β -Lactoglobulin-linoleate complexes: In vitro digestion and the role of protein in fatty acid uptake

Solène Le Maux,*†‡ André Brodkorb,‡ Thomas Croguennec,*† Alan A. Hennessy,‡ Saïd Bouhallab,*† and Linda Giblin‡¹

*INRA, UMR1253 STLO, 65 rue de Saint Brieuc, F-35042 Rennes, France

†Agrocampus Ouest, UMR1253 STLO, 65 rue de Saint Brieuc, F-35042 Rennes, France

‡Teagasc Food Research Centre, Moorepark, Fermoy, Co. Cork, Ireland

ABSTRACT

The dairy protein β -lactoglobulin (BLG) is known to bind fatty acids such as the salt of the essential long-chain fatty acid linoleic acid (*cis,cis*-9,12-octadecadienoic acid, n-6, 18:2). The aim of the current study was to investigate how bovine BLG-linoleate complexes, of various stoichiometry, affect the enzymatic digestion of BLG and the intracellular transport of linoleate into enterocyte-like monolayers. Duodenal and gastric digestions of the complexes indicated that BLG was hydrolyzed more rapidly when complexed with linoleate. Digested as well as undigested BLG-linoleate complexes reduced intracellular linoleate transport as compared with free linoleate. To investigate whether enteroendocrine cells perceive linoleate differently when part of a complex, the ability of linoleate to increase production or secretion of the enteroendocrine satiety hormone, cholecystokinin, was measured. Cholecystokinin mRNA levels were different when linoleate was presented to the cells alone or as part of a protein complex. In conclusion, understanding interactions between linoleate and BLG could help to formulate foods with targeted fatty acid bioaccessibility and, therefore, aid in the development of food matrices with optimal bioactive efficacy.

Key words: β -lactoglobulin, linoleate, in vitro digestion, bioaccessibility

INTRODUCTION

Bovine β -lactoglobulin (BLG) is the major whey protein in bovine milk. It is a globular protein with a monomeric molecular weight of 18.4 kDa, consisting of 162 AA (Kontopidis et al., 2004). β -Lactoglobulin is a member of the lipocalin family, which are able to bind small hydrophobic molecules, including FA and hydrophobic vitamins (Pérez and Calvo, 1995; Wang et al., 1997; Jiang and Liu, 2010). All members of the

lipocalin family contain a β -barrel, shaped into a calyx, composed of 8 antiparallel β -strands (Flower, 1996). It has been suggested that BLG binds hydrophobic ligands in its internal calyx, in a crevice near the α -helix on the external surface of the β -barrel and near the interface of BLG dimers (Wang et al., 1998; Wu et al., 1999; Yang et al., 2008). However, the binding sites and stoichiometries of ligands have been controversial (Wang et al., 1998; Wu et al., 1999; Kontopidis et al., 2002). β -Lactoglobulin may modify the transport through the gastric tract of hydrophobic substances naturally present in bovine milk (Pérez and Calvo, 1995; Ragona et al., 2000). β -Lactoglobulin was reported to enhance pregastric lipase activity by binding FA that inhibit the enzyme (Perez et al., 1992). The binding of hydrophobic ligands has been shown to modify protein digestibility, as such interactions alter the accessibility of the protein to digestive enzymes (Puyol et al., 1993; Mandalari et al., 2009).

Previously, the salt of the essential long-chain fatty acid (LCFA) linoleic acid (LA; *cis,cis*-9,12-octadecadienoic acid, n-6, 18:2) was shown to bind BLG (Le Maux et al., 2012). Linoleic acid represents 1 to 3% (wt/wt) of the FA in milk fat (Jensen, 2002). The mechanisms by which LCFA are absorbed by cells remains controversial. Recent studies suggest that LCFA are taken up by intestinal epithelial cells by both active transport via specific FA transporters and by passive diffusion (Stremmel, 1988; Trotter et al., 1996; Abumrad et al., 1998). However, uptake and bioavailability of FA may be altered depending on the food matrix (Kushibiki et al., 2001; Mu, 2008; Singh et al., 2009). We observed that linoleate, the salt form of LA, bound to BLG was less bioaccessible compared with free linoleate (Le Maux et al., 2012). After uptake in enterocyte cells, FA are bound to proteins for their intracellular trafficking and thio-esterification (Niot et al., 2009). They are rapidly esterified into triacylglycerols in the endoplasmic reticulum. In the golgi, these triacylglycerols form chylomicrons (lipid droplets) before being released into the cytoplasm and finally into circulation for uptake by other cells (Niot et al., 2009). Fatty acids also trigger

Received February 11, 2013.

Accepted April 1, 2013.

¹Corresponding author: Linda.Giblin@teagasc.ie

internal signals in the intestinal epithelium to allow the body to control food intake for example, secretion of satiety hormones by specialized enteroendocrine cells.

Cholecystokinin (**CCK**) is a peptide hormone known to have a satiety effect as it influences the digestive processes in the gut (Gibbs et al., 1973). Cholecystokinin has been associated with delay of gastric emptying, stimulation of gallbladder contraction, increases in pancreatic enzyme secretion, and reduced food intake (Purhonen, 2008). Cholecystokinin is secreted by I enteroendocrine cells located predominantly in the proximal small intestinal mucosa in response to intraluminal nutrients such as FA and proteins (Liddle, 2000). Relatively little is known concerning the mechanisms whereby nutrients influence CCK synthesis and secretion. Several studies have shown that peptides and FA can use an external receptor to induce CCK secretions (Choi et al., 2007; Tanaka et al., 2008; Shah et al., 2011). In vivo and in vitro studies indicate that proteins increase CCK secretion (Beucher et al., 1994; Cordier-Bussat et al., 1997; Nishi et al., 2001). This effect is further improved when proteins are hydrolyzed. However, individual AA do not contribute to CCK release (Liddle et al., 1985). Secretion of CCK by FA is dependent on FA structure, chain length, degree of unsaturation, and whether it is in the free or bound form (Beard Shall et al., 1989; Douglas et al., 1990; Little et al., 2007).

This study investigated whether BLG-linoleate complexes alter BLG digestion, linoleate bioaccessibility, and intracellular transport of linoleate into intestinal epithelial cells Caco-2. To investigate whether enteroendocrine cells perceive linoleate differently when free or part of a complex, the production and secretion of the satiety hormone secretion CCK was measured in STC-1 cells.

MATERIALS AND METHODS

Materials

β -Lactoglobulin (96% purity) was obtained from Davisco Foods International Inc. (Eden Prairie, MN) and sodium linoleate (purity $\geq 98\%$) from Sigma-Aldrich (St. Louis, MO). All other chemicals and solutions were purchased from Sigma-Aldrich unless stated otherwise.

