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β-lactoglobulin as a molecular carrier of linoleate: characterisation and effects on intestinal epithelial cells *in vitro*

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ABSTRACT

The dairy protein β-lactoglobulin (βlg) is known to bind hydrophobic ligands such as fatty acids. In the present work, we investigated the biological activity *in vitro* of linoleate once complexed to bovine βlg. Binding of linoleate (C18:2) to bovine βlg was achieved by heating at 60 °C for 30 min at pH 7.4, resulting in a linoleate/βlg molar binding stoichiometry of 1.1, 2.1 and 3.4. Two types of binding sites were determined by ITC titrations. Binding of linoleate induced the formation of covalent dimers and trimers of βlg. The LD_{50} on Caco-2 cells after 24 hours was 58 μM linoleate. However cell viability was unaffected when 200 μM linoleate was presented to the Caco-2 cells as part of the βlg complex. The Caco-2 cells did not increase mRNA transcript levels of long chain fatty acid transport genes, *FATP4* and *FABPpm*, or increase levels of the cAMP signal, in response to the presence of 50 μM linoleate alone or as part of the βlg complex. Therefore, it is proposed that βlg can act as a molecular carrier and alter the bioaccessibility of linoleate/linoleic acid.

Key words: β-lactoglobulin; Sodium Linoleate/Linoleic Acid; Stoichiometry; Caco-2; Cytotoxicity

INTRODUCTION

Linoleic acid (LA, *cis,cis*-9,12-octadecadienoic acid, n-6, 18:2) is an essential long-chain fatty acid (LCFA). World Health Organization (WHO) and Food and Agriculture Organization of the United Nations (FAO) recommend an adequate intake of LA of 2 % of total energy.[1] LA is a precursor to long chain metabolites such as γ-linolenic acid, arachidonic acid and eicosapentaenoic acid.[2,3] Some of these polyunsaturated fatty acids such as LA or γ-linolenic acid have been shown to have anti-inflammatory properties.[4] Replacement of saturated fat with LA is advised to improve serum lipoprotein profiles and reduce the risk of developing cardiovascular coronary artery disease.[5] LA is also cytotoxic at high concentrations to cancerous cells *in vitro*.[6,7] LCFA are taken up by intestinal epithelial cells by both active transport via specific fatty acid (FA) transporters and passive diffusion.[8] However, uptake and bioavailability of fatty acids may be altered depending on the food matrix.[9,10]

Bovine β-lactoglobulin (βlg) is the major whey protein in bovine milk but absent in human milk. It is a globular protein with a monomeric molecular weight of 18.4 kDa, consisting of 162 amino acids.[11] Despite intensive studies on biological, chemical and physical properties of this protein, its biological function still remains unknown.[11-14] Structurally, βlg belongs to the lipocalin family[15], of which most are able to bind small hydrophobic molecules, such as FA, hydrophobic vitamins or curcumin.[11-19] βlg may be involved in transport, through the gastric tract, of hydrophobic substances naturally present in bovine milk, though clear evidence for this is lacking.[13,14] All members of the lipocalin family contain a β-barrel, shaped into a flattened calyx, composed of eight antiparallel β-strands. It has been suggested that βlg binds hydrophobic ligands in its internal calyx.[20,21] The existence of binding sites in a crevice near the α-helix on the external surface of the β-barrel has also been reported.[11,22-26] However, the binding sites and stoichiometry of several ligands have been controversial.[12,21,25]

βlg and LA are derived from food sources, βlg from milk and LA from many edible oils and fats, such as safflower oil, grape seed oil or corn oil and indeed milk fat.[27-29] On a daily basis, people in the Western world consume significant quantities of both βlg and LA. Little information is known on the interaction between the water-soluble form of LA, linoleate, and βlg. Indeed how this interaction impacts on protein structure and on LA biological properties. This study investigated the βlg-linoleate

complex formation in aqueous solution, the binding properties and the effect on protein structure. To investigate the bioavailability of the FA in the formed complexes, compared to FA alone, cytotoxicity was measured on intestinal epithelial cells *in vitro*. The active transport of FA in the cells was studied by two different methods. Intracellular cyclic adenosine 3',5'-monophosphate (cAMP) levels in viable Caco-2 cells were measured in the presence of linoleate alone or within βlg-linoleate complex, as an indication of active FA transport using cAMP signal transduction. Messenger RNA transcript levels of the LCFA transporter genes, Fatty Acid Binding Protein (*FABPpm*) and Fatty Acid Transport Protein 4 (*FATP4*), in the presence of linoleate alone or within βlg-linoleate complex were also measured.

MATERIALS AND METHODS

Materials

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

Isothermal Titration Calorimetry (ITC)

ITC was used to determine the interaction parameters between βlg and linoleate. ITC experiments were performed on a VP-ITC microcalorimeter (Microcal, Northampton MA). Solutions of βlg (0.163 mM) and linoleate (9.64 mM) in phosphate buffered saline (PBS; 0.01 M phosphate buffer, 2.7 mM KCl, 137 mM NaCl, pH 7.4) were degassed under vacuum before titration experiments. Measurements were performed at 60 °C. The reference cell was filled with PBS, and the sample cell (1.425 mL) was filled with βlg solution. βlg was titrated at 60 °C with 29 successive 10 µL injections of linoleate. The injection time was 20 s, and the time between injections was fixed at 600 s to allow thermodynamic equilibrium. During titrations, the solution in the sample cell was stirred at 310 rpm to ensure complete mixing of the solution. The control measurement was obtained by titrating sodium linoleate into the buffer. The first injection peak was ignored for the analysis. Data were analysed using MicroCal ORIGIN version 7.0 provided by the manufacturer: the

integrated area of each peak was plotted versus the linoleate/βlg molar ratio, providing binding constants.

Preparation of βlg-linoleate complexes

βlg-linoleate complex were prepared by heating a solution of βlg and sodium linoleate according to Lišková et *al.* (2011)[30] with the following modifications. Briefly, 0.163 mM βlg was dissolved in Phosphate Buffer Saline (pH 7.4) and sodium linoleate was added to reach final linoleate/βlg molar ratios of 5, 7.5 and 10. Solutions were heated for 30 min at 60 °C, then immediately cooled on ice. Samples were extensively dialysed against distilled water prior to freeze-drying. A control of sodium linoleate was dialysed using the same conditions. No FA was detectable by gas chromatography in the control.

