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Acute-on-chronic effects of fatty acids on intestinal triacylglycerol-rich lipoprotein metabolism

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Postprandial triacylglycerol (TAG) metabolism is an important metabolic state that has been associated with cardiovascular disease. The magnitude of the postprandial TAG response is determined by dietary fat composition, which alters intestinal and hepatic TAG-rich lipoprotein (TRL) metabolism. Caco-2 cell monolayers are morphologically and physiologically similar to the human intestinal enterocytes, hence they are a good model to study intestinal lipoprotein metabolism. To date only the acute effect of fatty acid composition on intestinal TRL metabolism in Caco-2 cells has been investigated. Little is known of the effect of habitual, or chronic, dietary fat composition on intestinal TRL metabolism. Using the Caco-2 cell model, the present study investigated the acute-on-chronic effect of fatty acid composition on TRL metabolism. Caco-2 cells were grown in the presence of 0·05 mM-palmitic acid (PA; 16 : 0), -oleic acid (OA; 18 : 1*n*-9),-eicosapentaenoic acid (EPA; 20 : 5*n*-3) or no fatty acid (control) for 19 d, then one of four acute treatments (control (bovine serum albumin (BSA; 5 g/l)) or BSA (5 g/l) plus 0·5 mM-PA, -OA or -EPA) were administered for 22 h. Significant acute × chronic interactions for the effect of fatty acid composition on cellular TAG:secreted *de novo* TAG, and cellular *de novo* TAG:*de novo* phospholipid were observed. Thus the effect of a fatty acid was determined by the duration of exposure to the fatty acid intervention. Acute PA treatment increased *de novo* TAG synthesis, but chronic PA supplementation did not. Acute and chronic OA treatments increased *de novo* TAG secretion. For EPA, chronic supplementation had the greatest effect on TAG synthesis and secretion. The acute-on-chronic effects of fatty acids on apolipoprotein B metabolism were relatively minor compared with the changes noted for TRL lipid composition. The present study shows that the Caco-2 cell model is valuable for studying intestinal TRL metabolism and that fatty acids modulate this process, the nature of which can be determined by the length of exposure of the cell to the fatty acid.

Cardiovascular disease: Fatty acids: Lipid metabolism: Triacylglycerol: Caco-2 cells

Postprandial triacylglycerol (TAG) metabolism is an important physiological state that can be associated with cardiovascular disease, whereby the magnitude and duration of postprandial TAG concentrations are positively related to the pathogenesis and progression of atherosclerosis (Weintraub *et al.* 1988; Patsch *et al.* 1992; Karpe *et al.* 1994, 1997). The postprandial TAG response is determined by genetic, physiological and nutritional factors, the details of which have been extensively reviewed elsewhere (Roche & Gibney, 1995, 2000). Both acute and chronic dietary fat composition is one of the most important nutritional determinants of postprandial TAG metabolism. In terms of the acute response, the greater the degree of unsaturation of the fatty acids presented in the test meal, the lower the postprandial TAG response (Zampelas *et al.* 1994). Chronic or habitual dietary fat composition also affects the magnitude of postprandial lipid metabolism. Dietary intervention studies show that the magnitude of the postprandial TAG response is greatest following a background saturated fatty acid (SFA) diet, less following an *n*-6 polyunsaturated fatty acid (PUFA)-rich diet and least following an *n*-3 PUFA-rich diet (Weintraub *et al.* 1988). Collaborative cross-cultural and dietary intervention studies by our research group show that the relative

Abbreviations: apo, apolipoprotein; BSA, bovine serum albumin; cB, non-secreted apolipoprotein B; cPL, non-secreted phospholipid; cTAG, non-secreted triacylglycerol; DPM, disintegrations/min; EPA, eicosapentaenoic acid; MUFA, monounsaturated fatty acid; OA, oleic acid; PA, palmitic acid; P_{app}, permeability coefficient; PL, phospholipid; PUFA, polyunsaturated fatty acid; sB, secreted apolipoprotein B; SFA, saturated fatty acid; sPL, secreted phospholipid; sTAG, secreted triacylglycerol; TAG, triacylglycerol; TRL, triacylglycerol-rich lipoprotein.

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proportion of monounsaturated fatty acids (MUFA) and SFA affects the nature of the postprandial response. Southern Europeans consuming a high-MUFA, low-SFA diet had significantly greater plasma TAG concentrations during the early postprandial state (2–3 h), which were then rapidly removed from the circulation, compared with Northern Europeans with a habitual low-MUFA, high-SFA diet, who were matched for age, gender and fasting lipid concentrations (Zampelas *et al.* 1998). In a subsequent dietary intervention trial, isoenergetic substitution of SFA for MUFA in the diet of Northern European males resulted in a greater increase in plasma TAG and TAG-rich lipoprotein (TRL) apolipoprotein (apo) B-48 levels during the early postprandial period (Roche *et al.* 1998). The significant alterations in plasma TAG and apo B-48 levels during the early postprandial phase suggested that dietary fatty acid composition altered gastrointestinal TRL metabolism.

