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Carotenoid exposure of Caco-2 intestinal epithelial cells did not affect selected inflammatory markers but altered their proteomic response

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Abstract

Carotenoid consumption has been linked to a number of beneficial health effects, including the reduction of chronic diseases such as cancer and cardiovascular complications. However, no data are available on their action on the intestinal epithelium, being exposed to the highest concentrations of carotenoids in the human body, and where they could act preventively on intestinal inflammatory diseases such as Crohn's disease and ulcerative colitis. The objective of the present study was to investigate whether lycopene and β-carotene in micelles (M), at concentrations that could be reached via the diet (10–25 μg/ml) could aid in the reduction of TNF-α plus IL-1β-induced inflammation of Caco-2 human epithelial cells. The impact on biomarkers of inflammation, including IL-8, NO and cyclo-oxygenase-2 (through PGE-2α), and the NF-κB and mitogen-activated protein kinase (MAPK) pathways of intracellular signalling cascades were evaluated compared with controls (empty M). Furthermore, proteomic analyses were conducted from total cellular protein extracts. The results revealed that isolated carotenoids had no statistical significant anti-inflammatory effect on the biomarkers observed, or on the regulation of NF-κB and MAPK. Nevertheless, analyses of the proteome suggested that fifteen proteins were significantly ($P<0{\cdot}05$, expression ratio $>1{\cdot}3$) differentially regulated following β-carotene exposure, participating mostly in metabolic activities including antioxidant mechanisms, such as glutathione *S*-transferase A1. Only one protein was differentially regulated by lycopene (profilin-1). To our knowledge, this is the first attempt to investigate pathways involved in the action of carotenoids on the intestinal epithelium.

Key words: Carotenoids: Inflammation: Intestinal epithelium: Caco-2 cells: Proteomics

Carotenoids are lipid-soluble, C-40-based pigments that can be synthesised by vegetables and fruits and certain fungi, but not by animals including humans. Of the approximately 700 identified carotenoids, only about fifty are of importance in the human diet[1]. In addition to the well-known pro-vitamin A activity of several carotenoids, it has been suggested that carotenoids have further beneficial effects, including the prevention of cancer[2,3], CVD[4,5] and diabetes[6,7]. Carotenoids can act as antioxidants with the potential to remove free radicals, either by a direct reaction with radicals, resulting in the formation of harmless products, or by disrupting radical chain reactions, avoiding further damage of cellular compounds, such as membrane lipids. There exists a growing awareness that oxidative stress and the resulting inflammation play an important role in the development of several chronic diseases[8], including cancer[9], diabetes[6] and inflammatory bowel diseases (IBD)[10]. It has been shown that reactive oxygen species can activate several signal transduction pathways such as the mitogen-activated protein kinase (MAPK) signalling cascade, as well as amplifying the activation of the nuclear transcription factor NF-κB[11], both resulting in enhanced formation of pro-inflammatory mediators such as cytokines[12].

Ulcerative colitis (UC) and Crohn's disease (CD) are the most predominant IBD, affecting over 2·2 million people of the European population, with a continuous increase over the past decades (www.efcca.org), with reasons remaining speculative, but including increased hygienic conditions and decreased immune stimulation[13]. Pathological complications of IBD may result following redox-mediated dysregulation of signalling cascades and/or gene expression[11]. Major intracellular signalling cascades that are responsible for the production of inflammatory molecules include NF-κB and MAPK, modulating action via their regulation and induction of target genes[14]. The resulting inflammation of the small (mainly CD) and large (both CD and UC) intestinal epithelium

Abbreviations: 2D-DIGE, two-dimensional differential in-gel electrophoresis; B, blank; CD, Crohn's disease; COX-2, cyclo-oxygenase-2; DMEM, Dulbecco's modified Eagle's medium; IBD, inflammatory bowel disease; JNK, c-Jun N-terminal kinases; M, micelles; MAPK, mitogen-activated protein kinase; S, stimulus; UC, ulcerative colitis.

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is chronic, has a low remission rate to medical treatment such as corticosteroids or non-steroidal anti-inflammatory agents[15,16], and may develop into cancer[15,16]. Thus, the search for preventive strategies targeting the onset of inflammation in the gut is paramount.