Determination of the FA content by gas chromatography (GC)

The FA content of the complexes was determined by GC following a protocol adapted from Palmquist and Jenkins (2003)[31]. Briefly, the internal standard tridecanoic acid (C13:0) was added to ~4 mg of complexes. FA were converted to fatty acid methyl esters (FAME) by the addition of 1.5 mL 10 % methanolic HCl and 1 mL hexane. The samples were vortexed and heated to 90 °C for 2 h. After cooling on ice, 1 mL hexane and 3 mL 10 % K_2CO_3 were added and samples were vortexed. After phase separation, the heptane phase (upper phase) containing the FAME were analysed as previously described by Coakley et *al.* (2003)[32], using a CP-SELECT CB column for FAME (100 m, 0.25 mm, 0,25 µm film thickness, Varian BV, Middelburg, the Netherlands), a Varian 3400 GLC (Varian, Walnut Creek, CA) and a flame ionization detector .

Gel Permeation HPLC

The concentration of monomers and aggregates were determined by gel-permeation-HPLC (GP-HPLC) using a TSK G SW guard column (7.5×7.5 mm, Tosoh Bioscience GmbH, Stuttgart, Germany) and a TSK G2000 SW column (7.5×600 mm, Tosoh Bioscience GmbH) connected to an HPLC system, consisting of a Waters 2695 Separations Module, a Waters 2487 Dual λ Absorbance Detector and an Empower Pro software (Waters, Milford, MA) to acquire and analyse data. 0.05 mg of protein was injected using a solution of 30 % acetonitrile (LabScan Analytical

Sciences, Dublin, Ireland) (v/v) and 0.1 % (w/v) trifluoracetic acid in Milli-Q® water (Millipore, Carrigtwohill, Ireland) as an eluent, at a flow rate of 0.5 mL/min. The use of acetonitrile ensured that native βlg was eluted in monomeric form. The method was calibrated using a set of protein molecular-weight standards (Sigma-Aldrich).

The proportions of monomers (including native and unfolded) of βlg were deduced from GP-HPLC data by integration of the peaks area. The proportion of βlg oligomers in samples was calculated by subtraction of the concentration of monomer from the initial protein concentration, determined by HPLC.

Polyacrylamide gel electrophoresis

Samples were analysed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) in order to determine the nature of the oligomers interaction. Mini-PROTEAN TGX precast Gels (4-20 % resolving gel, Bio-Rad Laboratories Inc., Hercules, CA) were used on a Mini Protean II system (Bio-Rad) according to the manufacturer's instructions. Samples were prepared under reducing (with β-mercaptoethanol) and non-reducing conditions. Protein was visualized by staining with Coomassie blue (Bio-Safe Coomassie Stain G-250, Bio-Rad). An Amersham Low Molecular Weight Calibration kit (14.4 to 97 kg/mol, GE Healthcare UK Limited, UK) was used as molecular weight standards.

Cell Culture

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. When fully differentiated, Caco-2 cells can mimic the enterocytes of the intestine.

Cells cultures were maintained in a humidified 37 °C incubator with a 5 % CO_2 in air atmosphere. Cells were routinely grown in 75 cm^2 plastic flasks in Dulbecco's modified Eagle medium (DMEM) containing 4.5 g/L glucose and 0.584 g/L L-glutamine. Media for subculture was supplemented with 10 % (v/v) foetal bovine serum (FBS), 100 U/mL penicillin and 100 mg/mL streptomycin. At 80 % confluency, cells were trypsinated with 0.25 % trypsin/EDTA, diluted 1:6 in media and reseeded. Media was changed three times a week. All cells used in these studies were between passage number 25 and 40.

Cytotoxicity assay

Cytotoxicity of test samples on Caco-2 cell proliferation was determined by MTS assay, using CellTiter 96 Aqueous One Solution Cell Proliferation Assay according to the manufacturer's intructions (Promega Corporation, Madison, Wisconsin). Briefly, Caco-2 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 penicillin and 100 mg/mL streptomycin). After 24 h, cells were treated with different concentrations of linoleate (0 to 150 μM), βlg (0 to 150 μM) or linoleate:βlg complexes (complex with a molar ratio of 1, 2 and 3, which contained 0 to 150 μM of linoleate) in serum-free media for 24 h. The One Solution Cell Proliferation reagent (20 μL) was then added to each well for a further 3 h. Viability was defined as the ratio of absorbance of treated cells to untreated cells (cells exposed to serum-free Media only) at 490 nm. Each cell exposure was repeated by six and intra-plate variation was accounted for by repeating the exposures on 3 different days (n=18). The Lethal Dose 50 (LD_{50}) values were 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 LD_{50}.

Real-Time Cell Analyzer (RTCA)

Cell growth was monitored in real time using the Real Time Cell Analyzer (RTCA) SP Instrument, the xCELLigence system (Roche Diagnostic Limited, West Sussex, UK). The RTCA system measures the impedance of the bottom of the well which is a function of cell number and cell morphology. As the cell numbers increase the impedance increases. Correspondingly if the cell morphology changes (cells swell or shrink), the impedance will also be affected. The RTCA software generates a cell index value based on the level of impedance. Caco-2 cells in serum-free media were seeded in 16 E-Plates (Roche Diagnostic Limited) at a cell density of 1×10^4 cells/well. After 24 h in a humidified 37 °C incubator with a 5 % CO_2 in air atmosphere, cells were treated for 48 h with different concentration of linoleate (0 to 100 μM), βlg (0 to 100 μM) or linoleate-βlg complexes (molar ratio of 3 linoleate/βlg containing 0 to 100 μM linoleate) in serum-free media. Data were analysed using the RTCA software 1.2 (Roche Diagnostic): Cell Index was plotted versus time and Effective Concentration 50 (EC_{50}) was determined by regression analysis of the cell index data versus the

concentration of the compound after 48 h. Using the software, the time dependent EC_{50} curves were determined by calculating the EC_{50} values at 20 time points within 48 h and these EC_{50} values were plotted versus time.