The human colon carcinoma Caco-2 cell line is an established model of the human small intestine because the cells spontaneously differentiate in culture into enterocytes and monolayers grown on permeable supports and are morphologically and physiologically similar to human intestinal enterocytes (Levy *et al.* 1995). The Caco-2 cell model allows investigation of the effects of dietary fat composition on intestinal TRL synthesis and secretion, that in turn contributes to postprandial TAG metabolism. Previous studies using Caco-2 cells have investigated the acute effects of fatty acids on TRL TAG and apo B synthesis and secretion (Field *et al.* 1988; Moberly *et al.* 1990; Murthy *et al.* 1990; Ranheim *et al.* 1992; van Greevenbroek *et al.* 1995, 1996, 1998, 2000). These acute studies investigated the effect of one dose of a fatty acid given to cells in serum-free media for no longer than 24 h. Such studies in the Caco-2 cell line have shown that oleic acid (OA; 18 : 1*n*-9) treatment induces a higher rate of TAG synthesis in Caco-2 cells compared with linoleic acid (18 : 2*n*-6) or palmitic acid (PA; 16 : 0) (Field *et al.* 1988). It was also shown that OA was the most efficient promoter of *de novo* TAG secretion followed by linoleic acid and PA (Field *et al.* 1988). Van Greevenbroek *et al.* (1996) confirmed that acute PA (0·5 mM) treatment reduces Caco-2 cell *de novo* TAG secretion compared with cells given either MUFA or *n*-6 PUFA treatment. Variation in the OA:PA ratios (final fatty acid concentration of 0·5 mM) exposed to Caco-2 cells also alters the secreted TRL:intermediate density lipoprotein and LDL-sized particles ratio by the cells (van Greevenbroek *et al.* 2000). The higher the OA:PA ratio administered to Caco-2 cells, the greater the proportion of *de novo* TAG found in the TRL density fraction. Also an acute dose of eicosapentaenoic acid (EPA; 20 : 5*n*-3) (0·25 mM) reduces *de novo* TAG synthesis and secretion, but had no effect on total TAG secretion from Caco-2 cells (Murthy *et al.* 1990).

To date, all *in vitro* studies have investigated the acute effect of fatty acids on Caco-2 cell TRL metabolism as a model of intestinal lipoprotein metabolism. We known from human intervention studies that chronic or habitual dietary fats, including SFA (Zampelas *et al.* 1994), MUFA (Roche *et al.* 1998) and PUFA (Weintraub *et al.* 1988), modulate postprandial TAG metabolism. Whilst part of that effect is due to altered hepatic lipoprotein metabolism, it is becoming increasingly clear that intestinal lipoprotein synthesis and secretion is also important. Therefore the present experiment was designed to investigate the acute-on-chronic effects of fatty acid composition on intestinal TRL metabolism using the Caco-2 cell model.

Materials and methods

Caco-2 cell culture

Adherent colon carcinoma cell line (Caco-2) was obtained from the European Collection of Animal Cell Cultures and was grown from passages 33 to 47. Cells were grown in media (consisting of Dulbecco's Modified Eagle Medium (High Glucose, with Glutimax and 4500 mg/l D-glucose; GibcoBRL, Paisley, UK), fetal calf serum (100 g/l; GibcoBRL), 1 × sodium pyruvate (GibcoBRL) and gentimycin (0·1 mg/ml; GibcoBRL). Caco-2 cells were grown in 75 cm^2 or 125 cm^2 flasks (Falcon; Becton Dickinson, Oxford, UK) at 37°C in a humidified atmosphere of 5 % (v/v) CO_2 until confluence was attained and were split by using trypsin–EDTA solution (GibcoBRL).

To investigate the acute-on-chronic effect of fatty acid supplementation on Caco-2 cell lipoprotein metabolism, the cells received a chronic fatty acid supplementation first, followed by an acute fatty acid treatment. For the chronic supplementation phase, the cells were seeded at a density of 3×10^5 cells per 24 mm polycarbonate transwells (growth area, 4·7 cm^2; pore size, 0·4 μm; Costar, Cambridge, MA, USA). The cells were grown for 19 d in media only (controls), or in media supplemented with fatty acids (0·05 mM-PA, -OA or -EPA). Sodium hexadecanoic acid (Na^+PA; Sigma-Aldrich Ireland Ltd, Tallaght, Dublin, Republic of Ireland), 9-octadecaenoic acid (OA; Sigma) and 5,8,11,14,17-eicosapentaenoic acid (EPA; Sigma) were stored at −20°C in ethanol. Before use, OA and EPA had Na ions added to them to make them soluble in media (van Greevenbroek *et al.* 1995) and Na^+PA, Na^+OA and Na^+EPA were dried. A 30 mM solution of each fatty acid was made in deionised water and added to the cell culture media (containing fetal calf serum) in the apical compartment of the transwells. The basolateral compartment did not receive fatty acid supplementation. This was done to mimic the intestine where the apical surface of enterocytes is exposed to dietary fats. Cells were fed every 2 d with fresh media or media containing fatty acids during the 19 d chronic fatty acid supplementation period. At day 19 the acute-on-chronic effect of fatty acid supplementation on *de novo* lipid synthesis was determined. First the cells were washed in 1 × PBS (GibcoBRL). Then the acute fatty acid treatment was administered. Fetal calf serum-free media (2·5 ml) was added to each basolateral compartment and fetal calf serum-free media (1·5 ml) containing one of four acute treatments (bovine serum albumin (BSA; 5 g/l)) only (control) or BSA (5 g/l) with 0·5 mM-PA, -OA or -EPA) was added to the apical compartment.

Before carrying out the main experiment, fatty acid-supplemented Caco-2 cells were characterised to investigate whether fatty acid supplementation altered Caco-2 cell

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growth and to determine how the fatty acids were assimilated into the lipid moieties of the supplemented cells compared with controls. Fatty acid supplementation with PA, OA and EPA at a concentration of 0·05 mM added to the apical compartment of the transwell did not alter cell growth and accumulated in the TAG and phospholipid (PL) fractions of the cells and hence these concentrations were used for the main experiments.