The implication of carotenoids in inflammatory processes in the human body is only marginally understood. It was noted that patients affected by early-stage IBD had low serum concentrations of micronutrients including Se and β-carotene[17], with similar results for several carotenoids in CD[10,18] and UC and CD subjects[19]. On the other hand, markers of oxidative stress or inflammation including TNF-α, IL-1β and lipid peroxidation were increased in adult subjects with CD[20,21], as was oxidative DNA damage[22]. Also increased NO was found as a mediator of inflammation in subjects with UC and CD[23,24], and was present in higher concentration in intestinal epithelial cells of IBD patients. Thus, it could be hypothesised that there exists a relationship between low antioxidant status and increased inflammation. However, carotenoid absorption is typically low[1] and carotenoid concentration decreases from the food matrix to the tissues. As the concentration of carotenoids is highest in the intestine, it is possible that their effect in this tissue is comparatively pronounced.

In the present study, the aim was to investigate the potential of carotenoids to interfere with inflammatory processes in the gut epithelium. For this purpose, differentiated human intestinal Caco-2 cells stimulated for inflammation with TNF-α and IL-1β were exposed to lycopene and β-carotene emulsified in artificial micelles, and inflammatory markers including IL-8, NO, cyclo-oxygenase-2 (COX-2) activity via PGE-2α measurement, as well as the signalling molecules NF-κB and MAPK were investigated. In addition, a proteomic study on extracted proteins from the whole cellular lysate following carotenoid exposure was performed using two-dimensional differential in-gel electrophoresis (2D-DIGE).

Materials and methods

Chemicals

All products were of analytical grade or higher. Unless otherwise stated, all chemicals including oleic acid, mono-olein, L-α-phosphatidylcholine, IL-8, lipopolysaccharide, TNF-α, sodium taurocholate hydrate, resazurine, arachidonic acid, penicillin/streptomycin mixture and non-essential amino acids were procured from Sigma-Aldrich. High-purity (18 mΩ) water was prepared with a purification system from Millipore and used throughout. Lycopene was purchased from Extrasynthèse and β-carotene was purchased from Dr Ehrenstorfer GmbH.

Cell culture

The TC-7 subclone (ATCC no. HTB-37) of the Caco-2 parental cell line was derived from a tumour isolated by J. Fogh[25] and was a generous gift from Monique Rousset (Nancy University). Cultures were routinely maintained in 75 cm^2 plastic flasks (Nunclon™; Nunc) at 37°C and 10 % CO_2 in a CB-210 CO_2 incubator (Binder GmbH). Cells were grown in Dulbecco's modified Eagle's medium (DMEM + GlutaMAX™; Gibco), supplemented with 1 % non-essential amino acids, 20 % heat-inactivated fetal bovine serum (Gibco), 1 % of a mixture of penicillin and streptomycin (10 000 units and 10 mg/ml, respectively) and subcultured weekly after reaching an approximately 80 % confluence. For all experiments, 1-week differentiated cells were used (passage 50-81). Transepithelial electrical resistance, a valid method for determining epithelial tightness and cellular differentiation, was used to evaluate the differentiation status of the cells, and no statistical significant differences were found compared with 2 weeks of differentiation.

Preparation of artificial micelles

For the preparation of artificial micelles, the original protocol of Biehler *et al.*[26] was adapted. In short, for the preparation of the medium, 0·11 g sodium taurocholate were weighed into a 100 ml glass vial, and 100 ml DMEM + glutamax (20 % fetal bovine serum, 1 % non-essential amino acids and 1 % penicillin and streptomycin) were added under a laminar flow box. In parallel, 12·8 mg L-α-phosphatidylcholine, 18·0 mg monoolein and 1·0 mg oleic acid were weighed on a six-digit balance (Metler Toledo) into another 100 ml glass vial. The vial was enwrapped with aluminium foil and kept on ice. Next, 4·0 mg of either lycopene or β-carotene were dissolved in 8 ml chloroform. Then, 2 ml (for 10 mg/l) or 5 ml (for 25 mg/l) of these solutions were added into the enwrapped 100 ml glass vial and the organic solvent was removed under a stream of N_2 during 5 min. Finally, 100 ml of the prepared medium described above were added and the mixture sonicated for a total of 30 min. After sonication, the solution appeared clear and no unsolubilised residues were visible. The last step consisted of a sterile filtration under a laminar flow box through a 0·22 μm filter (PALL Life Sciences). The mixture was then divided into aliquots and stored at −80°C until usage. In parallel, micelles without lycopene and β-carotene were prepared as negative controls.