Cyclic AMP Assay

A cAMP assay based on homogeneous time-resolved fluorescence, the cAMP HiRange kit (Cisbio Bioassays, Codolet, France), was performed according to manufacturer's instructions. Caco-2 cells were seeded into 96-well-half-area plates (Cruinn Diagnostics, Ireland) at a density of 1×10^5 cells/well in serum-free media. After an overnight incubation in a humidified 37 °C incubator with 5 % CO_2 in air atmosphere, media was aspirated and 25 µL serum-free media/IBMX (3-Isobutyl-1-methylxanthine) was then added to all wells and the plate was pre-incubated for 30 min at 37 °C, 5 % CO_2. Cells were incubated with 25 µL of linoleate (0 to 50 µM), βlg (0 to 50 µM) or linoleate-βlg complexes (molar ratio of 3 linoleate/βlg containing 0 to 50 µM linoleate) at room temperature for 30 min with gentle shaking. Forskolin (25 µL of 1 µM) was used as a positive control. Accumulation of the intracellular cAMP was measured using the cAMP HiRange kit following the manufacturer's instruction. Fluorescence was read using a FLUOstar Omega multi-mode microplate reader and analysed with the FLUOstar Omega software (BMG LABTECH GmbH, Ortenberg, Germany). Intracellular levels of cAMP (in the nM range) were determined by interpolating fluorescence readings from a cAMP standard curve generated in the same assay.

Messenger RNA levels of FABPpm and FATP4

For quantifying *FABPpm* and *FATP4* mRNA levels, real time PCR (RT-PCR) was performed in a LightCycler 480 instrument (Roche Diagnostic Limited) based on the principles of absolute quantification.

Caco-2 cells in serum-free media were seeded in 6-well plates at a density of 5.7×10^5 cells/well overnight in a humidified 37 °C incubator with 5 % CO_2 in air atmosphere. Cells were then exposed to linoleate (0 to 50 µM) or linoleate-βlg complexes (molar ratio of 3 linoleate/βlg containing 0 to 50 µM linoleate) for 4 h in serum-free media. A control was done with βlg (0 to 50 µM). Supernatant was removed and total RNA was extracted from the cells using the QIAGEN miRNeasy

Mini kit (QIAGEN Limited, West Sussex, UK). Quality and quantity of total RNA was measured by glyoxyl gel electrophoresis and spectrophotometrically using the NanoDrop 1000 (Thermo Fisher Scientific, Wilmington NC). First strand cDNA was generated from 1 µg total RNA using the Bioline cDNA synthesis kit (Bioline, London, UK).

All primers were designed across intron/exon boundaries and synthesised by Eurofins MWG Operon (Ebersberg, Germany). Primers for human *FABPpm* were designed using DNAstar Lasergene 8 software (DNAstar, Madison WI, USA) and based on the GenBank sequence (accession number NM002080). *FABPpm* forward primer sequence was 5'-CCGGAACAGTGGAAGGAAATAGC-3' and the reverse primer sequence was 5'-TTGAGGGGAGGGTTGGAATACAT-3'. The annealing temperature for amplification was 57 °C. The forward primer sequence used for human *FATP4* was 5'-CAGGGCGCCAACAACAAGAAGATT-3' and the reverse primer sequence was 5'-GCAAAGCGCTCCAGGTCACAGT-3', both designed from the accession number NM002080. The annealing temperature for amplification was 58 °C.

Plasmid standards for *FABPpm* and *FATP4* were created by cloning an amplified PCR product into the pCR4-TOPO vector using the TOPO-TA cloning system (Invitrogen, Life Technologies, Carlsbad CA) according to the manufacturers' instructions. The cloned amplicon was (a) identified by PCR amplification, using gene specific primer pairs, and/or digestion of plasmid DNA using the *Eco*RI restriction enzyme and (b) confirmed by sequencing (Beckman Coulter Genomics, Essex, UK). For RT-PCR standards, plasmid DNA was linearized and quantified using the Nanodrop 1000. Standard curve preparation involved creating a series of dilutions from 10^9 to 10^2 copies/µL.

For each 10 µl Lightcycler reaction, 1 µL of test cDNA or serially-diluted standard was used. The LightCycler 480 SYBR Green I Master kit (Roche Diagnostics Limited) was used for quantification according to the manufacturer's instructions using 0.5 µM of both the forward and reverse primers. All cDNA samples were tested in duplicates. Data were analysed using the LightCycler 480 Software (Roche Diagnostic Limited).

Statistical analysis

Results were compared using Minitab 15 statistical Software (Minitab Limited, Coventry, UK) and the ANOVA system with a Fisher's least significant difference comparison. Experiments were performed at least in triplicate.

RESULTS

Linoleate/βlg complexes were produced at 60 °C. The heating condition was below the denaturation temperature of βlg (~70 °C at neutral pH), in a temperature range where βlg is in the R-state.[33,34] βlg R-state is characterized by small changes in βlg tertiary structure, a slight expansion in its volume and an increase in accessible surface area compared to native βlg.[35] The salt of LA was used because it is fully soluble in water up to its critical micelle concentration (CMC: 2 mM[36]). Using the water-soluble form of LA permits direct contact between βlg and linoleate allowing complex formation whilst avoiding the use of ethanolic solutions to solubilise the FA.[37]

Interaction between linoleate and βlg

The thermodynamic parameters of linoleate binding to βlg were investigated by ITC. The changes in the enthalpy during the binding of βlg titrated with a solution of sodium linoleate was investigated at pH 7.4 and at 60 °C. The heat exchange from the interaction of βlg with linoleate is shown in Figure 1. Each peak represents the heat exchange within the system after an injection and indicates that linoleate-binding to βlg is an exothermic process. Figure 1B depicts peak integration corrected by control (titration of sodium linoleate in buffer). The energy released during the titration decreased as the molar ratio increased. The curve levelled off at a linoleate/βlg ratio of ~3. The saturation point of βlg by linoleate was above a molar ratio of 13. For linoleate/βlg molar ratios of 3 to 13, the released heat did not plateau. Instead the heat decreased by 1.5 kcal/mol of linoleate. The binding titration curve was best fitted according to a "two set of binding sites model", which yielded the thermodynamic constants for the two sites: $k_{a1}= (2.70\pm2.03)\times10^5$ M^{-1}, $n_1=0.62\pm0.004$; $k_{a2}=(5.91\pm3.85)\times10^3$ M^{-1}, $n_2=5.75\pm0.51$, where k_a is the association constant and n the stoichiometry. The return to thermodynamic equilibrium was very slow (>1500 s). A

longer equilibrium time did not affect the results (data not shown). The slow return to thermodynamic equilibrium could be due to the occurrence of other structural events, i.e. formation of oligomers (see below). No heat change was observed during direct injection of sodium linoleate in buffer solution (control sample).

