). A value of ±15% of control value (i.e. 100%) can be taken as a benchmark for toxicity. Values are means with their standard errors for at least three independent experiments. The effect of concentration of individual fatty acids on cell survival was analysed using one-way ANOVA. Mean values were significantly different from those of the control incubation (Tukey's multiple comparison test): *$P<0{\cdot}01$.

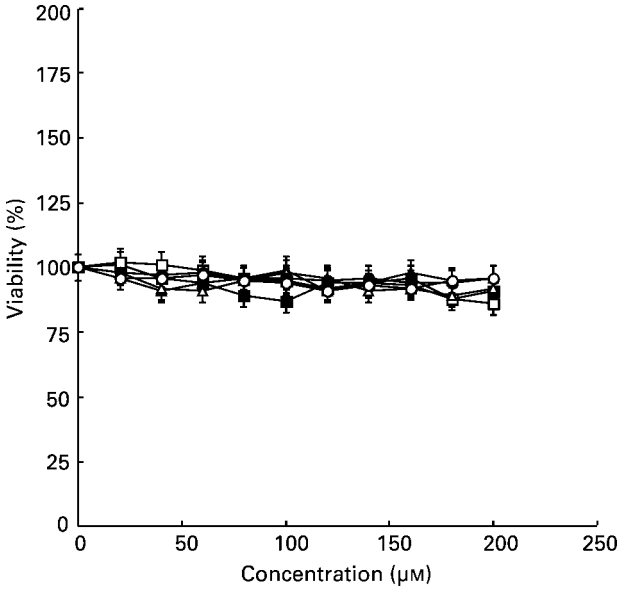

Fig. 2. Effect of 24 h incubation with fatty acids (○, 10 : 0; ●, 12 : 0; △, 18 : 2; ▲, conjugated linoleic acid (CLA) mixed blends; □, *cis*-9,*trans*-11 CLA; ■, *trans*-10,*cis*-12 CLA) on survival of Caco-2 cells, measured by 3-amino-7-dimethylamino-2-methylphenazine hydrochloride (Neutral Red) assay. For details of procedures, see p. 640. Results are expressed as surviving fraction relative to vehicle control (ethanol). A value of ±15% of control value (i.e. 100%) can be taken as a benchmark for toxicity. Values are means with their standard errors for at least three independent experiments. The effect of concentration of individual fatty acids on cell survival was analysed using one-way ANOVA. No significant ($P > 0{\cdot}05$) concentration effects were found.

values (Figs 1 and 2). However, 18:2 at concentrations >100 μM significantly ($P<0{\cdot}01$) reduced MTT metabolism relative to control values, whereas 10:0 at concentrations >160 μM significantly ($P<0{\cdot}01$) increased MTT metabolism, indicating the degree of stress the cells were undergoing (Fig. 1). On the other hand, increasing the concentration of 18 : 2 or 10 : 0 above 80 μM (and up to 200 μM) had no effect on cell viability, as determined with the Neutral Red uptake assay (Fig. 2). In addition, exposure of Caco-2 cells to 1,25$(OH)_2D_3$ (positive control for the Ca transport experiments) (10^{-10}–10^{-6} M) for 24 h had no effect on cell viability, using the MTT and Neutral Red assays.

Brush-border membrane-associated enzyme activities, markers of cell differentiation, in Caco-2 cells grown for 22 d with and without fatty acids (80 μM) are shown in Table 1. As expected, differentiation of Caco-2 cells significantly increased the activity of all three marker enzymes (alkaline phosphatase ($P<0{\cdot}001$), sucrase–isomaltase ($P<0{\cdot}05$), and aminopeptidase N ($P<0{\cdot}05$)), relative to undifferentiated control cells. However, treatment with the various fatty acids (80 μM) for 22 d had no effect on enzyme activity, relative to differentiated control cells.