Complex Preparation

Linoleate-BLG complexes were prepared by mixing a solution of BLG and sodium linoleate adapted from Lišková et al. (2011) and described by Le Maux et al. (2012). Under these conditions complexes with 1, 2, and 3 linoleate bound to BLG are formed. β -Lactoglobulin without FA was used as a BLG control.

In Vitro Digestion

The in vitro adult digestion model was adapted from Dupont et al. (2009). Briefly, BLG samples were dissolved in simulated gastric fluid (0.15 M NaCl; pH 2.5) and the pH was adjusted to 2.5 with 0.5 M HCl solution. Porcine gastric mucosa pepsin (Sigma-Aldrich P7000; activity: 837 U/mg of protein calculated using hemoglobin as a substrate) was added to give 182 U of pepsin/mg of BLG. The final concentration of BLG was 0.05 mM in a 20-mL solution. During this gastric digestion, aliquots (1 mL) were removed at regular intervals over a 60-min period. Pepsinolysis was stopped by raising the pH to 7.0 using 0.5 M NaOH. The pH was adjusted to 6.5 for duodenal proteolysis. Duodenal digestion components were dissolved in simulated duodenal fluid (0.15 M NaCl; pH 6.5). They were added to give final concentrations as follows: 4 mM sodium taurocholate, 4 mM sodium glycodeoxycholate, 26.1 mM Bis-Tris buffer pH 6.5, 0.4 U/mg of BLG for α -chymotrypsin (Sigma-Aldrich C4129; activity 59 U/mg of protein using benzoyltyrosine ethyl ester as substrate), and 34.5 U/mg of BLG for trypsin (Sigma-Aldrich T0303; activity 14,476 U/mg of protein using benzoylarginine ethyl ester as substrate). Aliquots of 1 mL were removed over a 30-min period of duodenal digestion. Proteolysis was stopped by addition of an excess of soybean Bowman-Birk trypsin or α -chymotrypsin inhibitor (100 μ L of 5 g/L in simulated duodenal fluid), which ensured inhibitor excess. The final solution was freeze-dried.

Gel Permeation-HPLC

The relative proportion of protein monomers, aggregates and peptides in the digested samples were determined by gel permeation-HPLC (**GP-HPLC**) using in tandem a TSK G spiral-wound guard column (7.5 $\times$ 7.5 mm, Tosoh Bioscience GmbH, Stuttgart, Germany) and a TSK G2000 spiral wound column 130 (7.5 $\times$ 600 mm, Tosoh Bioscience GmbH) connected to an HPLC system (Waters 2695 Separations Module, Waters 2487 Dual λ Absorbance Detector, at 214 and 280 nm; Waters, Milford, MA) with Empower Pro software (Waters). A quantity of 0.05 mg of protein was injected using a solution of 30% (vol/vol) acetonitrile (LabScan Analytical Sciences, Dublin, Ireland) and 0.1% (wt/vol) trifluoroacetic acid in Milli-Q water (Millipore, Carrigtwohill, Ireland) as an eluent at a flow rate of 0.5 mL/min. The method was calibrated using a set of protein molecular weight standards (Sigma-Aldrich). Digestion solutions without BLG or FA served as control chromatograms and were subtracted from chromatograms generated from test samples to reduce background noise. Chromatograms were integrated and

peaks expressed by size (>30 kDa, 30–10 kDa, 10–1 kDa, and <1 kDa) as a percentage of protein content.

Extraction and Determination of the FA Content in Digested Samples by GC

Fatty acid concentration in samples was determined by GC. To determine FA concentration in digested complexes, total FA were extracted and methylated according to the protocol of Palmquist and Jenkins (2003) with some modifications described by Le Maux et al. (2012). Fatty acid methyl esters (**FAME**) were quantified using a CP-Select CB column (100 m, 0.25 mm, 0.25 μ m film thickness; Varian BV, Middelburg, the Netherlands) on a Varian 3400 GLC (Varian, Walnut Creek, CA), which was fitted with a flame ionization detector as previously described by Coakley et al. (2003).

Cell Culture

Cell lines were cultured in a humidified 37°C incubator with a 5% (vol/vol) CO₂ in air atmosphere.

Caco-2 Cell Line. The Caco-2 cell line was purchased from the European Collection of Cell Cultures (collection reference: ECACC 86010202) and was derived from human colonic adenocarcinoma cells. Cells were routinely grown in 75-cm² plastic flasks in Dulbecco's modified Eagle medium (**DMEM**) containing 4.5 g/L of glucose and 0.584 g/L of L-glutamine. Media for subculture was supplemented with 10% (vol/vol) fetal bovine serum, 100 U/mL of penicillin, and 100 mg/mL of streptomycin. At 80% confluency, cells were trypsinized with 0.25% trypsin-EDTA, diluted 1:6 in media, and reseeded. Media was changed 3 times a week. All cells used in these studies were between passage number 32 and 42.

STC-1 Cell Line. The STC-1 cell line was purchased from American Type Culture Collection (Gaithersburg, MD). This enteroendocrine cell line originated from a double transgenic mouse tumor (Rindi et al., 1990). Cells were routinely grown in 75-cm² plastic flasks in DMEM containing 4.5 g/L of glucose and 0.584 g/L of L-glutamine. Media for subculture was supplemented with 20% (vol/vol) fetal bovine serum, 100 U/mL of penicillin, and 100 mg/mL of streptomycin. Cells were passaged at 80% confluence. Cells at passage numbers of between 15 to 20 were used in this study.

Viability Assay

Cytotoxicity of test samples on Caco-2 and STC-1 cell proliferation was determined by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-

(4-sulfophenyl)-2H-tetrazolium (MTS) assay, using CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega Corporation Madison, WI) according to the manufacturer's instructions. Viability was defined as the ratio of absorbance of treated cells to untreated cells (cells incubated in media only) at 490 nm. Each cell exposure was repeated in triplicate, at a minimum.

For Caco-2 experiments, cells were seeded in 96-well plates at a cell density of 2×10^4 cells/well using serum-free media (DMEM only supplemented with 100 U/mL of penicillin and 100 mg/mL of streptomycin) 24 h before the experiment. The lethal dose 50 (**LD**₅₀) concentration of free linoleate is 58 μ M (Le Maux et al., 2012). As such, Caco-2 cells were exposed for 24 h to digested complexes (molar ratio of 1, 2, and 3 linoleate-BLG) containing 58 μ M linoleate.

Cytotoxicity assays were also performed on Caco-2 monolayers. Caco-2 cells were seeded in 96-well plates at a cell density of 6.5×10^4 cells/well using complete media. Initially, media was changed after 6 h, and then every 2 d up to 21 d. Complete media was changed to serum-free media 24 h before the experiment. Cells were exposed to 50 μ M linoleate for 24 h.