Cell viability

To test the viability of the cells, especially following exposure to artificial micelles, the resazurine assay was used. Resazurine is a water-soluble dye, which can pass through cellular membranes. In the mitochondria, the oxidised blue resazurine is then reduced to the highly fluorescent pink compound resorufin, its amount being directly proportional to cell viability. Cells were exposed for 24 h to artificially produced micelles with or without carotenoids, and to pro-inflammatory cytokines used to induce inflammation (100 ng TNF-α/ml together with 25 ng IL-1β/ml). After the treatment, cells were washed with basal medium (DMEM + GlutaMAX™, supplemented with 1 % non-essential amino acids, 20 % heat-inactivated fetal bovine serum, 1 % of a mixture of penicillin (10 000 units) and streptomycin (10 mg/ml), respectively) and incubated for 2 h with a medium containing 400 μM-resazurine

in the dark (37°C, 90 % air, 10 % CO_2). Fluorescence was measured in a BioTek Synergy 2 plate reader, with excitation at 530 nm (530 (25) nm filter) and emission recorded at 590 nm (590 (25) nm filter). Cell viability is expressed as mean cell viability (in %) compared with cells without treatment (set at 100 %).

Exposure experiments for studying IL-8, NO and PGE-2α, mitogen-activated protein kinase and NF-κB

Cells were grown on twenty-four-well plates (BD Biosciences) and six different treatments were performed (Table 1). To each well, a total volume of 10 μl TNF-α (10 μg/ml) and 10 μl IL-1β (25 μg/ml) was added. Each experiment was performed on three different days (except for NF-κB and MAPK on *n* 4 different days), with each individual treatment done as duplicates (IL-8, PGE-2α and NO). Secretion of IL-8, PGE-2 and NO is expressed as percentage of the positive control (blank (B) + micelles (M) + stimulus (S)).

In order to verify uptake of the carotenoids by cells, Caco-2 cells grown on six-well plates (BD Biosciences) and incubated for 4 h at 37°C with 2 ml of the prepared medium/micelle mixture, containing either lycopene (10 or 25 μg/ml) or β-carotene (10 or 25 μg/ml), were extracted and analysed as described earlier[26].

Determination of IL-8 secretion

IL-8 concentration in the supernatant was measured by an enzyme immunoassay kit from BD OptEIA (Becton Dickinson), according to the manufacturer's recommendation. In short, 50 μl ELISA diluent and 100 μl of the supernatant were added to a ninety-six-well plate, which was pre-coated with a monoclonal antibody for human IL-8. The plate was incubated for 2 h and washed before the addition of the monoclonal IL-8 antibody and streptavidin-horseradish peroxidase. The plate was again incubated for 1 h, washed with washing buffer and 100 μl substrate of horseradish peroxidase (tetramethylbenzidine; 3,3′,5,5′-tetramethylbenzidine) was added. Finally, following another incubation of 30 min, stop solution (1 M-phosphoric acid) was added and absorbance was read at 450 nm in a BioTek Synergy 2 plate reader. IL-8 concentration (pg/ml) was calculated using an external IL-8 standard curve.