Transepithelial electrical resistance measurements

Transepithelial electrical resistance is a measure of resistance across monolayers and is used to assess monolayer development. The transepithelial electrical resistance of the Caco-2 epithelial cell monolayers was measured with an EVOM epithelial voltometer and STX2 electrode (World Precision Instruments, Sarasota, FL) every 2 d before changing the media. The electrode was sterilised for 20 min before use in 70 % (v/v) ethanol, and rinsed in media before measuring each transwell. A potential of 199 mV was applied between the apical and basolateral compartments of the transwell and the resulting resistance (Ω) was measured (0–1999 Ω). At a concentration of 0·05 mM-fatty acid supplementation, transepithelial electrical resistance values were not significantly different between control and fatty acid-supplemented Caco-2 cells.

Viability assay

The viability of cells that received acute (0·5 mM-fatty acid and BSA (5 g/l) (Sigma) for 24 h) and chronic exposure (media or 0·5 mM; 0·25 mM; 0·1 mM or 0·05 mM-fatty acid for 17–21 d) to each fatty acid treatment was assessed. Cells were seeded at a density of 4×10^4 cells/well, in a ninety-six-well microtitre plate (Falcon). Each experiment was repeated eight times. The media was changed every second day over the course of cell growth. A 3-(4,5-dimethylthiazol-2-yl)-2,5-dephenyltetrazolium bromide–thiazolyl blue (Sigma) solution (5 mg/ml) in 1 × PBS (GibcoBRL) was made and stored at 4°C. For experimentation, media or fatty acid treatment (180 μl) was added to cells and cells were incubated for 24 h. Subsequently, 3-4,5-dimethylthiazol-2-yl)-2,5-dephenyltetrazolium bromide–thiazolyl blue solution (20 μl) was added to the media and the cells were incubated for an additional 4 h at 37°C. SDS (100 g/l) and HCl (0·01 M) were then added to the cells, which were then incubated overnight at 37°C before the absorbance was read at $\lambda = 490$ and $\lambda = 570$ nm (Elisa Plate Reader; Dynatech MR5000 Microplate Reader; Dynex, Peterborough, Eaton, UK). Two-sample *t* tests against the control were used to assess viability, with the null hypothesis that viability was not affected by supplementation. The null hypothesis was rejected if the viability of the cells was lower than 95 % of the control. Chronic supplementation with 0·1 mM or 0·05 mM of any fatty acid had no effect on cell viability. Viability of cells was not affected by acute fatty acid treatment of 0·5 mM and BSA (5 g/l) or of BSA (5 g/l) (control), for 24 h.

Proliferation assay

The CellTiter 96 Aqueous Non-Radioactive Cell Proliferation Assay kit (Promega, Madison, WI, USA) was used to test the proliferation of cells under different fatty acid treatments. Each experiment was repeated eight times. Cells were seeded at 10^5 cells/well in ninety-six-well microtitre plates (Falcon) and grown for 24 h in control media or media supplemented with chronic fatty acids (0·1 mM and 0·05 mM). The manufacturer's protocol was followed. Then 3-(4,5-dimethyl-2-yl)-5-(3-carboxymethoxyphenyl-2-(4-sulfophenyl)-2H-tetrazolium, inner salt–phenazine methosulfate solution (20 μl) was added to each well and the cells were incubated for 90 min at 37°C. The absorbance at $\lambda = 490$ nm was read using an ELISA plate reader (Dynatech MR5000 Microplate Reader; Dynex). Two-sample *t* tests against the control were used to test for differences in the proliferation rate. The results showed that cells given either 0·1 mM- or 0·05 mM-fatty acid proliferate at the same rate as the control cells.

Translocation assay: paracellular and intracellular permeability

The permeability of the Caco-2 cell monolayers following the different fatty acid supplementations was measured using two radioactive marker molecules, [^{3}H]proprananol (15–30 ci/mmol; Amersham Pharmacia, Amersham, Bucks, UK), a lipophilic intracellular marker, and [^{14}C]mannitol (50–62 ci/mmol; Amersham Pharmacia), a paracellular marker, as previously described (Roche *et al.* 2001). Briefly, cells were seeded as for chronic supplementation phase and grown for either 6 or 14 d with or without the addition of PA, OA or EPA (0·1 mM and 0·05 mM). Experimentation (*n* 5) was carried out using Hank's balanced salt solution (GibcoBRL) at day 6 or 14 post-seeding in six-well plates (Falcon). Both [^{3}H]proprananol and [^{14}C]mannitol, each emitting 150 000 disintegrations/min (DPM), were added to the apical compartment of the transwell. Then the translocation of the intracellular and paracellular markers was assessed at 15, 30, 45, 60, 90 and 120 min. Basolateral Hank's balanced salt solution (1 ml) was removed from each well of the six-well plates to vials containing 10 ml scintillation fluid (Lumagel safe, Lumac LSC; Packard, Groningen, The Netherlands). DPM were counted for 10 min at radioactivity windows of 0·0 to 12·0 for [^{3}H]proprananol and 12·0 to 156 for [^{14}C]mannitol (TriCarb 2100Tr, Liquid Scintillation Analyzer; Packard). The apparent permeability coefficient (P_{app}) was calculated according to the equation:

$$P_{app}\ (\mathrm{cm} \times \mathrm{sec}) = (\mathrm{d}Q/\mathrm{d}t\ (\mathrm{s}))/A(\mathrm{cm}^2) \times C_o,$$

where dQ/dt is the slope of the graph of the DPM *v.* time in s, A is the area of the transwell (4·7 cm^2) and C_o is the initial DPM (150 000 DPM) added to the transwell. P_{app} values for fatty acid-supplemented cells were assessed and found to be similar to control cells for both the paracellular and intracellular markers. This suggests that fatty acid supplementation of 0·1 mM or 0·05 mM had no effect on tight-junction formation and lipid transport in Caco-2 cells.