For STC-1 experiments, STC-1 cells were seeded in 96-well plates at a cell density of 2×10^5 cells/well using complete media 24 h before the experiment. The STC-1 were treated with different concentrations of linoleate (0–200 μ M) and BLG (0–200 μ M) in serum-free media for 24 h. The LD₅₀ was determined using Graph-Pad Prism software 3.03 (GraphPad Software Inc., La Jolla, CA). The sigmoidal dose response with variable slope was used to fit the measured curves and calculate LD₅₀.

Caco-2 Transepithelial Transport

The Caco-2 cell line is derived from human colonic carcinoma and has similar characteristic to small intestine epithelial cells when cultured as a monolayer (Delie and Rubas, 1997; Yee, 1997).

For transepithelial transport experiments, Caco-2 cells were seeded at a density of 3×10^5 cells/well into permeable Transwell filter inserts (24 mm in diameter, 0.4 μ m pore size; Costar, Cambridge, MA). Cell culture media was changed every 2 d for 21 d, upon which time the cells were fully differentiated. Serum-free media was changed 24 h before experimentation. In these conditions, Caco-2 cells were exposed to media devoid of FA to minimize the effect of exogenous FA, and maximize the effect of added linoleate.

Cell monolayer integrity was confirmed by measuring transepithelial electrical resistance at 37°C using a Millicell-ERS meter (Millipore Corporation, Bedford, MA) according to the manufacturer's instructions. In-

serts with a transepithelial electrical resistance value $\leq 1,300 \Omega \cdot \text{cm}^2$ were discarded.

Transepithelial transport studies were adapted from Hubatsch et al. (2007). Briefly, inserts were washed 3 times with serum-free media. Serum-free media (2.5 mL) was added to the basal side. To the apical side was added 1.5 mL of tested compound (1, 2, and 3 linoleate-BLG complexes digested and undigested with a linoleate concentration of $50 \mu\text{M}$) in serum-free media containing 5.3 mM fluorescein sodium salt. Fluorescein acts as an indicator of paracellular transport across the Caco-2 monolayer (Gilman and Cashman, 2007). Plates were then incubated in a humidified 37°C incubator on an orbital shaker at 60 rpm to minimize the effect of the unstirred layer. After 4 h, apical and basal media were removed for analysis. Six replicates of each experiment were performed.

Determination of Paracellular Transport in Monolayer Experiments

Fluorescein concentration in the basal chamber was determined by detecting fluorescence in a $50\text{-}\mu\text{L}$ aliquot. Fluorescence was detected with a Synergy Biotek plate reader (BioTek Instruments Inc., Winooski, VT) at excitation and emission wavelengths of 485 and 535 nm, respectively. Fluorescein concentration was determined by interpolating fluorescence readings from a fluorescein standard curve. The concentration of fluorescein in the basal chamber was expressed as a percentage of the total fluorescein added to the apical side of the inserts. Paracellular transport was always inferior to 0.1%.

Extraction and Determination of the FA Content in Monolayer Experiments by GC

Fatty acid concentration in the apical and basal chambers was determined by GC. Total FA were extracted according to the method of the ISO standards 14156:2001 (ISO, 2001). The FA C13:0 (0.75 mg) was employed as an internal standard. Following removal of the diethyl ether (Fisher Scientific, Pittsburgh, PA) by heating at 45°C under nitrogen, extracted FA were converted to FAME by base-catalyzed methylation with 2 mL of 0.5 N sodium methoxide at ambient temperature for 10 min. This was followed by acid-catalyzed methylation using an adaptation of the method of previously described by Palmquist and Jenkins (2003). The concentrated FAME was transferred to clean glass GC vials and quantified as described previously.

The amount of linoleate remaining in the apical chamber and its transport in the basal chamber were corrected by the paracellular transport and the control (cells with media only). The corrected linoleate data

was then expressed as a percentage of the initial linoleate ($50 \mu\text{M}$) applied to the apical side.

Confocal Fluorescence Microscopy

Intracellular lipid accumulation was imaged using confocal microscopy. Caco-2 cells were seeded into 8-well chambered glass coverslips (Labtek, Nunc, Roskilde, Denmark) at a density of 5×10^4 cells/well with serum-free media 24 h before experimentation. Cells were then treated with $50 \mu\text{M}$ of linoleate or $50 \mu\text{M}$ of linoleate in a complex with a molar ratio of 3 linoleate-BLG. Following 4 h of exposure, cells were gently washed with PBS and stained with $4 \mu\text{M}$ of Nile Red (prepared in PBS; Sigma-Aldrich) to highlight intracellular lipid droplets (Greenspan et al., 1985). Cells were examined using a Nikon C1Si laser scanning confocal imaging system on inverted microscope TE2000-E (Nikon, Champigny-sur-Marne, France) equipped with a helium-neon laser emitting at 543 nm. Fluorescence emission was acquired with a 590/50 nm filter. Average fluorescence intensity was calculated from 9 images.

CCK Experiments on STC-1 Cells

To assess the effect of samples on CCK secretion in vitro, the STC-1 cell line was employed (Pasman et al., 2008, Hand et al., 2010). The CCK assay was adapted from Hand et al. (2010). Briefly, STC-1 cells were seeded into 12-well plates at a cell density of 2×10^6 cells/well in complete media and incubated overnight. On the day of the experiment, media was removed and cells were washed 3 times with serum-free media and then incubated for 30 min with serum-free media at 37°C . Media was removed and 400 μL of serum-free media containing $5 \mu\text{M}$ of linoleate, free or complexed to BLG (molar ratio 1 to 3), either digested or undigested were added. All CCK experiments were performed in triplicate.

After 4 h of incubation, 360 μL of the supernatant was removed, 40 μL of 10% BSA (prepared in PBS) was added and the solution was centrifuged at $900 \times g$ for 5 min at 4°C to remove cellular debris. The supernatant was collected and stored at -80°C before analysis by ELISA.

For determination of CCK mRNA levels, cells were washed with PBS. Cells were lysed using QIAzol Lysis Reagent (Qiagen Ltd., West Sussex, UK) and RNA was isolated from cell suspensions using the Qiagen miRNeasy Mini kit (Qiagen Ltd.) according to the manufacturers' instructions. The RNA was quantified spectrophotometrically using the Nanodrop 1000 (Thermo Fisher Scientific, Waltham, MA) and the integrity assessed by electrophoresis in a 1.5% glyoxyl gel

with 1× glyoxyl buffer (Ambion, Applied Biosystems, Foster City, CA). Complementary DNA synthesis was prepared from 1 µg of RNA using the Qiagen QuantiTect reverse transcription kit (Qiagen Ltd.).