βlg-linoleate complex formation: stoichiometry and oligomerisation of the protein

βlg-linoleate complexes were prepared by heating 0.16 mM βlg at 60 °C for 30 min at pH 7.4 in the presence of 5, 7.5 and 10 molar equivalent of linoleate. To remove excess of unbound FA, samples were extensively dialysed prior to freeze-drying.

Final stoichiometry of linoleate/βlg was determined from freeze-dried complexes using GC. A comparison of molar ratios of linoleate/βlg before the reaction, after dialysis and freeze-drying is shown in Figure 2A. The amount of linoleate bound to βlg increased by increasing the initial ratio of linoleate/βlg. For an initial linoleate/βlg molar ratio of 5, 7.5 and 10, the amount of linoleate bound to one protein after dialysis was 1.05±0.10, 2.14±0.06 and 3.35±0.47 moles, respectively. Consequently, complexes with a linoleate/βlg molar ratio of 1, 2 and 3 were formed.

As shown by GP-HPLC analysis (Figure 2B and 2C), the presence of linoleate induced oligomerisation of the protein. The amount of oligomers increased significantly with the molar ratios of linoleate/βlg. In the absence of linoleate (control), the amount of oligomers in βlg samples was 12 %. In the presence of linoleate (3 linoleate/βlg), the amount of oligomers reached up to 45 % of total protein concentration. The oligomers were mainly dimers and trimers of βlg as shown in Figure 2C and confirmed by SDS-PAGE experiment (see below). However, no significant change in the protein secondary structures was associated with oligomer formation, as indicated by FTIR (data not shown).

These results were confirmed by SDS-PAGE. Under non-reducing conditions, the SDS-PAGE analysis of native βlg and heated βlg (at 60 °C) (Figure 2C) showed a major band corresponding to the βlg monomer with small amount of dimers and trimers. In the presence of linoleate, the intensity of the bands corresponding to βlg dimers and trimers intensified. Under reducing conditions, no difference was observed between native βlg, heated βlg and linoleate/βlg complexes with a molar ratio of 1, 2 and 3 linoleate/βlg (Figure 2C). This indicated that dimers and trimers were covalently bound by disulfide links.

Cytotoxicity on Caco-2 cells

To elucidate the effect of βlg:linoleate on human epithelial cell viability, the human colonic adenocarcinoma cells, Caco-2, were exposed to βlg, linoleate or complexes for 24 h (Figure 3A). LD_{50}, the concentration required to decrease the cell viability by 50 %, was then calculated. βlg was not toxic to Caco-2 cells at the concentrations tested (0 to 150 μM), as measured by MTS assay. In contrast, the LD_{50} of linoleate was 58.04±4.21 μM. Linoleate:βlg complexes, where corresponding linoleate concentration varied from 0 to 150 μM, had no cytotoxic effect on Caco-2 cells after 24 h incubation.

Toxic effects of βlg, linoleate or complexes in real time over 48 h were studied using RTCA (Figure 3B). The cell index, which is function of the impedance at the bottom of the well, was measured. Cell index relates to cell viability and/or cell morphology. Results showed a decrease of cell index by 1.95when the concentration of βlg increased from 0 to 100 μM, compared to the control cells without compound. However the parallel MTS assay showed no change in cell viability (Figure 3A). Taken together, this allowed the authors to conclude that βlg alters cell morphology rather than cell viability. Interestingly, at the low concentrations of 5, 10 and 25 μM linoleate, an increase of cell index was observed for linoleate alone compared to control (Figure 3B-linoleate). At concentrations of 50, 75 and 100 μM, linoleate cell index decreased by 2.54 (for 100 μM linoleate) compared to control. However, the response of Caco-2 cells to βlg-linoleate complexes differed to linoleate alone at equivalent molar concentrations. A decrease in cell index was observed after 3 h of exposure to 100 μM of linoleate whereas the 3 linoleate/βlg complex (100 μM linoleate) required 12.5 h exposure to decreased the cell index (cf. arrows on Figures 3B-linoleate and 3B-cplx). βlg-linoleate complexes containing 5, 10, 25 and 50 μM linoleate increased Caco-2 cell index after 48 h. βlg-linoleate complexes containing 100 μM of linoleate in the complex decreased the cell index by 0.49 after 48 h. EC_{50}, the concentration required to obtain 50 % of the maximum effect, was calculated at different time points. After 48 h, EC_{50} was 35 μM for linoleate alone and 98 μM when linoleate was part of a 3 linoleate/βlg complex. A time dependent EC_{50} was calculated to indicate EC_{50} changes as a function of time (Figure 3C). At 13 h, the EC_{50} was reached with 74 μM linoleate alone. In contrast, it took 30 h for the EC_{50} to reach 78 μM linoleate when 3 linoleate was complexed to βlg.

Cellular Response to βlg, linoleate and βlg-linoleate complexes

Cyclic AMP is a ubiquitous intercellular/intracellular messenger which may be involved in active FA uptake by cells.[38] Cyclic AMP levels were measured in Caco-2 cells exposed to linoleate, βlg, or complexes at non-toxic concentrations (50 μM linoleate). No changes were detected in cAMP levels by incubation with linoleate (0 to 50 μM), βlg (0 to 50 μM) or linoleate-βlg complexes (0 to 50 μM linoleate) as measured by a FRET-based time-resolved fluorescence assay. Messenger RNA transcript levels of the FA transporter genes, *FATP4* and *FABPpm*, in Caco-2 cells were investigated as an indication of active transport of linoleate across the cell membrane. Messenger RNA levels of *FATP4* and *FABPpm* were not significantly increased in Caco-2 cells after 4 h incubation with linoleate (0 to 50 μM) or linoleate-βlg complexes with a concentration of 0 to 50 μM linoleate (Supplementary Figure 1).