Treatment of fully differentiated Caco-2 cell monolayers with 10 nM-1,25$(OH)_2D_3$ for 24 h significantly ($P<0{\cdot}001$) increased the total transepithelial Ca transport by about 2·5-fold compared with control values (Table 2 and 3). In addition, 10 nM-1,25$(OH)_2D_3$ significantly increased both transcellular (by about 2·7-fold; $P<0{\cdot}001$) and paracellular (by about 1·5-fold; $P<0{\cdot}01$) Ca transport compared with that in control monolayers (Table 2 and 3). TEER was significantly reduced (by about 18–39%; $P<0{\cdot}001$) by treatment with 1,25$(OH)_2D_3$ for 24 h, relative to control values (Tables 2 and 3).

Treatment of Caco-2 cell monolayers with 80 μM-18 : 2, 80 μM-10 : 0, 80 μM-12 : 0, 80 μM-CLA (blend of isomers), 80 μM-*cis*-9,*trans*-11 CLA or 80 μM-*trans*-10,*cis*-12 CLA for 24 h had no effect on total transepithelial, transcellular or paracellular Ca transport, or on TEER (Table 2).

Treatment of Caco-2 cell monolayers with 80 μM-18 : 2, 80 μM-CLA (blend of isomers) or 80 μM-*cis*-9,*trans*-11 isomer of CLA for 22 d had no effect on total transepithelial, transcellular or paracellular Ca transport or on TEER (Table 3). While total transepithelial and transcellular Ca transport in Caco-2 monolayers was unaffected by treatment with 80 μM-10 : 0, 80 μM-12 : 0, or 80 μM-*trans*-10,*cis*-12 CLA for 22 d, paracellular Ca transport (as indicated by fluorescein transport) was significantly increased (by about 1–5-fold; $P<0{\cdot}001$) by treatment with all three fatty acids (Table 3). In addition, TEER was significantly ($P<0{\cdot}05$) reduced by all three fatty acids, relative to control values (Table 3).

Table 1. Brush-border membrane-associated enzyme activities (mU/min per mg protein) in Caco-2 cells grown for 22 d with and without fatty acids (80 μM)*

(Mean values with their standard errors)

		Enzyme activities					
		Alkaline phosphatase		Sucrase–isomaltase		Aminopeptidase N	
Treatment	*n*	Mean	SE	Mean	SE	Mean	SE
Undifferentiated cells	3	3·35[a]	1·64	7·64[a]	1·58	22·05[a]	3·74
Differentiated cells							
Control	3	181·96[b]	7·23	16·85[b]	0·37	33·16[b]	3·25
Capric acid (10:0)	3	164·32[b]	8·21	15·51[b]	1·25	33·11[b]	4·51
Lauric acid (12:0)	3	173·47[b]	6·87	18·15[b]	2·01	32·23[b]	3·21
Linoleic acid (18:2)	3	185·36[b]	5·17	17·65[b]	3·18	31·09[b]	1·09
CLA (mixed isomers)	3	170·98[b]	3·29	17·48[b]	5·21	31·98[b]	2·65
9,11 CLA	3	169·01[b]	4·91	16·36[b]	2·30	35·19[b]	2·00
10,12 CLA	3	172·58[b]	6·87	17·87[b]	1·55	34·22[b]	3·74
Statistical significance of effect (one-way ANOVA): *P*		<0·001		<0·05		<0·05	

CLA, conjugated linoleic acid; 9,11 CLA, *cis*-9,*trans*-11 CLA; 10,12 CLA, *trans*-10,*cis*-12 CLA.
[a,b]Mean values within a column with unlike superscript letters were significantly different (ANOVA followed by Tukey's multiple comparison test, $P<0·05$).
* For details of procedures, see p. 640.

Discussion

Caco-2 cells have been suggested as a suitable *in vitro* model for predicting Ca absorption in man (Giuliano & Wood, 1991; Fleet & Wood, 1999). In culture, Caco-2 cells spontaneously differentiate and form a polarised 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 & Wood, 1991) and have Ca transport kinetics that suggest the presence of both a saturable and non-saturable Ca transport pathway, similar to observations in the intestines of animals and human subjects (Fleet & Wood, 1999). $1,25(OH)_2D_3$ treatment induces the saturable component of Ca transport (Giuliano & Wood, 1991) and induces accumulation of calbindin D^{9K} and 24-hydroxylase mRNA in these cells (Fleet *et al.* 1996).