Determination of Cholecystokinin Secretion Using ELISA. A CCK (26–33, nonsulfated) fluorescent ELISA immunoassay kit (Phoenix Pharmaceutical Inc., Burlingame, CA) was used according to manufacturer's instructions. The CCK content was determined using 25 µL of supernatant. Fluorescence (excitation: 337 nm; emission: 460 nm) was read using a FLUOstar Omega multi-mode microplate reader and analyzed with the FLUOstar Omega software (BMG Labtech GmbH, Ortenberg, Germany). Concentration of CCK in samples was determined by extrapolation to a standard curve. Data were expressed as a percentage of the control value (cells with media only). The STC-1 cells do not produce a detectable level of gastrin, so the detected signal was attributed to CCK (McLaughlin et al., 1998); experiments were performed in triplicate.

Determination of CCK mRNA Using Real-Time PCR. The CCK mRNA was quantified using Lightcycler and SyBr green technology (Roche Diagnostics Ltd., Mannheim, Germany) and by the principles of relative quantification using the housekeeping gene ubiquitin-conjugating enzyme (*E2D2*). Protocol and primers for murine CCK were previously described by Hand et al. (2010). The target (CCK) to reference (*E2D2*) ratio expression was calculated for each treatment and normalized compared with untreated (media alone) controls (Hand et al., 2010); experiments were performed in triplicate.

Statistical Analysis

Where appropriate, results were compared using the R software package version 2.15.1 (R Foundation for Statistical Computing, Vienna, Austria) and the ANOVA system with a Tukey's least significant difference comparison. Any *P*-values <0.05 were deemed to be statistically significant.

RESULTS

Protein Digestion

Gastric and duodenal in vitro digestion was performed on native BLG, BLG control, and linoleate-BLG complexes (linoleate-BLG molar ratio of 1, 2, and 3; Figure 1). As linoleate-BLG complexes had similar chromatograms, only the 3 linoleate-BLG molar ratio complex was compared with BLG control. The GP-HPLC chromatogram was divided into 4 fractions: protein oligomers [molecular weight (M_w) ≥ 30 kDa],

protein monomers (30 kDa ≥ M_w ≥ 10 kDa), large peptides (10 kDa ≥ M_w ≥ 1 kDa), and small peptides (M_w ≤ 1 kDa).

The initial HPLC profiles (before in vitro digestion) of BLG control and complexes were different regarding oligomer and monomer categories. The proportion of oligomers in the initial samples was 15% for BLG control and 31% for complex with a molar ratio of 3 linoleate-BLG. These BLG oligomers were rapidly hydrolyzed during the gastric digestion. Their proportion decreased from 31 to 7% and from 15 to 5% within the first minute of gastric digestion for the BLG control and 3 linoleate-BLG complex, respectively. The proportion of monomers decreased from 85 to 61% for BLG control and from 65 to 45% for a complex with a molar ratio of 3 linoleate-BLG. Small and large peptides were produced during the gastric digestion for all the samples. They represent about 10% for BLG control and 20% for the 3 linoleate-BLG complex at the end of the gastric digestion process.

During the duodenal digestion, the proportion of BLG monomers decreased from 61 to 53% for the BLG control sample, whereas it remained unchanged in samples with linoleate. A decrease in the proportion of small peptides (<1 kDa) was observed for all the samples prior to the addition of enzymes of the duodenal digestion. For example, a decrease from 20 to 12% of small peptides was observed for the 3 linoleate-BLG complex. Concomitantly, an increase of oligomers (M_w ~66 kDa) was detected. As no change in monomer and large peptide populations was observed, these oligomeric species were attributed to the aggregation of small peptides. This result was confirmed by SDS-PAGE experiments, which showed the formation of aggregated molecules that disappeared after chemical reduction (data not shown).

Cytotoxicity of In Vitro Digested Complexes on Caco-2 Cells

Exposure of Caco-2 cells to the digested complexes containing 58 µM of linoleate, the LD₅₀ of linoleate on Caco-2 cells in these conditions (Le Maux et al., 2012), resulted in a significant decrease in Caco-2 viability after 24 h compared with digested BLG alone (Figure 2). Cells exposed to digested BLG control had a viability of 90.8 ± 1.8%, whereas cells exposed to digested complexes had a viability of 71.7 ± 2.6%, 75.0 ± 1.7%, and 67.9 ± 3.2% for 1, 2, and 3 linoleate-BLG, respectively. Digested complexes did provide a protective effect to the cells, compared with free linoleate. However, undigested complexes, similar to BLG alone, had minimal effect on cell viability under the experimental conditions used (96.6 ± 5.6%; Le Maux et

Figure 1. Characterization of in vitro gastric and duodenal digestion of β -lactoglobulins (BLG) alone or in complexes with linoleate determined by gel permeation-HPLC. Proportion of BLG monomers (solid black line), aggregates (dotted black line), peptides (1–10 kDa; solid gray line), and small peptides (<1 kDa; dotted gray line) are represented. (A) Native BLG, (B) BLG control, (C) 1 linoleate-BLG, (D) 2 linoleate-BLG, and (E) 3 linoleate-BLG.

al., 2012). Consequently, the level of cell viability with digested complexes is intermediate between undigested complexes and free linoleate.

Uptake of Linoleate by Caco-2 Cells

Uptake by Caco-2 cells of linoleate free or bound to BLG was followed both by the use of Caco-2 cell

monolayers, which mimic the intestinal barrier, and by confocal imaging. Levels of linoleate in the apical and basal chamber of the Caco-2 monolayer was quantified by GC analysis (Figure 3). Preliminary cytotoxic assays demonstrated that 4 h of exposure of differentiated Caco-2 cells to 50 μ M of linoleate free or in complex did not disturb the Caco-2 monolayer (data not shown).

Figure 2. Viability of Caco-2 cells exposed to digested β -lactoglobulins (BLG)-linoleate complexes for 24 h. Cells (2×10^4 cells/well) were treated with digested complexes (molar ratio of 1, 2, and 3 linoleate-BLG each of which contained $58 \mu M$ of linoleate). Cell viability is calculated as a percentage of viability of cells incubated in media alone. Fatty acids = linoleate; 3X = 3 BLG-linoleate complex; D β -LG = digested BLG control; D1X, D2X, and D3X = linoleate-BLG digested complexes with a molar ratio of 1, 2, and 3, respectively. Results represent mean $\pm$ SD ($n = 6$). Multiple comparisons of means were performed using Tukey contrasts ($P < 0.001$). Different letters indicate values that are significantly different from each other.

