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 45Ca, 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<0.01, 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 Ca2+. A problem in the case of Ca, however, is that the enhanced paracellular epithelial permeability has...
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 \textit{trans}-10,\textit{cis}-12 isomer of CLA for 14 d had a significantly increased transepithelial transfer of \([^{14}\text{C}]\)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 \textit{cis}-9,\textit{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 \textit{trans}-10, \textit{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 (\textit{cis}-9,\textit{trans}-11 and \textit{trans}-10,\textit{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. \(^{45}\text{Ca}\) (as \(^{45}\text{Ca}\) in an aqueous solution of CaCl\(_2\), with a specific activity of 1·85 MBq/mg Ca) was purchased from Nensure\textsuperscript{TM}, Boston, MA, USA, Fluorescein sodium salt, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 3-amino-7-dimethylamo-2-methylphenazine hydrochloride (Neutral Red), 1,25 dihydroxycholecalciferol (1,25(OH)\(_2\)D\(_3\)), CLA (mixed isomers) and linoleic acid (18:2), 10:0 and 12:0 were purchased from Sigma-Aldrich Ireland Ltd. Isomers of CLA (\textit{cis}-9,\textit{trans}-11 CLA, \textit{trans}-10,\textit{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\textsuperscript{TM} 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)\(_2\)D\(_3\), 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 \textit{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 vs. 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 ≥800 Ω·cm² 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)₂D₃ (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 of the methods of Giuliano & Wood (1991) and Fleet & Wood (1994). Transepithelial transport of Ca was studied.

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>0.05) 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.
values (Figs 1 and 2). However, 18:2 at concentrations >100 μM significantly (P<0.01) reduced MTT metabolism relative to control values, whereas 10:0 at concentrations >160 μM significantly (P<0.001) 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)2D3 (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<0.001), sucrase–isomaltase (P<0.001) and aminopeptidase N (P<0.005), 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)2D3 for 24 h significantly (P<0.001) 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)2D3 significantly increased both transcellular (by about 2.7-fold; P<0.0001) and paracellular (by about 1.5-fold; P<0.001) Ca transport compared with that in control monolayers (Tables 2 and 3). TEER was significantly reduced (by about 18–39%; P<0.0001) by treatment with 1,25(OH)2D3 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<0.001) by treatment with all three fatty acids (Table 3). In addition, TEER was significantly (P<0.001) reduced by all three fatty acids, relative to control values (Table 3).
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 polarized epithelial monolayer with tight junctions and express a differentiated cell phenotype consistent with absorptive small intestine-like enterocytes (Pinto et al. 1983; Yee, 1997). In particular, these cells have a functional vitamin D receptor (Giuliano & 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)2D3 treatment induces the saturable component of Ca transport (Giuliano & Wood, 1991) and induces accumulation of calbindin D9K 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)2D3 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)2D3 on transepithelial Ca transport in Caco-2 cells reported an enhanced rate of transcellular, but not paracellular Ca transport (Giuliano & Wood, 1991).

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)*

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Enzyme activities</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Alkaline phosphatase</td>
</tr>
<tr>
<td></td>
<td>Mean SE</td>
</tr>
<tr>
<td>Undifferentiated cells</td>
<td>3 3.35a 1.64</td>
</tr>
<tr>
<td>Differentiated cells</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>3 181.96b 7.23</td>
</tr>
<tr>
<td>Capric acid (10:0)</td>
<td>3 164.32b 8.21</td>
</tr>
<tr>
<td>Lauric acid (12:0)</td>
<td>3 173.47b 6.67</td>
</tr>
<tr>
<td>Linoleic acid (18:2)</td>
<td>3 185.36b 5.17</td>
</tr>
<tr>
<td>CLA (mixed isomers)</td>
<td>3 170.98b 3.29</td>
</tr>
<tr>
<td>9,11 CLA</td>
<td>3 169.01b 4.91</td>
</tr>
<tr>
<td>10,12 CLA</td>
<td>3 172.58b 6.87</td>
</tr>
</tbody>
</table>

Statistical significance of effect (one-way ANOVA): P<0.001, 0.05, 0.05

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*

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Calcium transport</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total transepithelial</td>
</tr>
<tr>
<td></td>
<td>nmol/well per min</td>
</tr>
<tr>
<td></td>
<td>Mean SE</td>
</tr>
<tr>
<td>Control</td>
<td>0.149a 0.009</td>
</tr>
<tr>
<td>1,25(OH)2D3</td>
<td>0.365b 0.018</td>
</tr>
<tr>
<td>Capric acid (10:0)</td>
<td>0.145b 0.009</td>
</tr>
<tr>
<td>Lauric acid (12:0)</td>
<td>0.158b 0.011</td>
</tr>
<tr>
<td>Linoleic acid (18:2)</td>
<td>0.162a 0.011</td>
</tr>
<tr>
<td>CLA (mixed isomers)</td>
<td>0.152a 0.006</td>
</tr>
<tr>
<td>9.11 CLA</td>
<td>0.161a 0.008</td>
</tr>
<tr>
<td>10,12 CLA</td>
<td>0.148a 0.009</td>
</tr>
</tbody>
</table>

Statistical significance of effect (one-way ANOVA): P<0.0001, 0.0001, 0.0001, 0.0014, 0.0001

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)*

(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).