Determination of cyclo-oxygenase-2 activity

Cells were treated as described above; however, to induce COX-2 activity, cells were additionally incubated for 10 min with arachidonic acid (10 mM in PBS). Concentration of PGE-2α in the supernatant was determined using the PGE-2 enzyme immunoassay kit from Cayman Chemical following the manufacturer's instructions. Briefly, 50 μl of culture medium, PGE-2-acetylcholinesterase conjugate and PGE-2α monoclonal antibodies were added to a ninety-six-well plate supplied with the kit. This goat polyclonal anti-mouse IgG antibody pre-coated plate was incubated for 18 h at 4°C. The plate was then washed to remove unbound reagents and 200 μl reagent (substrate for the acetylcholinesterase) was added. The plate was again incubated for 90 min at room temperature and absorbance read at 412 nm in a BioTek Synergy 2 plate reader. The concentration of PGE-2α (pg/ml) was determined by external calibration curves.

Determination of NO secretion

NO is unstable and is rapidly oxidised into nitrate (NO_3^-) and nitrite (NO_2^-). The measurement of NO (as NO_3^- and NO_2^-) was carried out by the Nitric Oxide Assay Kit from Calbiochem (Merck KGaA) and was based on the Griess assay (40). The Griess reagent, however, does not measure nitrate, therefore the NADPH-dependent enzyme, nitrate reductase, was used to convert the nitrate to nitrite before quantification. Briefly, 85 μl of culture medium were added to a ninety-six-well plate. Then nitrate reductase (10 μl) and NADH (10 μl) were added to the wells. The plate was incubated for 20 min at room temperature. Next, the Griess reagent (100 μl) was added and the plate was shaken for 5 min before reading absorbance at 540 nm in a BioTek Synergy 2 plate reader. The concentration of NO (μM) present in the samples was calculated by means of an external standard curve.

Determination of NF-κB

Cells were grown in 75 cm^2 plastic flasks (Nunclon™; Nunc) and five different treatments (with *n* 4 replicas each) were performed. Cells were treated as described above; only lycopene at 10 μg/ml was omitted due to reagent limitations. The nuclear extract was obtained from the cultured cells by using the Cayman Nuclear Extraction kit (Cayman Chemical). Briefly, 10^7 cells were collected and centrifuged at 300 ***g*** for 5 min at 4°C. The supernatant was discarded and the cells were resuspended in PBS containing phosphatase inhibitors, and centrifuged again. This step was repeated before the addition of hypotonic buffer (500 μl). The cells were then incubated on ice for 15 min, before detergent addition (10 % Nonidet P-40 Assay Reagent, 50 μl) and centrifuged for

Table 1. Exposure of 1-week differentiated Caco-2 epithelial cells to various carotenoid treatments (*n* 6 per treatment)

Exposure group	Treatment of cells
Blank	Untreated: no inflammatory stimuli or micelles
Blank + micelles (negative control)	24 h with empty micelles
Blank, micelles + stimuli (positive control)	TNF-α, IL1-β* (28 h) and empty micelles (4–28 h)
Blank, micelles, stimuli + all-*trans*-lycopene (10 or 25 μg/ml)	TNF-α, IL1-β (28 h) and micelles containing 10 or 25 μg lycopene/ml (4–28 h)
Blank, micelles, stimuli + all-*trans* β-carotene (10 or 25 μg/ml)	TNF-α, IL1-β (28 h) and micelles containing 10 or 25 μg β-carotene/ml (4–28 h)

* 25 ng IL-1β/ml and 100 ng TNF-α/ml simultaneously.

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30 s at 4°C. The supernatant containing the cytosolic fraction was transferred to a 1·5 ml microcentrifuge tube and stored at −80°C until analysis. Pellets were resuspended in 50 µl nuclear extraction buffer (containing protease and phosphatase inhibitors and dithiothreitol) and incubated on ice (15 min). This step was repeated before centrifugation at 14 000 ***g*** (10 min, 4°C). The supernatant was transferred to a 1·5 ml microcentrifuge tube and was either further examined or stored at −80°C until analysis.