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Fatty acid compositional analysis

The lipid compositions of cellular and secreted TAG and PL fractions were assessed following PA, OA and EPA supplementation. On day 19 post-seeding (the media being changed every 2 d) the Caco-2 cell basolateral media (approximately 3 ml) was transferred to a sterile 15 ml tube (Falcon) and stored (− 80°C) before lipid isolation. Apical media was removed from the cells, which were washed in 1 × PBS (GibcoBRL). Then 1 ml 1 × PBS (GibcoBRL) was added to the apical compartment of the transwell and the cells were scraped off the membranes and transferred to 1·5 ml tubes (Starstead, Wexford, Republic of Ireland). The cells were centrifuged at 132 000 rpm for 2 min and the supernatant fraction was removed. The cells were re-suspended in 1 ml 1 × PBS and stored (− 80°C) until subsequent lipid isolation. The lipid in the basolateral media and cellular fraction was extracted using the Folch method, as previously described (Gibney & Daly, 1994). The isolated lipids were stored at − 80°C. The TAG and PL fractions of lipid in the basolateral media and cellular were isolated by TLC, as previously described (Roche & Gibney, 1997), and stored (− 80°C). The basolateral lipids could not be visualised on the TLC plate and so cellular and basolateral lipid fractions isolated from each transwell were loaded beside each other on the TLC plate. This was done because it was observed that when Caco-2 cells were supplemented with different fatty acids, the TAG fractions isolated from these cells would run at different rates. Methyl esters of fatty acids for GLC analysis were generated using the BF_3 method (Shishehbor *et al.* 1998). The fatty acid composition of the TAG and PL fractions of the cells and basolateral medium were analysed using a Shinadzu GC-14A Series GLC (Mason Technologies, Dublin, Republic of Ireland) fitted with a BP 21 GC polar Al coated silica column (Scientific Glass Engineering Europe Ltd., Milton Keynes, Bucks., UK). The TAG and PL samples derived from the cells and basolateral medial were re-suspended in 80–100 μl hexane, of which 65–80 μl was injected onto the GC column at a 1:60 split ratio and analysed according to conditions described previously (Shishehbor *et al.* 1998). A diagram (Fig. 1) of the differences between cellular fatty acid composition in fatty acid-supplemented Caco-2 cells (0·05 mM) and control cells shows that supplementing Caco-2 cells with PA, OA and EPA altered cellular fatty acid composition in a fatty acid-specific manner. Investigation of fatty acid supplementation at concentrations of 0·5, 0·25, 0·1 mM as well as 0·05 mM showed that cellular fatty acid composition was altered in a dose-dependent manner. PA supplementation increased cellular 16 : 0 levels and decreased 18 : 1*n*-9 levels. OA supplementation led to an increase in cellular 18 : 1*n*-9 levels. EPA supplementation increased cellular 20 : 5*n*-3 levels and decreased 18 : 1*n*-9 levels. These findings confirm the uptake of the chronic fatty acid supplements by Caco-2 cells.

De novo triacylglycerol and phospholipid synthesis

The acute-on-chronic effect of fatty acid supplementation on *de novo* lipid synthesis was determined in Caco-2 cells following 19 d of chronic fatty acid supplementation. The cells were washed in 1 × PBS (GibcoBRL) and the acute fatty acid treatments were administered. Fetal calf serum-free media (2·5 ml) was added to each basolateral compartment and fetal calf serum-free media (1·5 ml) containing one of four acute treatments (BSA (5 g/l) only (control) or BSA (5 g/l) with 0·5 mM-PA, -OA or -EPA) was added to the apical compartment. Immediately, 13 μl (400 ng, 1 443 000 DPM) of [^{14}C]glycerol (140–168 mCi/mmol; Amersham Pharmacia) was added to each apical

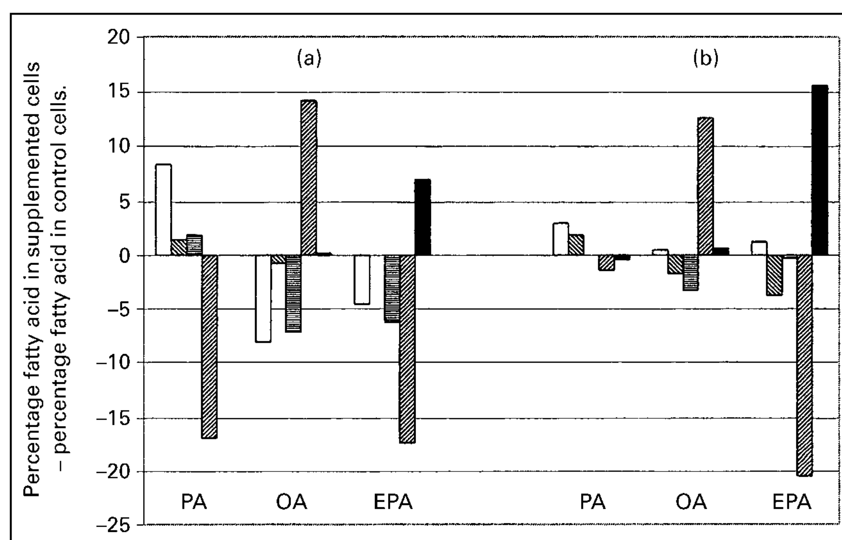

Fig. 1. Difference between cellular triacylglycerol (a) and phospholipid (b) compositions in Caco-2 cells after fatty acid supplementation (0·05 mM) with palmitic acid (PA), oleic acid (OA) or eicosapentaenoic acid (EPA) compared with Caco-2 cells not supplemented with fatty acids. (□), 16 : 0; (▧), 16 : 1; (▤), 18 : 0; (▨), 18 : 1; (■), 20 : 5*n*-3.