As expected, in the present study, exposing Caco-2 cell monolayers in culture to 10 nM-$1,25(OH)_2D_3$ for 24 h stimulated total transepithelial Ca transport, but, unexpectedly, both transcellular and paracellular Ca transport were enhanced. While several studies of the stimulatory effect of $1,25(OH)_2D_3$ on transepithelial Ca transport in Caco-2 cells reported an enhanced rate of transcellular, but not paracellular Ca transport (Giuliano

Table 2. Effect of 24 h exposure to 1,25-dihydroxycholecalciferol (10 nM) or fatty acids (80 μM) on calcium transport in Caco-2 cell monolayers in culture*

(Mean values with their standard errors)

		Calcium 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	9	0·149[a]	0·009	0·33[a]	0·02	0·122[a]	0·007	0·060[a]	0·004	1107[a]	28
$1,25(OH)_2D_3$	9	0·365[b]	0·018	0·81[b]	0·04	0·328[b]	0·016	0·082[b]	0·004	912[b]	19
Capric acid (10:0)	9	0·145[a]	0·009	0·32[a]	0·02	0·119[a]	0·007	0·058[a]	0·004	1104[a]	25
Lauric acid (12:0)	9	0·158[a]	0·011	0·34[a]	0·02	0·129[a]	0·009	0·064[a]	0·004	1092[a]	34
Linoleic acid (18:2)	9	0·162[a]	0·011	0·36[a]	0·02	0·135[a]	0·009	0·060[a]	0·004	1120[a]	20
CLA (mixed isomers)	9	0·152[a]	0·006	0·34[a]	0·01	0·125[a]	0·005	0·060[a]	0·002	1106[a]	25
9,11 CLA	9	0·161[a]	0·008	0·36[a]	0·02	0·134[a]	0·006	0·060[a]	0·004	1051[a]	26
10,12 CLA	9	0·148[a]	0·009	0·33[a]	0·02	0·118[a]	0·006	0·067[a]	0·004	1087[a]	31
Statistical significance of effect (one-way ANOVA): *P*		<0·0001		<0·0001		<0·0001		0·0014		<0·0001	

TEER, transepithelial electrical resistance (after 24 h exposure to the different treatments); $1,25(OH)_2D_3$, 1,25-dihydroxycholecalciferol; CLA, conjugated linoleic acid; 9,11 CLA, *cis*-9,*trans*-11 CLA; 10,12 CLA, *trans*-10,*cis*-12 CLA.
[a,b]Mean values within a column with different superscript letters were significantly different (ANOVA followed by Tukey's multiple comparison test, $P<0·05$).
* For details of procedures, see p. 640.
† Transcellular transport is total calcium transport corrected for paracellular (fluorescein) transport (for details see p. 641).

Table 3. Effect of 24 h exposure to 1,25-dihydroxycholecalciferol (10 nM) and long-term (22 d) exposure to fatty acids (80 μM) on calcium transport in Caco-2 cell monolayers in culture*

(Mean values with their standard errors)

		Calcium transport									
		Total transepithelial				Transcellular		Paracellular			
		(nmol/well per min)		(%/h)		(nmol/well per min)†		(%/h)		TEER (Ω/cm^2)	
Treatment	*n*	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Control	9	0·141[a]	0·008	0·31[a]	0·02	0·118[a]	0·007	0·042[a]	0·003	1877[a]	62
$1,25(OH)_2D_3$	9	0·356[b]	0·010	0·79[b]	0·02	0·309[b]	0·010	0·062[b]	0·004	1145[b]	19
Capric acid (10 : 0)	9	0·146[a]	0·006	0·32[a]	0·01	0·112[a]	0·006	0·060[b]	0·004	1595[c]	38
Lauric acid (12 : 0)	9	0·158[a]	0·009	0·35[a]	0·02	0·119[a]	0·009	0·065[b]	0·003	1539[c]	49
Linoleic acid (18 : 2)	9	0·155[a]	0·008	0·34[a]	0·02	0·124[a]	0·009	0·056[a]	0·004	1726[a,c]	45
CLA (mixed isomers)	9	0·145[a]	0·008	0·32[a]	0·02	0·111[a]	0·004	0·053[a]	0·003	1731[a,c]	54
9,11 CLA	9	0·155[a]	0·011	0·34[a]	0·02	0·118[a]	0·006	0·056[a]	0·004	1839[a]	65
10,12 CLA	9	0·145[a]	0·007	0·32[a]	0·02	0·115[a]	0·008	0·064[b]	0·004	1633[c]	26
Statistical significance of effect (one-way ANOVA): *P*		<0·0001		<0·0001		<0·0001		0·0008		<0·0001	