The concentration of NF-κB was measured by the NF-κB (p65) ELISA kit (Cayman Chemical) following the manufacturer's protocol. In short, 90 µl of complete transcription factor buffer and 10 µl of the nuclear extract were added to a ninety-six-well plate and incubated overnight at 4°C. The plate was pre-coated with a consensus double-stranded DNA sequence containing the NF-κB response element. After incubation, the plate was washed five times with 200 µl wash buffer, and the primary antibody added. The plate was covered and incubated for 1 h at room temperature. Following incubation, the plate was again washed with wash buffer and a second antibody conjugated to horseradish peroxidase was added. The plate was covered and incubated for 1 h at room temperature. After incubation, the plate was washed with 200 µl wash buffer, and 100 µl developer solution was added. The colorimetric development was monitored between 15 and 45 min, and 100 µl stop solution was added. The absorbance was read at a wavelength of 450 nm in a BioTek Synergy 2 plate reader.

Determination of mitogen-activated protein kinase activation

Cells were grown and exposed as described for NF-κB (with n 4 replications for each of the five exposures). The nuclear extract was obtained by following the cell extraction protocol of the p38 MAPK Immunoassay Kit (Invitrogen). The cells were collected in PBS by scraping them from the culture flasks. Then, two washing steps with PBS followed by centrifugation for 1 min at 16 500 ***g*** were performed and the supernatant was discarded. The cell pellet was lysed in cell extraction buffer for 30 min on ice and was vortexed every 10 min. The extract was then transferred to a 1·5 ml microcentrifuge tube and centrifuged for 10 min at 18 000 ***g*** at 4°C. The lysate was divided into aliquots into 1·5 ml microcentrifuge tubes. The nuclear extracts (100 µl) were added to anti-p38 antibody pre-coated ninety-six-well plates. After 2 h of incubation, the wells were washed with wash buffer (200 µl) and 100 µl anti-p38 MAPK antibody was added. The plate was incubated for another 1 h and after an additional washing step with 200 µl wash buffer, 100 µl horseradish peroxidase-labelled anti-rabbit IgG were added. After a third incubation and washing step with 200 µl wash buffer, a chromogen (tetramethylbenzidine) was added and 30 min later, the reaction was stopped by adding 100 µl stop solution and the absorbance read at 450 nm in a BioTek Synergy 2 plate reader. The concentration of MAPK (pg/ml) present in the samples was calculated by external calibration curves.

Proteomic analyses following carotenoid exposure of Caco-2 cells

For 2D-DIGE, an earlier protocol[27] was adapted. Unless otherwise stated, materials were from GE Healthcare. For each treatment (B, B + M, B + M + S, lycopene 10 µg/ml and β-carotene 10 µg/ml or 25 µg/ml), four TC175 flasks (Nunclon™; Nunc) were used to obtain sufficient biological cellular material. The total protein extract from the Caco-2 cells was obtained by using the extraction protocol of the p38MAPK immunoassay kit from Invitrogen. The protein concentration was determined by the Bradford method and a 1 mg/ml bovine serum albumin standard[28]. The pH of the extracted protein samples was adjusted to 8·5 with 3 M-Tris, and each extract was labelled by the minimal labelling process, following the manufacturer's instructions. Briefly, 30 µg of proteins were labelled with either 240 pmol Cy3 or Cy5 protein labelling dye, respectively. In addition, an internal standard was created by pooling aliquots of all protein extracts obtained, and 30 µg labelled with Cy2 dye. For the labelling, 240 pmol of dye were added to each tube, briefly centrifuged and vortexed and incubated for 30 min on ice in the dark. To stop the labelling reaction, 1 µl of 10 mM-lysine was added; samples were briefly centrifuged, vortexed and incubated for 10 min on ice in the dark. Afterwards, the Cy3- and Cy5-labelled extracts were combined with the Cy2-labelled internal standard (Table 2). The volume was adjusted to 450 µl with sample buffer (urea (7 m), thio-urea (2 m), (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (0·5 %) and bromophenol blue (traces)), and then 9 µl of biolyte pH 3–10 ampholyte buffer (Bio-Rad, Nazareth-Eke, Belgium) and 2·7 µl of destreak reagent were added. A total of 90 µg proteins (30 µg per sample and 30 µg of internal sample) were thus loaded on each strip and 2D-DIGE was performed. The use of an internal standard reduced the possibility of erroneous conclusions due to different concentration loads or differences between conditions of each strip or gel. The samples were added on an Immobiline DryStrip (24 cm, pH 3–10 non-linear; BioRad) and incubated overnight at room temperature to achieve optimal passive rehydration of the strip and sample loading. Proteins were then subjected to isoelectric focusing on an IPGphore III at 20°C. Mineral oil was added on the strips to prevent evaporation. The voltage was stepwise increased from 30 to 10 000 V during the first 21 h and then stabilised at 10 000 V for 8 h (about 120 kV h of the total current applied in total to each strip). Following the first dimension separation, strips were equilibrated for 15 min in equilibration buffer (2D Gel DALT; Gelcompany) containing also urea and dithiothreitol, and then for another 15 min in the same buffer containing iodoacetamide instead of dithiothreitol. After equilibration, strips were loaded on precast gels (2D gel DALT NF 12·5 %; Gelcompany) for second-dimensional separation, carried out on an Ettan DALT II system with 0·5 W/gel for 2 h and then 2·5 W/gel for 14 h at 25°C. The gels were scanned using a Typhoon 9400 scanner (Molecular Dynamics Inc.) and analysed by the DeCyder 2D Differential Analysis version 7.0. The protein images were produced by excitation of gels at 488, 532 and 633 nm (Cy2, Cy3