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compartment, and cells were incubated for 22 h at 37°C. The basolateral media for total lipid determination was harvested into 15 ml tubes (Falcon) and frozen immediately. Cells were washed in 1 × PBS (GibcoBRL) and scraped into 1·5 ml eppendorfs (Starstead). The cells were centrifuged at 13 200 rpm for 2 min and the supernatant fraction was removed. The cells were re-suspended in 1 × PBS (GibcoBRL) and stored at −80°C until lipid isolation. Lipids were isolated from the basolateral media and cellular fraction using the Folch method (Gibney & Daly, 1994). The TAG and PL fractions from the basolateral media and cells were separated by TLC (Roche & Gibney, 1997), the TAG and PL fractions were scraped into 10 ml liquid scintillation fluid (Lumagel safe, Lumac.LSC; Packard) and counted for 10 min (TriCarb 2100Tr, Liquid Scintillation Analyzer; Packard).

Immunoprecipitation and apolipoprotein B analysis

The acute-on-chronic effect of fatty acid supplementation on *de novo* apo B synthesis was determined in Caco-2 cells following 19 d of chronic fatty acid supplementation. Acute fatty acid treatments were administered to Caco-2 cells as described earlier. Then 10 μl (22 200 000 DPM) of [^{35}S]methionine (40–500 mCi/mmol; Amersham Pharmacia) was added to each apical compartment and the cells were incubated for 22 h at 37°C (Murthy *et al.* 1998). The basolateral media was harvested into 15 ml tubes (Falcon) and protease inhibitors were added (Leupeptin (1 mg/ml), Pepstatin (1 mg/ml), Aprotinin (1 mg/ml), phenylmethanesulfonyl fluoride (10 mg/ml) and Benzamidine (1 mg/ml); Sigma). The cells were washed in 1 × PBS (GibcoBRL) (4°C), then 200 μl Boehringer Mannheim wash 1 (Protein A-Agarose protocol; Boehringer Mannheim GmbH, Ingleheim, Germany) with protease inhibitors was added to the cells, which were scraped into eppendorf tubes (Starstead). Cells were centrifuged to remove cellular debris and the supernatant fraction was transferred to a new tube. Cellular and basolateral fractions were pre-cleared (removal of any other antibodies) using the Protein A-Agarose protocol (Boehringer Mannheim GmbH), and anti-human apo B antibody (ICN, OH, USA) was added. The immunoprecipitation of apo B-48 and apo B-100 was carried out using the Protein A-agarose method (Boehringer Mannheim GmbH) according to the manufacturer's instructions. The apo B-100 and apo B-48 isoforms were separated on 4 % running, 2 % stacking SDS–PAGE gels. The Prestained Protein Large Molecular Weight Ladder (GibcoBRL) was loaded on the SDS–PAGE gels along with the samples. The gels were run in 1 × running buffer (25 mM-Tris.Cl, 250 mM-glycine, SDS (1 g/l), pH 8·3) for approximately 90 min at 100 mV. Gels were dried on a gel dryer before autoradiography film (Hyperfilm; Amersham Pharmacia) was put on the gels. Films were developed after 2 d. The quantity of both forms of apo B, apo B-100 and apo B-48 were measured using an instant imager (Instant Imager, Model A202401; Packard, Reading, Berks, UK). The apo B-100 (500 kDa) and apo B-48 (230 kDa) proteins were identified by comparison with the apo B-100 (Sigma) and apo B-48 (internal laboratory standard) standards and the Prestained Large Molecular Weight Protein Ladder (GibcoBRL).

Statistical analysis

The objective of the present study was to examine the acute-on-chronic effects of fatty acid composition on Caco-2 cell TRL synthesis and secretion. The original data, the mean and standard error DPM values of the lipid analysis are presented in Table 1. To investigate *de novo* TAG, PL and apo B synthesis and secretion the following ratios were used as metabolic indicators of

Table 1. Disintegrations per minute obtained for lipid fractions*
(Mean values with their standard errors)

Lipid fraction counted...		cTAG		sTAG		cPL		sPL	
Chronic treatment	Acute treatment	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Control	BSA (5 g/l)	10 056	937	516	40	25 460	2787	448	203
Control	PA (0·5 mM) + BSA (5 g/l)	45 829	4087	4857	883	47 161	7553	830	301
Control	OA (0·5 mM) + BSA (5 g/l)	20 436	551	6417	1226	39 460	4607	889	324
Control	EPA (0·5 mM) + BSA (5 g/l)	16 019	4397	1068	274	40 881	1776	909	191
PA (0·05 mM)	BSA (5 g/l)	9603	772	842	118	19 355	1379	180	26
PA (0·05 mM)	PA (0·5 mM) + BSA (5 g/l)	34 871	3882	2555	291	19 973	3826	379	94
PA (0·05 mM)	OA (0·5 mM) + BSA (5 g/l)	23 533	1779	3870	241	24 554	1305	359	24
PA (0·05 mM)	EPA (0·5 mM) + BSA (5 g/l)	8718	1146	1237	326	26 015	3912	437	169
OA (0·05 mM)	BSA (5 g/l)	9992	882	778	53	20 949	2251	143	14
OA (0·05 mM)	PA (0·5 mM) + BSA (5 g/l)	38 687	2732	3137	465	28 799	3247	455	70
OA (0·05 mM)	OA (0·5 mM) + BSA (5 g/l)	21 768	2044	4588	270	25 497	1368	377	25
OA (0·05 mM)	EPA (0·5 mM) + BSA (5 g/l)	9639	926	878	139	27 385	4106	591	206
EPA (0·05 mM)	BSA (5 g/l)	4059	655	400	74	18 756	1950	165	40
EPA (0·05 mM)	PA (0·5 mM) + BSA (5 g/l)	24 886	1416	1609	177	38 677	1610	404	82
EPA (0·05 mM)	OA (0·5 mM) + BSA (5 g/l)	15 795	1687	2589	151	24 702	1241	302	7
EPA (0·05 mM)	EPA (0·5 mM) + BSA (5 g/l)	7440	1785	763	239	25 155	3719	410	96

cTAG, non-secreted triacylglycerol; sTAG, secreted triacylglycerol; cPL, non-secreted phospholipids; sPL, secreted phospholipids; BSA, bovine serum albumin; PA, palmitic acid; OA, oleic acid; EPA, eicosapentaenoic acid.
* For details of treatments and procedures, see p. 662.