Therefore, this sublethal concentration was selected for the Caco-2 transepithelial transport experiments. It was observed that the presence of BLG did not affect the transport of linoleate through the basal chamber as the basal level of linoleate was similar for all the samples (i.e., $3.86 \pm 0.7\%$). However, the molar ratio of linoleate-BLG complexes affected the proportion of linoleate remaining in the apical chamber. As shown in Figure 3, $4.9 \pm 0.9\%$ linoleate remained in the apical chamber when cells were exposed to $50 \mu M$ of free linoleate, compared with $10.4 \pm 0.4\%$, $13.7 \pm 0.3\%$, and $15.3 \pm 2.5\%$ linoleate when cells were exposed to complexes of 1, 2, and 3 linoleate-BLG, respectively. Interestingly, the proportion of linoleate remaining in the apical chamber was similar for all digested samples ($12.2 \pm 0.6\%$). Hence, binding of BLG with linoleate significantly decreased the uptake of linoleate by the cells.

Lipid accumulation in Caco-2 cells was measured by Nile Red fluorescence via confocal imaging (Figure 4). Cells were incubated for 4 h with $50 \mu M$ of free linoleate or in a 3 linoleate-BLG complex. We observed a significant increase in the amount of lipid accumulated in the cells exposed to free linoleate compared with the cells exposed to linoleate complexed to BLG. Fluorescence

Figure 3. Transport of linoleate across the Caco-2 monolayer after 4 h of exposure to $50 \mu M$ of linoleate free or bound to β -lactoglobulins (BLG; undigested and digested complexes with a molar ratio of 1, 2, or 3). Proportion of linoleate in apical or basal chamber was expressed as the percentage of the initial linoleate content added to cells. Linoleate was measured by GC in the apical chamber (black bars) and in the basal chamber (gray bars). Fatty acids = linoleate; 1X, 2X, and 3X = undigested linoleate-BLG complexes with a molar ratio of 1, 2, and 3, respectively; D1X, D2X, and D3X = digested linoleate-BLG complexes with a molar ratio of 1, 2, and 3, respectively. Results represent mean $\pm$ SD ($n = 6$). Multiple comparisons of means were performed using Tukey contrasts ($P < 0.05$). Different letters indicate values that are significantly different from each other.

intensity was 333 ± 25 and $2,203 \pm 570$ AU after 4 h of incubation with bound and free linoleate, respectively. For both treatments, the formation of lipid droplets was observed in the confocal images, with larger lipid droplets in cells exposed to free linoleate (Figure 4).

Effect of Linoleate-BLG on the Regulation of the Satiety Hormone CCK, Secretion, and mRNA Level

To determine if linoleate bound to BLG can alter downstream cellular response, the enteroendocrine cell line STC-1 was incubated with free linoleate or complexes of BLG-linoleate. Preliminary experiments showed that BLG was not cytotoxic to STC-1 cells at the concentrations tested (0 – $200 \mu M$) after 24 h, as measured by MTS assay (data not shown). In contrast, STC-1 cells were sensitive to linoleate with an LD_{50} of $12.68 \pm 2.56 \mu M$ after 24 h (data not shown).

No significant difference was observed in the levels of CCK peptide secreted from STC-1 cells exposed for 4 h to untreated control, free linoleate, and undigested and digested complexes (data not shown). In contrast, all the samples tested increased CCK mRNA transcripts levels compared with untreated control after 4 h (Figure 5). No significant difference was observed between the normalized mRNA response of undigested 1, 2, and 3 linoleate-BLG molar ratios used. In the same manner, digested complexes did not show significant difference between the 3 linoleate-BLG molar ratios. Normalized mRNA levels were 1.007 ± 0.004 for free linoleate and

Figure 4. Confocal analysis of Caco-2 cells (5×10^4 cells/well) exposed for 4 h to $50 \mu\text{M}$ of linoleate-free or bound in a complex with a 3 linoleate-β-lactoglobulin (BLG) molar ratio. Lipid droplets were stained with Nile Red dye (Sigma-Aldrich, St. Louis, MO). (A) Fluorescence intensity of Nile Red in Caco-2 cells. (B) Confocal imaging depicting the difference in the level of fluorescence from cells exposed to free (B1) or bound (B2) linoleate (bar = $5 \mu\text{m}$). Results represent mean $\pm$ SD ($n = 9$ pictures). Multiple comparisons of means were performed using Tukey contrasts ($P < 0.01$). Different letters indicate values that are significantly different from each other. Color version available in the online PDF.

increased to 1.041 ± 0.013 and 1.072 ± 0.014 when cells were exposed 3 linoleate-BLG complex undigested and digested, respectively ($P < 0.05$). Normalized CCK mRNA levels were 1.027 ± 0.016 and 1.059 ± 0.009 for undigested and digested BLG control, respectively.

DISCUSSION

Digested complexes of BLG-linoleate were found to be more cytotoxic to Caco-2 cells compared with undigested complexes but less cytotoxic than free linoleate (Le Maux et al., 2012). Linoleate has to be transported into the cell to be cytotoxic (Lu et al., 2010); this would infer that a portion of the linoleate remains bound to

Figure 5. Cholecystokinin (CCK) mRNA levels in 2×10^6 STC-1 cells/well exposed for 4 h to linoleate-β-lactoglobulin (BLG) complexes with a linoleate concentration of $5 \mu\text{M}$. Normalized CCK fold change was calculated by (treated CCK/*E2D2*)/(untreated CCK/*E2D2*), where *E2D2* is the housekeeping gene. FA = linoleate; BLG = BLG control; 1X, 2X, and 3X = undigested linoleate-BLG complexes with a molar ratio of 1, 2, and 3, respectively; Dβ-LG = digested BLG control; D1X, D2X, and D3X = linoleate-BLG digested complexes with a molar ratio of 1, 2, and 3, respectively. Results represent mean $\pm$ SD ($n = 3$). Multiple comparisons of means were done using Tukey contrasts ($P < 0.05$). Different letters indicate values that are significantly different from each other.

the protein or peptides after in vitro gastro-duodenal digestion. Even if it is assumed that linoleate dissociates from the protein under gastric conditions (pH 2.5), complexes could reform under duodenal digestion conditions (pH 6.5; Ragona et al., 2000). The interaction between FA and BLG is pH-dependent; at a low pH (below pH 6) FA are released from the BLG, but the interaction is reversible when the pH is increased. As up to 50% of BLG monomers remained after digestion, it is highly probable that reformed complexes consisted of linoleate and undigested monomers.

In its native state, BLG conformation is resistant to pepsin hydrolysis (Guo et al., 1995). However, BLG was hydrolyzed faster under gastric digestion conditions when the FA to BLG molar ratio was increased. This is probably due to the increased proportion of oligomers in the sample (Le Maux et al., 2012). During the duodenal digestion, no hydrolysis was observed for linoleate-BLG complexes. Only the BLG control undergoes peptic hydrolysis during duodenal digestion, suggesting a protective effect of linoleate. Puyol et al. (1993) demonstrated that the binding of palmitic acid to BLG has a protective effect on the protein against hydrolysis, whereas the binding of retinol does not. Mandalari et al. (2009) also demonstrated that phosphatidylcholine could protect BLG from hydrolysis, possibly because the binding sites are located close to protease cleavage sites. Furthermore, Mandalari et al. (2009) observed that this protective effect was only effective if the protein was in its native form.