TEER, transepithelial electrical resistance (after 24 h exposure to 1,25-dihydroxycholecalciferol and long-term (22 d) exposure to fatty acids); $1,25(OH)_2D_3$, 1,25-dihydroxycholecalciferol; CLA, conjugated linoleic acid; 9,11 CLA, *cis*-9,*trans*-11 CLA; 10,12 CLA, *trans*-10,*cis*-12 CLA.

[a,b,c]Mean values within a column with different superscript letters were significantly different (ANOVA followed by Tukey's multiple comparison test, $P<0·05$).

* For details of procedures, see p. 640.

† Transcellular transport is total calcium transport corrected for paracellular (fluorescein) transport (for details see p. 641).

& Wood, 1991; Fleet & Wood, 1994, 1999; Fleet *et al.* 1996), Chirayath *et al.* (1998) have reported that 10 nM-$1,25(OH)_2D_3$ for up to 72 h stimulated paracellular, but had no effect on transcellular, Ca transport. The dual stimulatory effect of $1,25(OH)_2D_3$ on both processes of Ca transport observed in the present study deserves further investigation, especially since at moderate to high dietary Ca intakes, paracellular Ca transport is dominant (Bronner & Pansu, 1999). The significant enhancement of total transepithelial Ca transport by $1,25(OH)_2D_3$ in the present study, irrespective of the mechanism, acted as a positive control for our experiments to investigate the influence of two medium-chain fatty acids (namely 10 : 0 and 12 : 0) and CLA on Ca transport in the Caco-2 model.

In the present study, short-term (24 h) exposure of Caco-2 cells in culture to 10 : 0, 12 : 0, 18 : 2, CLA blend, or the *cis*-9,*trans*-11 and *trans*-10,*cis*-12 isomers of CLA had no effect on the total transepithelial, transcellular or paracellular transport of Ca. The effect of 24 h exposure to medium-chain fatty acids or isomers of CLA on Ca absorption or paracellular permeability has not been reported elsewhere. Some studies which have investigated the effect of sodium salts of medium-chain fatty acids, such as 10:0 and 12 : 0, on paracellular permeability have demonstrated an enhancing effect after as little as 2 min following exposure of Caco-2 cells to the fatty acids (Anderberg *et al.* 1993; Lindmark *et al.* 1998). The longest reported exposure time of the Caco-2 cells to the fatty acids is only 60 min (Anderberg *et al.* 1993; Lindmark *et al.* 1995, 1998). Interestingly, Lindmark *et al.* (1998) showed that while 10 : 0 and 12 : 0 at a concentration of 13·00 and 0·75 mM respectively stimulated paracellular permeability over the course of a 40 min experiment, the stimulatory effect of 10 : 0 and 12 : 0 at lower concentrations (5·000 and 0·375 mM respectively), while evident initially at 2–4 min, disappeared after 20–40 min. Therefore, the lack of effect of 80 μM-10:0 and 80 μM-12:0 on paracellular permeability in Caco-2 cells after exposure for 24 h in the present study may reflect a complete restoration of the epithelial integrity at the time the transport studies were performed. Alternatively, the concentration of 10 : 0 and 12 : 0 (80 μM) may have been too low to evoke a stimulatory effect on paracellular permeability over 24 h, even though this stimulatory effect was evident after 22 d of exposure in the present study. For example, Lindmark *et al.* (1995) investigated the effect of a concentration range of 12 : 0 (60–5000 μM) on paracellular permeability across Caco-2 cell monolayers after 60 min and reported that the lowest concentration of 12 : 0 which stimulated permeability was 580 μM.