intestinal lipid and apo B metabolism:non-secreted TAG:secreted TAG (cTAG:sTAG), cTAG:non-secreted PL (cTAG:cPL), sTAG:secreted PL (sTAG:sPL), non-secreted apo B-100:secreted apo B-100 (cB100:sB100) and cB100:non-secreted apo B-48 (cB100:cB48). The acute-on-chronic effect of fatty acid composition on Caco-2 cell TAG and apo B metabolism was analysed by ANOVA (Data Desk 6.0; Data Descriptions Inc., New York, NY) for each variable in the cells that received chronic fatty acid supplementation followed by acute fatty acid treatment. *Post hoc* statistical analysis to identify the significant acute, chronic and acute × chronic effects of fatty acid composition was determined using protected least-significant difference tests. For the sake of clarity, the acute fatty acid intervention is referred to as a treatment, whilst the chronic fatty acid intervention is referred to as supplementation.

Results

Cellular triacylglycerol:secreted triacylglycerol

A significant acute × chronic interaction (P=0·0236) was observed for cTAG:sTAG (Table 2). This demonstrates that the composition of the chronic fatty acid supplement determined the effect of the acute fatty acid treatments on cTAG:sTAG. The effect of chronic fatty acid supplementation alone is shown in the cells that received the acute BSA treatment. Chronic EPA supplementation reduced cTAG:sTAG compared with non-supplemented (control) cells (P=0·004) following acute BSA treatment. Likewise following the acute EPA treatment, cTAG:sTAG was significantly (P=0·015) reduced in the cell that had received chronic EPA supplementation. However, following acute PA treatment cTAG:sTAG was significantly increased when the cells had received chronic EPA supplementation compared with control supplemented cells (P=0·05). In all cases EPA supplementation decreased cellular *de novo* TAG synthesis by approximately 2-fold compared with non-supplemented cells (Table 1). In addition, the acute PA treatment following chronic EPA supplementation reduced *de novo* TAG secretion (Table 1). In the cells that did not receive a chronic fatty acid supplement (controls), the acute effect of fatty acid treatment alone can be observed. Acute PA (P=0·007) and OA (P=0·0001) treatments significantly reduced cTAG:sTAG compared with chronic control cells that received the acute BSA treatments. When cells received chronic PA supplementation, the acute OA treatment significantly reduced (P=0·03) cTAG:sTAG compared with acute BSA-treated cells. These effects were due to increased *de novo* TAG secretion (Table 1). Also the acute EPA treatment, following chronic PA supplementation, reduced cTAG:sTAG but this difference was only significantly different from the acute PA treatment (P=0·023). This difference was due to lower cellular TAG levels in the acute EPA-treated cells (Table 1). Following chronic OA supplementation, acute OA treatment significantly reduced cTAG:sTAG compared with acute BSA (P=0·006), PA (P=0·003) and EPA (P=0·014) treatments, by increasing *de novo* TAG secretion (Table 1). In contrast, chronic EPA supplementation caused acute PA treatment to significantly increase cTAG:sTAG compared with acute BSA (P=0·029), OA (P=0·001) and EPA (P=0·014) treatments due to increased TAG synthesis following acute PA treatment (Table 1).

Cellular triacylglycerol:cellular phospholipids

A significant acute × chronic interaction (P=0·0001) for cTAG:cPL (Table 3) shows that the acute response to a fatty acid treatment was affected by the composition of the chronic fatty acid supplement. The acute PA treatment significantly increased cTAG:cPL compared with the acute BSA (P=0·000) and EPA treatments (P<0·001), irrespective of chronic fatty acid composition, which was due to increased *de novo* TAG synthesis over *de novo* PL synthesis. The magnitude of this increase in cTAG:cPL was affected by the composition of the chronic fatty acid supplement. Compared with the control cells that did not receive a chronic fatty acid supplement, chronic PA and OA supplementation before the acute PA treatment increased the cTAG:cPL values (P=0·000 and P=0·001, respectively), which was due to increased *de novo* TAG synthesis over *de novo* PL synthesis (Table 1). Caco-2 cells that received the acute OA treatment also had

Table 2. The effect of chronic fatty acid supplementation followed by acute fatty acid treatments on non-secreted triacylglycerol:secreted triacylglycerol*

(Mean values with their standard errors for four or five samples)

Acute treatment...	BSA (5 g/l)		PA (0·5 mM) + BSA (5 g/l)		OA (0·5 mM) + BSA (5 g/l)		EPA (0·5 mM) + BSA (5 g/l)		ANOVA†	
Chronic supplementation	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Effect	P
Control	16·99^{a1}	2·68	8·88^{bc1}	0·85	3·68^{b1}	0·66	14·21^{ac1}	5·44	MSE = 14·05	
PA (0·05 mM)	11·76^{ab12}	2·08	13·83^{a12}	1·65	5·91^{c1}	0·93	7·59^{bc2}	2·33	Acute	0·0001
OA (0·05 mM)	12·01^{a12}	0·65	12·7^{a12}	0·67	4·38^{b1}	0·56	11·12^{a12}	1·14	Chronic	0·4678
EPA (0·05 mM)	8·29^{a2}	1·06	14·25^{b2}	1·38	5·08^{a1}	0·44	7·54^{a2}	1·28	Acute × Chronic	0·0236

BSA, bovine serum albumin; PA, palmitic acid; OA, oleic acid; EPA, eicosapentaenoic acid.
a,b,c Mean values within a row with unlike superscript letters were significantly different (acute treatment effect) (P<0·05).
1,2 Mean values within a column with unlike superscript numbers were significantly different (chronic supplementation effect) (P<0·05).
* For details of treatments and procedures, see p. 662.
† Least-squared difference tests were used to identify the fatty acids responsible for acute × chronic interaction.