DISCUSSION

βlg and linoleate formed complexes that protected Caco-2 cells from the cytotoxic effects of linoleate (Figure 3). Intracellular cAMP levels (Figure 4), mRNA *FATP4* and *FABPpm* levels were unaffected by the presence of linoleate either alone or in a protein complex. SDS-PAGE and HPLC analysis of the complexes revealed the formation of intermolecular disulfide bonds between protein molecules, which increased with higher molar ratios of linoleate/βlg (Figures 2B and 2C). These observations, combined with those from ITC (Figure 1), allowed us to suggest a binding mechanism between βlg and linoleate which modifies the cytotoxic effect of the FA.

Linoleate interacted with βlg via two different binding sites with respective affinity constants of 2.7×10^5 and 5.9×10^3 M^{-1}. These association constants are similar to those reported for other hydrophobic ligands.[39,40] Spector and Fletcher (1970)[40] reported two binding sites with association constant in the order of 10^5 M^{-1} and 10^3 M^{-1} for the binding of βlg to palmitate, oleate, stearate and laurate. Concomitantly to linoleate binding, we also observed the formation of covalent protein oligomers, i.e. dimers and trimers, that could explain the stoichiometry value (n = 0.62) determined

from ITC experiments. This value could result from a mixture of complexes such as $(\beta lg)_2$-$(linoleate)_1$ (n = 0.5) and $(\beta lg)_3$-$(linoleate)_2$ (n = 0.67). This hypothesis is consistent with the simple shape of the ITC peaks, with slow return to equilibrium attributed to the induced oligomerisation step. Recently, a crystallographic structure of native βlg/linoleate complex, showing the fatty acid located at the protein calyx (stoichiometry = 1) was published.[41] Our results suggest that heating of βlg and linoleate mixture may lead to the formation of other types of complexes.

βlg has a weak aptitude to aggregation below the temperature of denaturation.[42] At 60 °C, the presence of linoleate increased the formation of disulfide-linked dimers and trimers without formation of larger aggregates. This work confirms previous work in our laboratory[30] where binding of sodium oleate to βlg at 60 °C decreased monomeric βlg and increased the formation of dimers and trimers. Protein aggregation in the presence of lipids has also been reported for other protein systems.[43] βlg oligomerisation into covalent dimers and trimers may be triggered by slight structural changes induced by linoleate binding to βlg monomers, as suggested by previous studies.[44,45]

From the presented results, we propose a hypothetic binding mechanism where the interaction of the FA with the protein and the oligomerisation of βlg take place in a single step:

- At 60 °C, native βlg monomers reversibly unfold to form non-native R-state monomers.[33]
- Negatively charged linoleate molecules interact with positively charged regions at the surface of βlg monomers, as suggested from the exothermic signal of ITC experiments. Consequently, the formation of a linoleate: βlg complex would favour additional hydrophobic interactions between proteins.
- Two or three βlg molecules were then non covalently “cross-linked” by one or two linoleate molecules bound to a high affinity binding site this favours the formation of βlg oligomers by intermolecular disulfide bonds, making the unfolding irreversible after cooling.

This assumption is in agreement with the single peak structure showing a slow return to thermodynamic equilibrium of ITC results. It probably indicates that several physico-chemical phenomena can contribute simultaneously to the measured signal including: (i) binding of linoleate to βlg, (ii) conformational changes of βlg following

444 binding of linoleate molecules, (iii) oligomerisation of the protein βlg and (iv)
445 counterion release.
446 The protection provided by βlg:linoleate complexes to Caco-2 cells from
447 linoleate differs to βlg:Conjugated Linoleic Acid (CLA) complex. Although the
448 different CLA isomers display varying effects on biological functions,[46,47] a 2.46
449 c9,t11-CLA/βlg molar ratio complex resulted in a 30 % increase in cytotoxicity after
450 48 h of exposure to c9,t11-CLA at a concentration of 100 μM, compared to c9,t11-
451 CLA alone.[17] HAMLET/BAMLET (Human/Bovine Alpha-lactalbumin Made LEthal
452 to Tumor cells), a complex formed of oleic acid (OA) and α-lactalbumin (αla), is
453 more cytotoxic than OA on its own.[48] OA and LA by themselves exhibit cytotoxic
454 effects on various cell lines.[48,49] However, binding these FA to proteins such as βlg,
455 modify their cytotoxic effect compared to the FA on its own. Indeed OA/αla complex
456 is ~40 % more cytotoxic to human larynx carcinoma cells compared to free OA.[48]
457 However, recent studies based on direct measurement of OA content in the incubation
458 mixture would argue that OA alone or involved in a complex have comparable
459 cytotoxicity effects on various cells, with the protein alone having no effect.[30,50]
460 Frapin et *al*. (1993)[39] showed that the structural constraints imposed by the double
461 bonds of FA only weakly affects the interaction of FA with βlg. The reduced
462 cytotoxic effect observed with linoleate/βlg complexes may relate to the solubility of
463 the FA. OA has a poor solubility in aqueous solution, its CMC is between 20 and 69
464 μM at pH 8.3 at the temperature and salt concentration tested by Knyazeva et *al.*
465 (2008).[48] Therefore in the absence of protein, the amount of OA available to the cells
466 would be low. The binding of OA to βlg or other proteins such as αla (HAMLET,
467 BAMLET) increased the solubility of OA (Joseph J. Kehoe, personal communication)
468 and possibly its bioavailability. The solubility of FA increases with the number of
469 C=C double bonds in the aliphatic chain.[51] Consequently, the solubility of LA (C18:2)
470 is higher than that of OA (C18:1). Under the experimental conditions used by Collin
471 et *al*. (2010)[36], sodium linoleate has a CMC of 2 mM. Hence, the binding of linoleate
472 to βlg is unlikely to alter solubility but potentially alter linoleate uptake by altering
473 levels of free FA.
474 The cytotoxicity of linoleate was concentration dependent, in agreement with
475 that observed with LA.[7] Norman et *al*. (1988)[52] showed that sodium linoleate was
476 more cytotoxic to the epithelial mouse cells, Ehrlich Ascites Tumor, than emulisified
477 LA. This effect may be explained by the higher solubility of sodium linoleate in

aqueous bioassays with greater access to the Caco-2 cells. Prior to uptake, LCFA enters a low pH microclimate at the enterocyte surface. As this local pH is below their pK_a, protonation of LCFA will occur with LCFA entering in the FA form rather than the salt form.[53] To date, it is not well understood how intestinal cells metabolize LA. A previous study showed that FA cytotoxic effect was initiated by mitochondrial apoptotic pathway with cytochrome C release, indicating that uptake of LA is essential for its cytotoxic effect.[6] FA cytotoxicity may also occur by an alteration of the cellular n-6 to n-3 polyunsaturated FA ratio adversely affecting membrane permeability and fluidity.[8]