During duodenal in vitro digestion, we observed the appearance of aggregates greater than 30 kDa and a

concomitant decrease in the population of small peptides less than 1 kDa. The proportions of large peptides and monomers remained constant. These aggregates disappeared under reducing conditions. This suggests that a fraction of low- M_w peptides aggregate, under duodenal conditions, via disulfide bonds. Aggregates were observed for all complexes and BLG control samples, suggesting that the presence of linoleate did not influence peptide aggregation. Jiang and Liu (2010) also observed the formation of aggregates of around 36 kDa during trypsin treatment of conjugated LA-BLG, whereas Bateman et al. (2011) showed formation of BLG fibrils after pepsin hydrolysis and a subsequent pH increase to 6.9.

Caco-2 cells are an appropriate model to investigate FA transport across the intestinal gut (Puyol et al., 1995; Riihimäki-Lampén, 2009). Our results showed a decrease in the transport of the linoleate, when bound to BLG, into and out of the Caco-2 monolayer. Confocal microscopy confirmed that more linoleate accumulated in the cells when free, compared with when linoleate was complexed to BLG. This is in agreement with Riihimäki-Lampén (2009), who demonstrated that free retinol and free palmitic acid were transported with more efficiency from the apical to the basal side of Caco-2 monolayer than when complexed to BLG. However, no such change was observed for cholesterol. Opposite results were found for conjugated LA (Jiang and Liu, 2010). These authors showed that the level of conjugated LA in cells was significantly greater when conjugated LA was provided to the cells as a BLG-conjugated LA complex compared with free conjugated LA. The low solubility of conjugated LA and the concentration range used in the various studies could explain the discrepancy.

Our results suggest that a considerable quantity of FA was metabolized or stored by the cell. Riihimäki et al. (2008) and Puyol et al. (1995) showed that a palmitic acid-bovine BLG complex was principally stored in the cell (more than 90% after 24 h; Puyol et al., 1995) and its transport in the basal chamber was low (i.e., 5–7% in the presence of bovine BLG; Puyol et al., 1995; Riihimäki et al., 2008). However, our confocal analysis showed a substantial difference in lipid accumulation in cells compared with our monolayer data. This might be accounted for by our use of Nile Red dye, which fluoresces better in lipid droplets (Greenspan et al., 1985). This confocal result suggests that linoleate from the BLG-linoleate complexes is slower to form lipid droplets.

When in complexes, an increase in linoleate uptake was observed with increasing concentrations of BLG content using the Caco-2 monolayer. As linoleate concentration was constant (50 μM of linoleate), BLG

concentration was 3 times smaller for a linoleate-BLG molar ratio of 3 compared with a molar ratio of 1. Native BLG has been shown to be transported into the cells (Caillard and Tome, 1995); this could explain why the kinetics of linoleate transport from the medium to the cells is affected by BLG concentration. However, some authors report the existence of another mechanism involving a pH-dependent dissociation of the ligand bound to BLG immediately before transport into the cell, as the pH of the environment close to the cells is more acidic (pH <5; Ragona et al., 2000; Niot et al., 2009).

Digestion of linoleate-BLG complexes seems to affect linoleate uptake by Caco-2 cells. Linoleate remaining in the apical chamber increased when the linoleate-BLG complex with a molar ratio of 1 was digested compared with undigested. This could be explained by the fact that, after digestion, the BLG monomer concentration was reduced by a factor of 2. Under these conditions, the concentration of residual BLG monomers was sufficient to load linoleate (as indicated for complexes with a linoleate-BLG molar ratio of 2). However, transport of FA to the cells facilitated by BLG would be lower in the digested sample, as the amount of BLG monomer was lower. Interestingly, the concentration of linoleate remaining in the apical chamber decreased when the complex with linoleate-BLG molar ratio of 3 was digested compared with undigested. Under this condition, it is possible that the concentration of residual BLG monomer after digestion was too low to bind all the linoleate molecules in the sample increasing the amount of free linoleate. As free linoleate is quickly transported to the cells, an overall faster transport is observed for digested complex than undigested.

The ability of STC-1 cells to produce and secrete CCK is thought not to require internalization of the FA (Choi et al., 2007; Tanaka et al., 2008; Shah et al., 2011). After 4 h of exposure to the studied protein or FA complexes, no effect on CCK secretion was detected, whereas mRNA CCK levels differed between samples. This supports that CCK synthesis and secretion are distinct. This was highlighted by Hand et al. (2010), who showed that after STC-1 cells were exposed to 100 μM of LA, differences were observed between the mRNA CCK levels and CCK content in the cell or secreted after either 30 min or 72 h. We found that digested samples induced higher CCK mRNA levels than undigested samples. This is probably due to protein digestion, as it has been reported that protein hydrolysis leads to higher CCK response compared with undigested protein (Beucher et al., 1994). The CCK mRNA levels were unaffected by the addition of linoleate to BLG. However, the CCK mRNA data seem contradictory with the cytotoxicity and the transport

experiments. Indeed, these experiments demonstrated that less linoleate was free in the presence of BLG. Thus, we would expect less linoleate to be in contact with the cell membrane and, therefore, a lower CCK response. However, the denaturation of the BLG is likely to have exposed unfolded proteins, which may result in an increase in CCK mRNA compared with the native protein. Although the CCK mRNA response is significant, the difference is so small that it may not be biologically relevant.

This study has demonstrated that BLG modifies linoleate transport and FA metabolism in Caco-2 cells by altering FA bioaccessibility. Binding of linoleate to BLG induces protein oligomerization and, consequently, influences protein susceptibility to digestion. Digested linoleate-BLG complexes delayed the cytotoxicity of the FA, suggesting that part of FA is still in a complexed form. Binding of linoleate to BLG potentially alters linoleate uptake by altering levels of FFA. However, no effect on CCK secretion was observed when linoleate was free or bound to BLG. This work highlights the importance of investigating food matrices to understand digestion and bioaccessibility of individual components. Research such as this aims to optimize foods for bioactive delivery.

ACKNOWLEDGMENTS

S. Le Maux is currently supported by a Teagasc Walsh Fellowship and the Department of Agriculture, Fisheries and Food (Dublin, Ireland; FIRM project 08/RD/TMFRC/650). We also acknowledge funding from IRCSET-Ulysses Travel Grant (Dublin, Ireland). The authors express their gratitude to Marie-Noëlle Madec (INRA, Rennes, France) for her assistance with confocal imaging.

REFERENCES

Abumrad, N., C. Harmon, and A. Ibrahim. 1998. Membrane transport of long-chain fatty acids: Evidence for a facilitated process. *J. Lipid Res.* 39:2309–2318.