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Table 3. The effects of chronic fatty acid supplementations followed by acute fatty acid treatments on non-secreted triacylglycerol:non-secreted phospholipids*

(Mean values with their standard errors for eight to ten samples)

Acute treatment...	BSA (5 g/l)		PA (0·5 mM) + BSA (5 g/l)		OA (0·5 mM) + BSA (5 g/l)		EPA (0·5 mM) + BSA (5 g/l)		ANOVA†	
Chronic supplementation	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Effect	*P*
Control	0·401[a1]	0·026	1·099[b1]	0·148	0·564[a1]	0·059	0·391[a1]	0·102	MSE=0·045	
PA (0·05 mM)	0·495[a1]	0·018	1·576[c3]	0·072	0·961[b2]	0·06	0·363[a1]	0·042	Acute	0·0001
OA (0·05 mM)	0·496[a1]	0·042	1·448[c3]	0·16	0·86[b2]	0·086	0·394[a1]	0·052	Chronic	0·0001
EPA (0·05 mM)	0·21[a1]	0·022	0·645[b2]	0·026	0·634[b1]	0·043	0·283[a1]	0·042	Acute × Chronic	0·0001

BSA, bovine serum albumin; PA, palmitic acid; OA oleic acid; EPA eicosapentaenoic acid.
[a,b,c]Mean values within a row with unlike superscript letters were significantly different (acute treatment effect) ($P<0·05$).
[1,2,3]Mean values within a column with unlike superscript numbers were significantly different (chronic supplementation effect) ($P<0·05$).
* For details of treatments and procedures see p. 662.
† Least-squared difference tests were used to identify the fatty acids responsible for acute × chronic interaction.

increased cTAG:cPL, but this was only significant in the cells that received chronic PA and OA supplementation compared with the control ($P=0·000$ and $P=0·001$, respectively); again this was due to increased *de novo* TAG synthesis (Table 1). In response to both the acute PA and acute OA treatments, cTAG:cPL was increased to a greater extent after chronic PA supplementation than after chronic OA supplementation; because acute PA treatment increased cellular *de novo* TAG levels more than acute OA treatment (Table 1). In cells that received chronic EPA supplementation before the acute PA treatment, the cTAG:cPL values were significantly decreased compared with the acute BSA ($P=0·000$) treatment because of increased cellular *de novo* PL production compared with *de novo* TAG synthesis (Table 1).

Secreted triacylglycerol:secreted phospholipids

Although no significant acute-on-chronic interaction ($P=0·344$) was observed for sTAG:sPL, both significant chronic ($P=0·002$) and acute ($P=0·0001$) effects were observed (Table 4). *Post hoc* tests showed that chronic PA and OA supplementation increased sTAG:sPL compared with the control ($P=0·03$ and $P=0·02$, respectively) and chronic EPA-supplemented cells ($P=0·002$ and $P=0·001$, respectively). This difference was due to less secreted *de novo* PL (Table 1). The significant effect of acute fatty acid treatments on sTAG:sPL was due to fatty acid-specific alterations in *de novo* TAG and PL secretion. The acute PA and OA treatments increased sTAG:sPL compared with the acute control BSA-treated cells ($P=0·001$ and $P=0·000$, respectively) by increasing secreted *de novo* TAG levels (Table 1). This increase in *de novo* TAG secretion levels was greater following the mean acute OA treatment compared with the mean acute PA treatment ($P=0·000$). In contrast, the acute EPA treatment decreased sTAG:sPL compared with BSA-treated cells ($P=0·05$), which was due to increased *de novo* PL secretion (Table 1) following the mean acute EPA treatment.

Cellular apolipoprotein B-100:secreted apolipoprotein B-100 and cellular apolipoprotein B-100:cellular apolipoprotein B-48

No significant effect of fatty acid supplementation, treatment or interaction on cB100:sB100 or cB100:cB48 was observed in the present study (data not shown).

Discussion

The important finding of the present study is that the composition of fatty acids presented as either an acute treatment (<24 h) or a chronic supplement (19 d) had a significant effect on intestinally derived TRL metabolism. Whilst these studies have been conducted in the Caco-2 cell model, they provide important information in terms of understanding how habitual (chronic) dietary fat composition determines the postprandial TAG response to acute test meals. The present study showed that a fatty acid could have a different effect on TRL metabolism depending on whether it is presented as an acute treatment or a chronic supplement. The results of the present study suggest that acute PA treatment and OA treatment were associated with an increase in cellular *de novo* TAG levels (cTAG:cPL). Cellular *de novo* TAG levels were increased by approximately 4-fold following acute PA treatment and 2-fold following acute OA treatment compared with acute BSA treatment, and were augmented when the acute treatments were preceded by chronic PA or OA supplementation. These data agree and extend those of van Greevenbroek *et al.* (1996), who showed that acute PA treatment also increased cellular TAG levels in Caco-2 cells. Also in hepatoma G2 cells acute PA treatment increased cellular TAG levels, compared with OA (Arrol *et al.* 2000). It is important to note that Caco-2 cells preferentially synthesise TAG rather than PL following PA exposure; this effect was attributed to increased utilisation of diacylglycerol to synthesis for TAG rather than PL (Trotter & Storch, 1993).