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Table 2. Experimental design for the proteomic experiment employing two-dimensional differential in-gel electrophoresis following six different carotenoid treatments (*n* 4 replica each, no. 1–4)*

Gel no.	Cy2	Cy3	Cy5
1	Pooled internal standard	Blank no. 1†	β-Carotene 25 μg/ml no. 3‡
2	Pooled internal standard	Blank no. 2†	β-Carotene 25 μg/ml no. 4‡
3	Pooled internal standard	Blank/micelles no. 1§	β-Carotene 10 μg/ml no. 3‡
4	Pooled internal standard	Blank/micelles no. 2§	β-Carotene 10 μg/ml no. 4‡
5	Pooled internal standard	Blank/micelles/stimulus no. 1‖	Lycopene 10 μg/ml no. 3¶
6	Pooled internal standard	Blank/micelles/stimulus no. 2‖	Lycopene 10 μg/ml no. 4¶
7	Pooled internal standard	Lycopene 10 μg/ml no. 1¶	Blank/micelles/stimulus no. 3‖
8	Pooled internal standard	Lycopene 10 μg/ml no. 2¶	Blank/micelles/stimulus no. 4‖
9	Pooled internal standard	β-Carotene 10 μg/ml no. 1‡	Blank/micelles no. 3§
10	Pooled internal standard	β-Carotene 10 μg/ml no. 2‡	Blank/micelles no. 4§
11	Pooled internal standard	β-Carotene 25 μg/ml no. 1‡	Blank no. 3†
12	Pooled internal standard	β-Carotene 25 μg/ml no. 2‡	Blank no. 4†

* Each cellular protein extract obtained following exposure (30 μg) was labelled with one of the three fluorophores (Cy2, Cy3 or Cy5). The pooled internal standard contained equal amounts of protein extracts from each treatment and was labelled with Cy2; the other samples were labelled with Cy3 or Cy5.
† Without stimuli and micelles.
‡ β-Carotene (10 or 25 μg/ml), cells stimulated with the TNF-α/IL1-β mixture (28 h) exposed to micelles containing either 10 or 25 μg β-carotene/ml (24 h).
§ Without stimuli but with empty micelles (negative control).
‖ With stimuli and empty micelles (positive control), i.e. TNF-α plus IL1-β at 100 and 25 ng/ml (28 h) and exposed to empty micelles for 24 h.
¶ Lycopene (10 μg/ml), cells stimulated with the TNF-α/IL1-β mixture (28 h) exposed to micelles containing 10 μg lycopene/ml (24 h).