Bateman, L., A. Ye, and H. Singh. 2011. Re-formation of fibrils from hydrolysates of beta-lactoglobulin fibrils during in vitro gastric digestion. *J. Agric. Food Chem.* 59:9605–9611.

Beard Shall, K., Y. Morarji, S. R. Bloom, G. Frost, J. Domin, and J. Calam. 1989. Saturation of fat and cholecystokinin release: Implications for pancreatic carcinogenesis. *Lancet* 334:1008–1010.

Beucher, S., F. Levenez, M. Yvon, and T. Corring. 1994. Effects of gastric digestive products from casein on CCK release by intestinal cells in rat. *J. Nutr. Biochem.* 5:578–584.

Caillard, I., and D. Tome. 1995. Transport of beta-lactoglobulin and alpha-lactalbumin in enterocyte-like Caco-2 cells. *Reprod. Nutr. Dev.* 35:179–188.

Choi, S., M. Lee, A. L. Shiu, S. J. Yo, G. Halldén, and G. W. Aponte. 2007. GPR93 activation by protein hydrolysate induces CCK transcription and secretion in STC-1 cells. *Am. J. Physiol. Gastrointest. Liver Physiol.* 292:G1366–G1375.

Coakley, M., R. P. Ross, M. Nordgren, G. Fitzgerald, R. Devery, and C. Stanton. 2003. Conjugated linoleic acid biosynthesis by human-derived *Bifidobacterium* species. *J. Appl. Microbiol.* 94:138–145.

Cordier-Bussat, M., C. Bernard, S. Haouche, C. Roche, J. Abello, J. A. Chayvialle, and J. C. Cuber. 1997. Peptones stimulate cholecystokinin secretion and gene transcription in the intestinal cell line STC-1. *Endocrinology* 138:1137–1144.

Delie, F., and W. Rubas. 1997. A human colonic cell line sharing similarities with enterocytes as a model to examine oral absorption: Advantages and limitations of the Caco-2 model. *Crit. Rev. Ther. Drug Carrier Syst.* 14:221–286.

Douglas, B. R., J. B. Jansen, A. J. de Jong, and C. B. Lamers. 1990. Effect of various triglycerides on plasma cholecystokinin levels in rats. *J. Nutr.* 120:686–690.

Dupont, D., G. Mandalari, D. Molle, J. Jardin, J. Léonil, R. M. Faulks, M. S. J. Wickham, E. N. C. Mills, and A. R. Mackie. 2009. Comparative resistance of food proteins to adult and infant in vitro digestion models. *Mol. Nutr. Food Res.* 54:767–780.

Flower, D. R. 1996. The lipocalin protein family: Structure and function. *Biochem. J.* 318:1–14.

Gibbs, J., R. C. Young, and G. P. Smith. 1973. Cholecystokinin decreases food intake in rats. *J. Comp. Physiol. Psychol.* 84:488–495.

Gilman, J., and K. D. Cashman. 2007. The effect of marine oil-derived n-3 fatty acids on transepithelial calcium transport in Caco-2 cell models of healthy and inflamed intestines. *Br. J. Nutr.* 97:281–288.

Greenspan, P., E. P. Mayer, and S. D. Fowler. 1985. Nile red: A selective fluorescent stain for intracellular lipid droplets. *J. Cell Biol.* 100:965–973.

Guo, M. R., P. Fox, A. Flynn, and P. Kindstedt. 1995. Susceptibility of beta-lactoglobulin and sodium caseinate to proteolysis by pepsin and trypsin. *J. Dairy Sci.* 78:2336–2344.

Hand, K. V., C. M. Bruen, F. O'Halloran, L. Giblin, and B. D. Green. 2010. Acute and chronic effects of dietary fatty acids on cholecystokinin expression, storage and secretion in enteroendocrine STC-1 cells. *Mol. Nutr. Food Res.* 54(Suppl. 1):S93–S103.

Hubatsch, I., E. G. E. Ragnarsson, and P. Artursson. 2007. Determination of drug permeability and prediction of drug absorption in Caco-2 monolayers. *Nat. Protoc.* 2:2111–2119.

ISO. 2001. Milk and milk products Extraction methods for lipids and liposoluble compounds. ISO 14156:2001. ISO Publications, International Organization for Standardization, Geneva, Switzerland.

Jensen, R. G. 2002. The composition of bovine milk lipids: January 1995 to December 2000. *J. Dairy Sci.* 85:295–350.

Jiang, H. R., and N. Liu. 2010. Self-assembled beta-lactoglobulin-conjugated linoleic acid complex for colon cancer-targeted substance. *J. Dairy Sci.* 93:3931–3939.

Kontopidis, G., C. Holt, and L. Sawyer. 2002. The ligand-binding site of bovine beta-lactoglobulin: Evidence for a function? *J. Mol. Biol.* 318:1043–1055.

Kontopidis, G., C. Holt, and L. Sawyer. 2004. Invited review: Beta-lactoglobulin: Binding properties, structure, and function. *J. Dairy Sci.* 87:785–796.

Kushibiki, S., K. Hodate, J. Kurisaki, H. Shingu, Y. Ueda, A. Watanabe, and M. Shinoda. 2001. Effect of beta-lactoglobulin on plasma retinol and triglyceride concentrations, and fatty acid composition in calves. *J. Dairy Res.* 68:579–586.

Le Maux, S., L. Giblin, T. Croguennec, S. Bouhallab, and A. Brodtkorb. 2012. Beta-lactoglobulin as a molecular carrier of linoleate: Characterisation and effects on intestinal epithelial cells in vitro. *J. Agric. Food Chem.* 60:9476–9483.

Liddle, R. A. 2000. Regulation of cholecystokinin secretion in humans. *J. Gastroenterol.* 35:181–187.

Liddle, R. A., I. D. Goldfine, M. S. Rosen, R. A. Taplitz, and J. A. Williams. 1985. Cholecystokinin bioactivity in human plasma. Molecular forms, responses to feeding, and relationship to gallbladder contraction. *J. Clin. Invest.* 75:1144–1152.

Lišková, K., M. A. E. Auty, V. Chaurin, S. Min, K. H. Mok, N. O'Brien, A. L. Kelly, and A. Brodtkorb. 2011. Cytotoxic complexes of sodium oleate with beta-lactoglobulin. *Eur. J. Lipid Sci. Technol.* 113:1207–1218.