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)2D3 treatment induces the saturable component of Ca transport (Giuliano & Wood, 1991) and induces accumulation of calbindin D9K 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)2D3 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)2D3 on transepithelial Ca transport in Caco-2 cells reported an enhanced rate of transcellular, but not paracellular Ca transport (Giuliano & Wood, 1991).
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)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Total transepithelial (nmol/well per min) Mean ± SE</th>
<th>Transcellular (nmol/well per min) Mean ± SE</th>
<th>Paracellular (µmol/well per min) Mean ± SE</th>
<th>TEER (Ω/cm²) Mean ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.141 ± 0.008</td>
<td>0.31 ± 0.02</td>
<td>0.042 ± 0.003</td>
<td>1877 ± 62</td>
</tr>
<tr>
<td>1,25(OH)₂D₃</td>
<td>0.359 ± 0.010</td>
<td>0.79 ± 0.02</td>
<td>0.062 ± 0.004</td>
<td>1145 ± 19</td>
</tr>
<tr>
<td>Capric acid (10:0)</td>
<td>0.146 ± 0.006</td>
<td>0.32 ± 0.01</td>
<td>0.060 ± 0.004</td>
<td>1585 ± 38</td>
</tr>
<tr>
<td>Lauric acid (12:0)</td>
<td>0.158 ± 0.009</td>
<td>0.35 ± 0.02</td>
<td>0.065 ± 0.003</td>
<td>1539 ± 49</td>
</tr>
<tr>
<td>Linoleic acid (18:2)</td>
<td>0.155 ± 0.008</td>
<td>0.34 ± 0.02</td>
<td>0.056 ± 0.004</td>
<td>1726 ± 45</td>
</tr>
<tr>
<td>CLA (mixed isomers)</td>
<td>0.145 ± 0.008</td>
<td>0.32 ± 0.02</td>
<td>0.053 ± 0.003</td>
<td>1731 ± 54</td>
</tr>
<tr>
<td>9,11 CLA</td>
<td>0.155 ± 0.011</td>
<td>0.34 ± 0.02</td>
<td>0.056 ± 0.004</td>
<td>1839 ± 65</td>
</tr>
<tr>
<td>10,12 CLA</td>
<td>0.145 ± 0.007</td>
<td>0.32 ± 0.02</td>
<td>0.064 ± 0.004</td>
<td>1633 ± 26</td>
</tr>
</tbody>
</table>

Statistical significance of effect (one-way ANOVA): *P < 0.0001

* 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)₂D₃ for up to 72 h stimulated paracellular, but had no effect on transepithelial, Ca transport. The dual stimulatory effect of 1,25(OH)₂D₃ 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)₂D₃ 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 \textit{trans}-10,\textit{cis}-12 isomer of CLA (but not \textit{cis}-9,\textit{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 \( \mu \text{M} \)-\textit{trans}-10,\textit{cis}-12 isomer for 14 d increased the paracellular epithelial permeability of Caco-2 cells, using \( ^{14}\text{C}\)mannitol as a marker, and reduced TEER, whereas exposure to 50 \( \mu \text{M} \)-\textit{cis}-9,\textit{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 paracellular 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 \textit{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 (\textit{trans}-10,\textit{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 \textit{trans}-10,\textit{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 transepacellular 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 transepacellular 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 \textit{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, transepithelial Ca transport (Auchere et al. 1998). Therefore, a 1.5-fold increase in paracellular Ca transport by 10:0, 12:0 and \textit{trans}-10, \textit{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 \textit{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 \textit{trans}-10,\textit{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 \textit{trans}-10,\textit{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 \textit{trans}-10,\textit{cis}-12 isomer of CLA (but not \textit{cis}-9, \textit{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 \textit{trans}-10,\textit{cis}-12 isomer (but not \textit{cis}-9,\textit{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 \( \mu \text{M} \), but in particular, by the ones with paracellular Ca transport-modulatory potential (i.e. 10:0, 12:0 and \textit{trans}-10,\textit{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.

\textbf{Conclusion}

The results of the present study showed that chronic, but not acute, exposure to 10:0, 12:0 and the \textit{trans}-10,\textit{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 relays 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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References