LCFA are hydrophobic and so uptake by enterocytes was thought to occur by diffusion. However, recent studies suggest the involvement of a protein-transfer mechanisms, with transport of LCFA reaching saturation at high concentrations in Caco-2 cells.[8,38,54] It is likely that an efficient LCFA uptake by cells requires both passive and facilitated transfer, possibly using a cAMP pathway.[38] However, in our study, no change in intracellullar cAMP levels was observed by viable intestinal cells exposed to different concentrations of linoleate and βlg-linoleate complexes (0 to 50 μM linoleate). Nevertheless, the requirement of cAMP in a facilitated LCFA uptake or metabolism is controversial and appears to depend on the FA and cell type used. Bovine oocytes treated with 100 μM LA for 6 or 24 h decreased intracellular cAMP levels.[55] A perfusion of 1 mmol/L plasma of eicosapentaenoic acid (EPA; C20:5 n–3) during 150 min decreased cAMP level by 0.27 nmol/g tumor on MCF-7 human breast cancer xenografts perfused in situ in nude rats.[56] In contrast, C6 glioma cells incubated with 100 μM EPA for 48h, increased cAMP levels by ~250 %.[57] The FA transporters, FABPpm and FAPT4 have been involved in the uptake of LCFA by intestinal cells.[54,58] Messenger RNA transcript levels of *FATP4* and *FABPpm* were not increased upon exposure to linoleate which suggests that either (a) there is sufficient quantities of FATP4 and FABPpm transporter proteins to transport linoleate or (b) these transporters are not involved in linoleate transport, at the concentrations tested.[39]

This study has demonstrated that βlg can bind at least three linoleate per βlg monomer at two different sets of binding sites. According to cell proliferation assays, linoleate can inhibit the viability of Caco-2 cells, but βlg-linoleate complexes appear to protect cells from the cytotoxicity effect of linoleate. This effect could be due to the relatively high solubility of linoleate. Caco-2 exposure to linoleate or βlg-linoleate

complexes did not modify intracellular cAMP levels or mRNA transcript levels of the LCFA transporter genes, *FABPpm* and *FAPT4*.

ABBREVIATIONS USED

αla, α-lactalbumin; βlg, β-lactoglobulin; cAMP, cyclic adenosine 3',5'-monophosphate; CLA, conjugated linoleic acid; CMC, critical micelle concentration; DMEM, Dulbecco's modified Eagle medium; EC_{50}, effective concentration 50; FA, fatty acids; FABPpm, fatty acid binding protein; FAME, fatty acid methyl ester; FATP4, fatty acid transport protein 4; FBS, foetal bovine serum; GC, gas chromatography; GP-HPLC, gel permeation high performance liquid chromatography; HAMLET/BAMLET, human/bovine α-lactalbumin made lethal to tumor cells; ITC, isothermal titration calorimetry; k_a, association constant; LA, linoleic acid; LCFA, long chain fatty acid; LD_{50}, lethal dose 50; n, reaction stoichiometry; OA, oleic acid; RTCA, real time cell analyzer; RT-PCR, real time polymerase chain reaction.

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SUPPORTING INFORMATION AVAILABLE

Supporting Information Available: Supplementary Figure 1, levels of *FABPpm* and *FATP4* mRNA transcripts in Caco-2 cells after 4h exposure to linoleate. This material is available free of charge via the Internet at http://pubs.acs.org.

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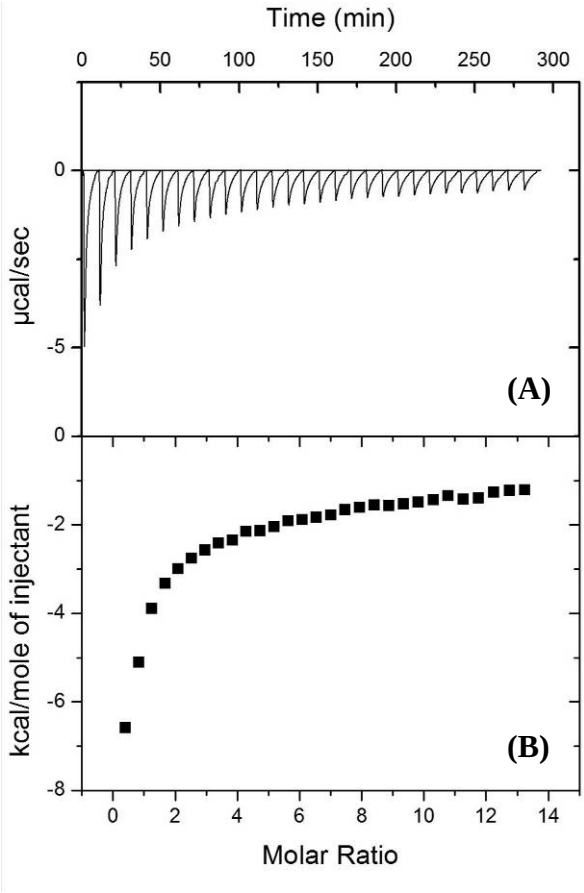
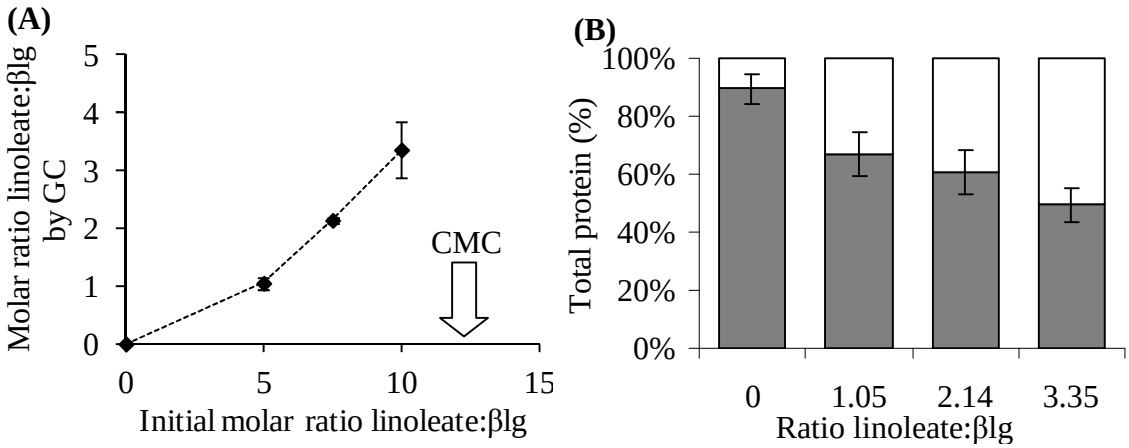


Figure 1: Microcalorimetric titration of βlg with linoleate in PBS buffer (pH 7.4) at 60 °C. (A) raw heat signal for the titration of βlg (0.16 mM) with 10 µL increments of 9.64 mM linoleate. (B) area under each peak integrated and plotted against the linoleate/βlg molar ratio.