- Little, T. J., A. Russo, J. H. Meyer, M. Horowitz, D. R. Smyth, M. Bellon, J. M. Wishart, K. L. Jones, and C. Feinle-Bisset. 2007. Free fatty acids have more potent effects on gastric emptying, gut hormones, and appetite than triacylglycerides. *Gastroenterology* 133:1124–1131.
- Lu, X., H. Yu, Q. Ma, S. Shen, and U. N. Das. 2010. Linoleic acid suppresses colorectal cancer cell growth by inducing oxidant stress and mitochondrial dysfunction. *Lipids Health Dis.* 9:1. <http://dx.doi.org/10.1186/1476-511X-9-1>
- Mandalari, G., A. M. Mackie, N. M. Rigby, M. S. J. Wickham, and E. N. Mills. 2009. Physiological phosphatidylcholine protects bovine beta-lactoglobulin from simulated gastrointestinal proteolysis. *Mol. Nutr. Food Res.* 53(Suppl. 1):S131–S139.
- McLaughlin, J. T., R. Lomax, L. Hall, G. Dockray, D. Thompson, and G. Warhurst. 1998. Fatty acids stimulate cholecystokinin secretion via an acyl chain length-specific, Ca²⁺ dependent mechanism in the enteroendocrine cell line STC-1. *J. Physiol.* 513:11–18.
- Mu, H. 2008. Bioavailability of omega-3 long-chain polyunsaturated fatty acids from foods. *Agro Food Ind. Hi Tec.* 19:24–26.
- Niot, I., H. Poirier, T. T. T. Tran, and P. Besnard. 2009. Intestinal absorption of long-chain fatty acids: Evidence and uncertainties. *Prog. Lipid Res.* 48:101–115.
- Nishi, T., H. Hara, T. Hira, and F. Tomita. 2001. Dietary protein peptic hydrolysates stimulate cholecystokinin release via direct sensing by rat intestinal mucosal cells. *Exp. Biol. Med.* (Maywood) 226:1031–1036.
- Palmquist, D. L., and T. C. Jenkins. 2003. Challenges with fats and fatty acid methods. *J. Anim. Sci.* 81:3250–3254.
- Pasman, W. J., J. Heimerikx, C. M. Rubingh, R. Van Den Berg, M. O'Shea, L. Gambelli, H. F. Hendriks, A. W. Einerhand, C. Scott, and H. G. Keizer. 2008. The effect of Korean pine nut oil on in vitro CCK release, on appetite sensations and on gut hormones in post-menopausal overweight women. *Lipids Health Dis.* 7:1. <http://dx.doi.org/10.1186/1476-511X-7-1>.
- Pérez, M. D., and M. Calvo. 1995. Interaction of beta-lactoglobulin with retinol and fatty acids and its role as a possible biological function for this protein: A review. *J. Dairy Sci.* 78:978–988.
- Perez, M. D., L. Sanchez, P. Aranda, J. Ena, R. Oria, and M. Calvo. 1992. Effect of beta-lactoglobulin on the activity of pregastric lipase. A possible role for this protein in ruminant milk. *Biochim. Biophys. Acta* 1123:151–155.
- Purhonen, A. K. 2008. Signals arising from the gastrointestinal tract that affect food intake. PhD Thesis. University of Kuopio, Kuopio, Finland.
- Puyol, P., M. Dolores Perez, L. Sanchez, J. M. Ena, and M. Calvo. 1995. Uptake and passage of beta-lactoglobulin palmitic acid and retinol across the Caco-2 monolayer. *Biochim. Biophys. Acta Biomembranes* 1236:149–154.
- Puyol, P., M. D. Perez, L. Mata, J. M. Ena, and M. Calvo. 1993. Effect of retinol and fatty acid binding by bovine beta-lactoglobulin on its resistance to trypsin digestion. *Int. Dairy J.* 3:589–597.
- Ragona, L., L. Zetta, F. Fogolari, H. Molinari, D. M. Pérez, P. Puyol, K. D. Kruif, F. Löhr, and H. Rüterjans. 2000. Bovine beta-lactoglobulin: Interaction studies with palmitic acid. *Protein Sci.* 9:1347–1356.
- Riihimäki, L., A. Galkinab, M. Finel, J. Heikura, K. Valkonen, V. Virtanen, R. Laaksonen, J. P. Slotte, and P. Vuorela. 2008. Transport properties of bovine and reindeer beta-lactoglobulin in the Caco-2 cell model. *Int. J. Pharm.* 347:1–8.
- Riihimäki-Lampén, L. 2009. Interactions of natural products with beta-lactoglobulins, members of the lipocalin family. PhD Thesis. University of Helsinki, Helsinki, Finland.
- Rindi, G., S. Grant, Y. Yiangou, M. Ghatei, S. Bloom, V. L. Bautch, E. Solcia, and J. Polak. 1990. Development of neuroendocrine tumors in the gastrointestinal tract of transgenic mice. Heterogeneity of hormone expression. *Am. J. Pathol.* 136:1349–1363.
- Shah, B. P., P. Liu, T. Yu, D. R. Hansen, and T. A. Gilbertson. 2011. TRPM5 is critical for linoleic acid-induced CCK secretion from the enteroendocrine cell line, STC-1. *Am. J. Physiol. Cell Ph.* XX:C210–C219.
- Singh, H., A. Ye, and D. Horne. 2009. Structuring food emulsions in the gastrointestinal tract to modify lipid digestion. *Prog. Lipid Res.* 48:92–100.
- Stremmel, W. 1988. Uptake of fatty acids by jejunal mucosal cells is mediated by a fatty acid binding membrane protein. *J. Clin. Invest.* 82:2001–2010.
- Tanaka, T., S. Katsuma, T. Adachi, T. Koshimizu, A. Hirasawa, and G. Tsujimoto. 2008. Free fatty acids induce cholecystokinin secretion through GPR120. *Naunyn-Schmiedeberg's Arch. Pharmacol.* 377:523–527.
- Trotter, P. J., S. Y. Ho, and J. Storch. 1996. Fatty acid uptake by Caco-2 human intestinal cells. *J. Lipid Res.* 37:336–346.
- Wang, Q., J. C. Allen, and H. E. Swaisgood. 1997. Binding of vitamin D and cholesterol to beta-lactoglobulin. *J. Dairy Sci.* 80:1054–1059.
- Wang, Q. W. Q., J. C. Allen, and H. E. Swaisgood. 1998. Protein concentration dependence of palmitate binding to beta-lactoglobulin. *J. Dairy Sci.* 81:76–81.
- Wu, S. Y., M. D. Pérez, P. Puyol, and L. Sawyer. 1999. Beta-lactoglobulin binds palmitate within its central cavity. *J. Biol. Chem.* 274:170–174.
- Yang, M. C., H. H. Guan, M. Y. Liu, Y. H. Lin, J. M. Yang, W. L. Chen, C. J. Chen, and S. J. T. Mao. 2008. Crystal structure of a secondary vitamin D₃ binding site of milk beta-lactoglobulin. *Proteins* 71:1197–1210.
- Yee, S. 1997. In vitro permeability across Caco-2 cells (colonic) can predict in vivo (small intestinal) absorption in man-fact or myth. *Pharm. Res.* 14:763–766.