The concentration of the CLA used in the present study (80 μM) was chosen as an estimate of luminal concentration of CLA in the human small intestine following a meal, based on an estimated daily dietary intake of about 150 mg (Lawson *et al.* 2001). A similar concentration of 18 : 2, 10 : 0 and 12 : 0 was used in the present study for comparative purposes, but also because of evidence that above 80 and 160 μM there was an adverse effect of 18 : 2 and 10 : 0 respectively on cell viability. Based on estimated daily dietary intakes of 445 and 890 mg for 10 : 0 and 12 : 0 respectively (US Department of Agriculture, Agricultural Research Service, 1997), the expected luminal concentrations of 10 : 0 and 12 : 0 in the human small intestine following a meal would be about 430 and 740 μM respectively, which are much higher than that used in the present study (80 μM).

In the present study, in contrast to the lack of effect of short-term (24 h) exposure of Caco-2 cells in culture to the various fatty acids, chronic exposure to 10 : 0 and 12 : 0 for 22 d significantly reduced TEER ($P<0·001$) and significantly ($P<0·001$) increased the paracellular transport of Ca by about 1·5-fold for both fatty acids, relative to

control values. Similarly, the *trans*-10,*cis*-12 isomer of CLA (but not *cis*-9,*trans*-11 isomer of CLA, CLA isomer blend, or 18 : 2) also enhanced paracellular transport (by about 1·5-fold, $P<0·001$) and reduced TEER ($P<0·001$), relative to control values. The effect of long-term (22 d) exposure to medium-chain fatty acids or CLA blend on Ca absorption or paracellular permeability has not been reported elsewhere. Roche *et al.* (2001) showed that exposure of Caco-2 monolayers to 50 μM-*trans*-10,*cis*-12 isomer for 14 d increased the paracellular epithelial permeability of Caco-2 cells, using [^{14}C]mannitol as a marker, and reduced TEER, whereas exposure to 50 μM-*cis*-9,*trans*-11 isomer of CLA and 18 : 2 had no effect on epithelial permeability or TEER. However, in that study, there were no significant differences in paracellular permeability or TEER after 21 d of exposure to the fatty acids. A reduction in TEER across Caco-2 cell monolayers is considered to reflect an effect on tight junction-mediated paracellullar ion permeability (McRoberts *et al.* 1990). While short-chain fatty acids, such as acetate, propionate and butyrate, have recently been shown to stimulate paracellular Ca transport individually *in vitro* in the epithelium of the rat caecum and colon (Mineo *et al.* 2001), this is the first report, to our knowledge, which shows that medium-chain fatty acids, as well as a specific isomer of CLA (*trans*-10,*cis*-12 CLA), can modulate paracellular Ca transport across intestinal epithelial cells. The increase in paracellular Ca transport brought about by exposure to these fatty acids, however, did not translate into a significantly increased total transepithelial Ca transport across the Caco-2 monolayer. It is likely that the lack of stimulatory effect of 10 : 0, 12 : 0 and *trans*-10,*cis*-12 isomer of CLA on total transepithelial Ca transport, despite significantly enhancing paracellular Ca transport in the present study, is due to the fact that the latter only contributed about 13·5 % to total Ca transport in these Caco-2 cells, with transcellular transport being the major component of overall transport. Thus, the increased paracellular transport did not have a major impact of the absolute amount of Ca transported (i.e. a 1·5-fold increase in paracellular Ca transport upon addition of fatty acids would translate into a maximal 8 % increase in total transepithelial Ca transport, which in the present study was well below the detectable change (16 %) in total transport, as determined using a power calculation). That paracellular Ca transport made only a minor contribution to overall Ca transport in the Caco-2 cell monolayers in the present study is in agreement with the findings of several other studies of Ca transport in Caco-2 cells, which show that the transcellular route of Ca transport is the dominant pathway (Giuliano & Wood, 1991; Fleet & Wood, 1994, 1999; Fleet *et al.* 1996). In contrast, the paracellular route of Ca absorption is generally thought to be predominant in the human intestine *in vivo* when Ca intake is adequate to high (Bronner, 1998; Bronner & Pansu, 1999), because moderate to high Ca intake leads to down-regulation of active, transcellular Ca transport (Auchere *et al.* 1998). Therefore, a 1·5-fold increase in paracellular Ca transport by 10 : 0, 12 : 0 and *trans*-10, *cis*-12 isomer of CLA could, in theory, have a significant impact on Ca absorption in intestinal cells that transport Ca predominantly by the paracellular route (such as human intestinal cells *in vivo*), but this needs to be investigated further.