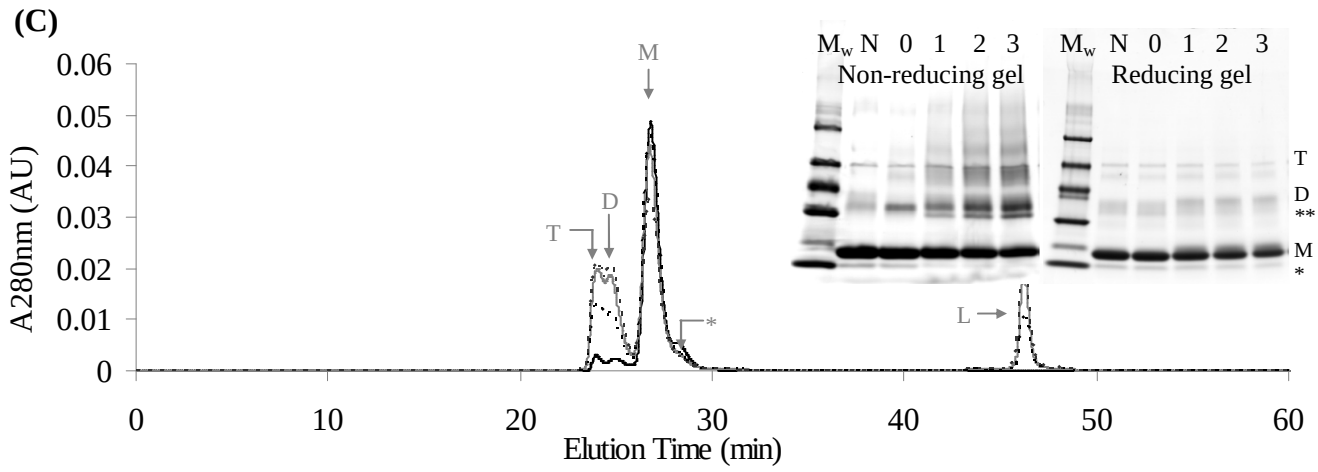


Figure 2: βlg-linoleate complexes formations: 0.16 mM β-lactoglobulin in the absence or presence of linoleate were heat treated at 60 °C for 30 min, extensively dialysed and freeze dryed. (A) Correlation of the molar ratios of linoleate/βlg added to the starting solutions with the molar ratios of linoleate/βlg that were detected by GC analysis in the βlg/linoleate samples after extensive dialysis and freeze-drying. The arrow represent the CMC of the sodium linoleate.[36] Complexes with a linoleate/βlg molar ratio of 1.05±0.10, 2.14±0.06 and 3.35±0.47 moles were formed, referred as complexes with a linoleate/βlg molar ratio of 1, 2 and 3. (B) Protein composition observed by GP-HPLC (shown if Figure 2C). The molar ratio of linoleate/βlg in dialysed solutions is indicated on the x-axis, the total protein content on the y-axis as follows: grey area, monomers; white area, oligomers. (C) Composition of solutions of βlg in presence or absence of linoleate observed by GP-HPLC chromatograms (30 % acetonitrile and 0.1 % TFA). Black line, βlg heated without sLA; dotted black line, complex of 1 linoleate/βlg molar ratio; Light grey line, complex of 2 linoleate/βlg molar ratio; dotted grey line, complex of 3 linoleate/βlg molar ratio. The inserts show SDS-PAGE profile of βlg-linoleate complexes under non-reducing (left gel) and reducing (right gel) conditions. M_w, molecular weight markers (14.4, 20.1, 30, 45, 66, 97 kDa); N, native βlg; 0, βlg control treated to complex formation conditions; lane 1, 2 and 3, complex with a final molar ratio of 1, 2 and 3 linoleate/βlg, respectively. T, βlg trimer; D, βlg dimer; M, βlg monomer; *, αla monomer; L, linoleate. A band with slightly lower molecular weight than βlg dimers was observed in the presence of linoleate under non reducing conditions (**). This maybe due to the formation of heterodimers of βlg and αla, as the electrophoretic band corresponding to αla monomers decreased in presence of linoleate.