As in previous studies (Field *et al.* 1988; van Greevenbroek *et al.* 1996) acute exposure to OA was associated with increased *de novo* TAG secretion (cTAG:sTAG and sTAG:sPL). Acute OA exposure to Caco-2 cells has been

Table 4. The effects of chronic fatty acid supplementations followed by acute fatty acid treatments and the mean acute and mean chronic effects for the different fatty acids on secreted triacylglycerol:secreted phospholipids*

(Mean values with their standard errors for four or five samples)

Acute treatment...	BSA (5 g/l)		PA (0·5 mM) + BSA (5 g/l)		OA (0·5 mM) + BSA (5 g/l)		EPA (0·5 mM) + BSA (5 g/l)		Mean chronic effect		ANOVA†	
Chronic supplementation	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Effect	P
Control	1·787	0·833	7·582	1·602	9·525	2·153	1·498	0·572	5·319[1]	1·158	MSE = 3·959	
PA (0·05 mM)	4·747	0·315	7·475	1·254	10·927	0·92	3·625	0·724	6·694[2]	0·825	Acute	0·0001
OA (0·05 mM)	5·622	0·663	7·142	0·839	12·462	1·472	1·858	0·367	6·771[2]	1·067	Chronic	0·0021
EPA (0·05 mM)	2·595	0·332	4·435	0·874	8·575	0·4	1·905	0·33	4·377[1]	0·711	Acute × Chronic	0·3436
Mean acute effect	3·815[a]	0·470	6·659[b]	0·626	10·370[c]	0·731	2·221[d]	0·317				

BSA, bovine serum albumin; PA, palmitic acid; OA, oleic acid; EPA, eicosapentaenoic acid.
[a,b,c,d]Mean values within a row with unlike superscript letters were significantly different (acute treatment effect) ($P<0·05$).
[1,2]Mean values within a column with unlike superscript numbers were significantly different (chronic supplementation effect) ($P<0·05$).
* For details of treatments and procedures, see p. 662.
† Least-squared difference tests were used to identify the fatty acids responsible for acute by chronic interaction.

shown to increase *de novo* TAG secretion compared with controls (van Greevenbroek *et al.* 1996) and have higher *de novo* TAG secretion levels than acute PA or linoleic acid treatments (Field *et al.* 1988). The present study suggests that acute rather than chronic OA intervention has the greatest effect on *de novo* TAG secretion, although chronic OA supplementation was associated with increased *de novo* TAG secretion following the acute BSA and OA treatments. In terms of explaining the effect of OA on *de novo* TAG secretion, van Greevenbroek *et al.* (1998) hypothesised that OA was a better substrate than PA for the microsomal triacylglycerol transfer protein complex, resulting in large TAG-rich chylomicrons being secreted more quickly from the Caco-2 cells. Field *et al.* (1988) showed that acute OA treatment (0·25 mM) increased *de novo* TAG levels in TRL secreted by Caco-2 cells. Also van Greevenbroek *et al.* (2000) demonstrated that TAG synthesised after acute OA treatment was assembled into TRL particles, whereas TAG synthesised after acute PA treatments was assembled in intermediate-density lipoprotein and LDL particles. Therefore it was hypothesised that in man a test meal rich in unsaturated fatty acid might form large chylomicrons that can be cleared rapidly from the circulation. In contrast, an SFA meal might form small chylomicrons that are secreted slowly by the intestinal enterocyte and are not cleared quickly from the circulation, thereby prolonging the postprandial lipid response (van Greevenbroek *et al.* 2000). Indeed the Caco-2 cell studies suggest concurrence with human trials that showed that habitual consumption of an OA-rich diet caused a very rapid rise in plasma TRL during the early postprandial phase, which were cleared efficiently from the circulation (Roche *et al.* 1998; Zampelas *et al.* 1998).

In the case of EPA, chronic fatty acid supplementation rather than acute EPA treatment resulted in significant effects on cTAG:sTAG and cTAG:cPL. Chronic exposure to EPA was associated with a 2-fold reduction in cellular *de novo* TAG levels (cTAG:sTAG). A previous study showed that acute (0·25 mM) EPA exposure (4 h) significantly reduced *de novo* TAG synthesis and secretion in Caco-2 cells (Murthy *et al.* 1990). Conversely, Ranheim *et al.* (1992) demonstrated that acute (0·6 mM) EPA treatment (<24 h) stimulated *de novo* TAG synthesis and secretion, an effect that was similar to that of acute OA treatment. Our study extends this information, suggesting that chronic EPA supplementation may reduce intestinal *de novo* TAG synthesis. The results of the present study highlight that the concentration and duration of EPA intervention may explain divergent results between the previously published acute studies. Chronic EPA exposure was found to increase cellular *de novo* PL levels (cTAG:cPL) and acute or chronic EPA supplementation increased *de novo* PL secretion (sTAG:sPL). This may be due to acute EPA treatment inhibiting diacylglycerol acyl transferase activity thus reducing the competition for the PL synthesis pathway (Ranheim *et al.* 1992). In human subjects chronic *n*-3 PUFA supplementation is an effective hypotriacylglycerolaemic agent (Roche & Gibney, 1996), which is largely attributable to reduced hepatic TAG synthesis and secretion (Harris *et al.* 1990). However, the present study suggests that chronic EPA may also modulate

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intestinal TAG metabolism by reducing cellular *de novo* TAG levels and by increasing PL synthesis due to inhibition of diacylglycerol acyl transferase activity.

In conclusion our study shows that the Caco-2 cell model is a suitable model to investigate the acute-on-chronic effects of fatty acid composition on intestinal TRL metabolism. The results show that particularly with regard to OA the Caco-2 cell model concurs with human studies. The study also highlights that fatty acids can have different effects depending upon whether they are presented in the short (acute treatment) or long (chronic supplementation) term.

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