It is not clear why in the present study exposure of Caco-2 cells for 22 d to 10 : 0, 12 : 0 and the *trans*-10,*cis*-12 isomer of CLA stimulated paracellular permeability, whereas exposure for 24 h had no effect. To test whether the enhanced paracellular permeability evident in Caco-2 monolayers after their chronic (22 d) exposure to 10 : 0, 12 : 0 and *trans*-10,*cis*-12 CLA in the present study was due to an effect of these fatty acids on Caco-2 cell differentiation, and thus development of functional tight junction, various differentiation marker enzymes were measured in the present study. There was, however, no effect of any of the test fatty acids on Caco-2 cell differentiation. Furthermore, the mechanism by which 10 : 0, 12 : 0 and the *trans*-10,*cis*-12 isomer of CLA (but not *cis*-9, *trans*-11 CLA isomer or 18 : 2, all as non-esterified fatty acids) enhanced paracellular Ca transport over 22 d in the present study is unclear. Roche *et al.* (2001) recently reported that the *trans*-10,*cis*-12 isomer (but not *cis*-9,*trans*-11 CLA isomer or 18 : 2) increased paracellular permeability, possibly by altering the cellular distribution of occludin (an integral structural protein component of the tight junction, expressed in polarised epithelial cell monolayers). The sodium salts of certain medium-chain fatty acids (at relatively high concentrations) have been shown to damage the intestinal epithelium by lysing the apical membrane and in that way increase the paracellular permeability (Duizer *et al.* 1998; Ward *et al.* 2000). However, at lower concentrations the same compounds increases paracellular permeability in a reversible mode that does not involve cell lysis, but rather via a specific and controlled modulation of tight junctions (Ward *et al.* 2000). In the present study, there was no evidence of any cytotoxic effects by any of the test fatty acids at a concentration of 80 μM, but in particular, by the ones with paracellular Ca transport-modulatory potential (i.e. 10 : 0, 12 : 0 and *trans*-10,*cis*-12 CLA). Therefore, their enhancing ability for paracellular permeability was more likely to be due to modulation of the tight junctions. While the molecular basis of the effects of sodium salts of medium-chain fatty acids (such as 10 : 0 and 12 : 0) on paracellular epithelial permeability are not completely understood: several mechanisms, including activation of phospholipase C-dependent inositol triphosphate–diacylglycerol signalling pathway, ATP depletion, tyrosine kinase–phosphatase pathway, depletion of extracellular Ca leading to a disruption of cell–cell adhesion, have been proposed and these have recently been reviewed by Ward *et al.* (2000). Whether the non-esterified acid forms of medium-chain fatty acids, as used in the present study, operate through the same mechanisms as the sodium salts of these fatty acids would need to be investigated.

Conclusion

The results of the present study showed that chronic, but not acute, exposure to 10 : 0, 12 : 0 and the *trans*-10,*cis*-12 isomer of CLA enhanced the paracellular route of Ca absorption in Caco-2 cells. The mechanism by which this occurred is unclear. Because the mode by which Caco-2

cells transports Ca across the epithelium is predominantly by the transcellular route, the influence of these fatty acids on total transepithelial Ca transport (and transcellular and paracellular transport) should be re-evaluated in an intestinal cell model which relies more heavily on the paracellular route of Ca transport. The effect of these fatty acids on the transport of other mineral ions should also be investigated.

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