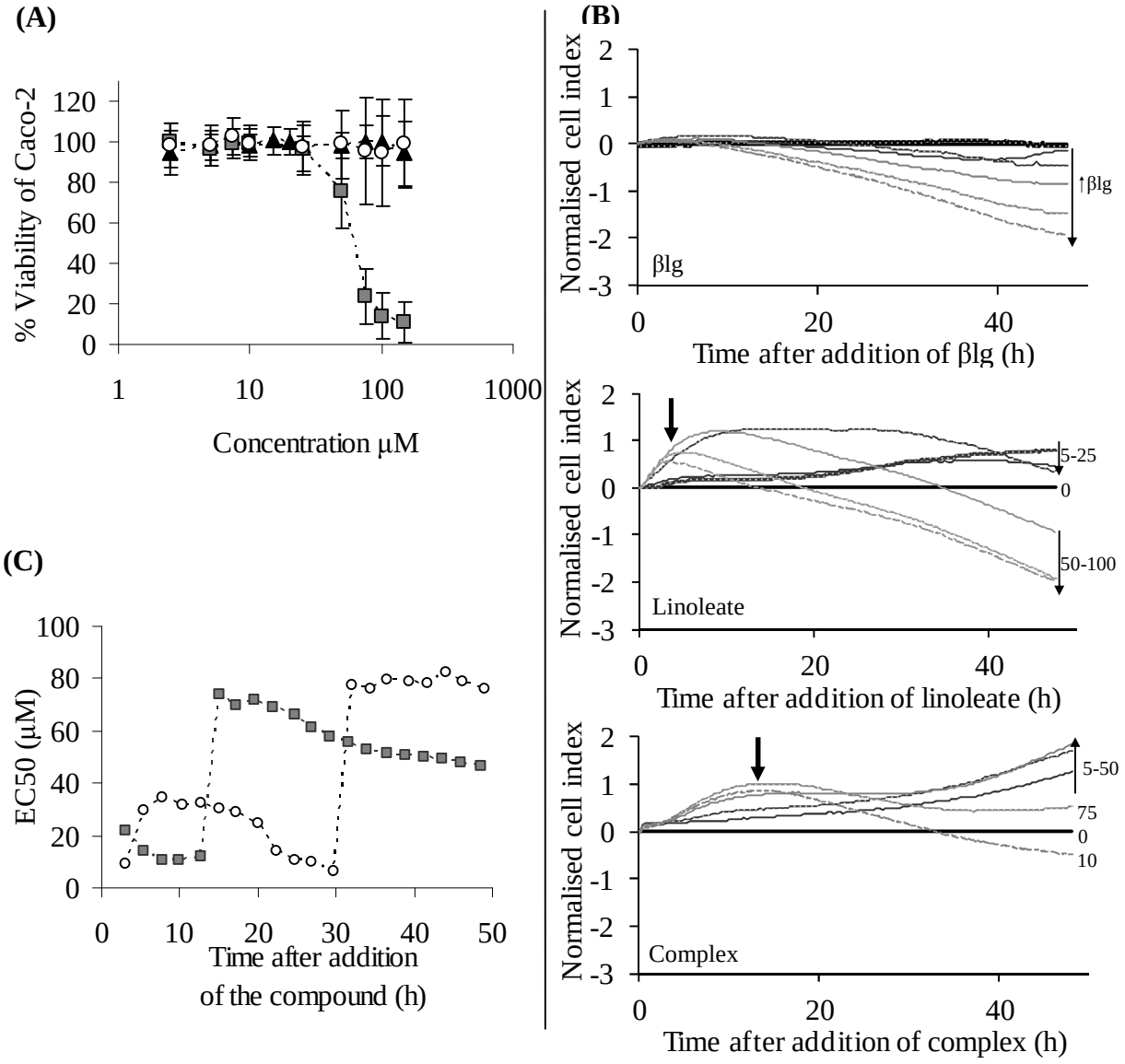


Figure 3: Cytotoxicity of βlg, linoleate and βlg-linoleate complexes on Caco-2 cells. Cytotoxicity activity was assessed using (A) MTS assay and (B, C) RTCA system. (A) % viability after 24 h on 2×10^4 Caco-2 cells compared to control cells. βlg concentration – ▲ –, linoleate concentration – □ – and linoleate concentration in the βlg-linoleate complexes– ○ – are given on the x-axis (0 to 150 µM). (B) Normalised cell index (difference between the cell index and the cell index without compound) over time in hours, 1×10^4 Caco-2 cells were exposed to 0 µM ——, 5 µM ~~~, 10 µM ——, 25 µM - - - -, 50 µM ——, 75 µM - - - -, 100 µM – – of βlg (B-βlg), linoleate (B-linoleate) and linoleate in the 3 linoleate-βlg complex (B-complex). The large arrows on B-linoleate and B-complex indicate the start of the normalised cell index decrease for the highest concentration. (C) Time dependence EC_{50} is based on the Figure B,

EC_{50} is calculated over a 48 h exposure to linoleate –□– or 3 linoleate-βlg complex–○–.

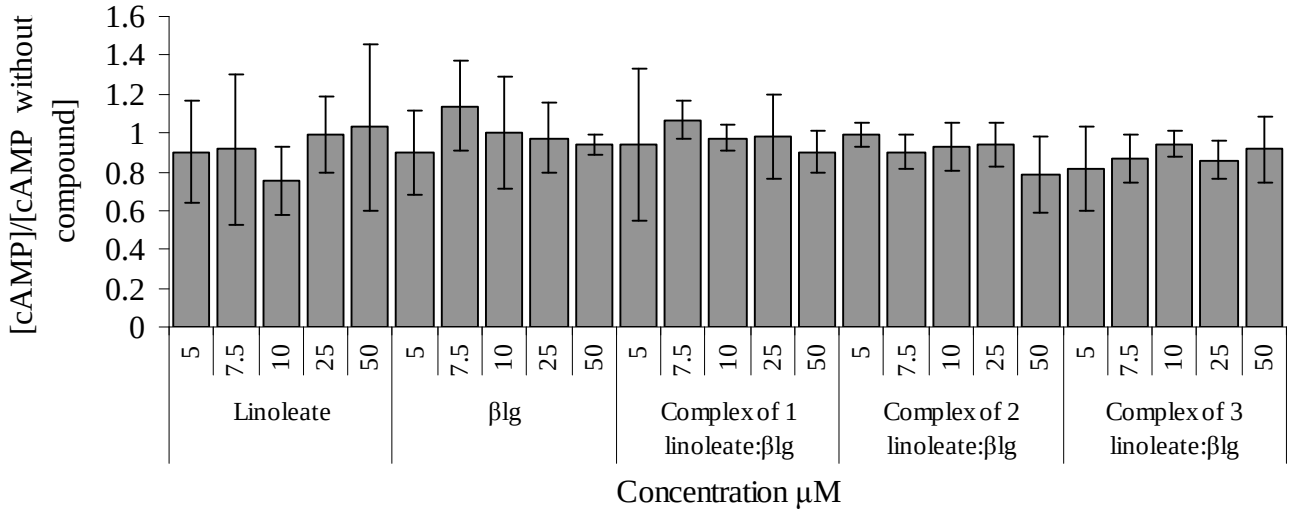


Figure 4: Relative intracellular cAMP levels in 1×10^5 Caco-2 cells treated with different concentrations of linoleate, βlg and βlg-linoleate complexes. cAMP without compound is defined as cAMP levels in Caco-2 cultured in medium without compound. No significant difference between samples and concentrations were found.

TABLE OF CONTENT GRAPHIC