and Cy5, respectively) and emission at 520, 610 and 670 nm (Cy2, Cy3 and Cy5, respectively) using the Typhoon 9400 scanner at a spatial resolution of 100 μm. Selected spots of interest (abundance of variation 1·3-fold, $P<0·05$) were located on a gel and a 'picking list' was generated. The spot picking, digestion and the spotting of the samples on matrix-assisted laser desorption/ionisation (MALDI) disposable targets plates (4800; Applied Biosystems) were done automatically using the Ettan Spot Handling Workstation. Peptide mass fingerprint and MS/MS analyses were carried out using the 4800 Proteomics Analyser (Applied Biosystems). Calibration was done with the peptide mass calibration kit 4700 (Applied Biosystems). Proteins were identified by the SWISS-PROT database (version 20100924 with 519 538 sequences) with 'Homo sapiens' as taxonomy, using GPS Explorer Software version 3.6 (Applied Biosystems) including MASCOT (Matrix Science, www.matrixscience.com). All searches were carried out allowing for a mass window of 150 ppm for the precursor mass and 0·75 Da for fragment ion masses. The search parameters allowed for carboxyamidomethylation of cysteine as fixed modification. Oxidation of methionine and oxidation of tryptophan (single oxidation, double oxidation and kynurenin) were set as variable modifications. Proteins with probability-based MOlecular Weight SEarch (MOWSE) scores ($P<0·01$) were considered as positively identified.

Statistical analysis

Original data were verified for normality by Q–Q plots and checked for equality of variance by boxplots. Wherever required, data were log-transformed for statistical evaluation. Univariate models were then created with the observed parameters (e.g. IL-8 concentration) as the dependent variable and the different carotenoids and their concentrations (also including the controls) as fixed factors. Following significant Fisher's *F* tests, individual *post hoc* tests were carried out to determine differences compared with the control (Dunnet's test). A *P* value below 0·05 (two-sided) was chosen to indicate significance. Unless otherwise stated, all data are expressed as mean percentages (compared with the positive control) and standard deviations.

Results

Cell viability, IL-8, PGE-2α and NO secretion following carotenoid exposure

The different carotenoid treatments (Table 1) did not result in a significant reduction of Caco-2 cell viability. Furthermore, treatment with micelles *per se* (B/M) and with pro-inflammatory cytokines (B/M/S) did not result in significant changes of cell viability (Table 3). Mean uptake of β-carotene into cells was approximately 7·2 %, and that of lycopene 4·3 %.

The addition of TNF-α and IL-1β resulted in a 4·1-fold induction of the IL-8 secretion compared with the negative control (B; Fig. 1). Neither empty micelles (B/M) nor the addition of lycopene or β-carotene (10 and 25 μg/ml) to the Caco-2 cells did result in any significant effect on the IL-8 secretion compared with the positive controls (Table 3). PGE-2 production was already high in unstimulated cells (B and B/M) compared with the positive control. The addition of TNF-α and IL-1β did not result in any further induction of COX-2 activity. The carotenoid treatment likewise did not show any significant effect (Table 3). Similarly, the NO secretion in unstimulated cells (B and B/M) was already high compared with the positive control. The addition of the pro-inflammatory cytokines and the carotenoid treatment had no further significant impact on NO secretion (Table 3).

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Table 3. Effect of lycopene (L) and β-carotene (BC) exposure of Caco-2 cells on different inflammatory markers (IL-8, cyclo-oxygenase-2 (COX-2) and NO), and on intracellular signalling cascades (NF-κB and mitogen-activated protein kinase (MAPK) activation), expressed as per cent compared with control and absolute values
(Mean values and standard deviations)

	Cell viability		IL-8 production			COX-2 activation via PGE-2α production			NO production			NF-κB activation		MAPK activation		
Cell exposure*	Mean† (%)	SD (%)	Mean‡ (%)	SD (%)	Mean (pg/ml)	Mean‡ (%)	SD (%)	Mean (pg/ml)	Mean‡ (%)	SD (%)	Mean (μM)	Mean§ (%)	SD (%)	Mean§ (%)	SD (%)	Mean (pg/ml)
Blank	95·4	7·2	24·2	16·8	18·4	106·1	12·9	275·4	87·5	18·7	2·4	30·6	19·0	98·6	5·4	176·9
Blank/micelles	82·5	6·8	11·1	19·9	8·5	97·8	32·5	177·3	106·1	18·9	2·9	30·3	9·8	95·9	5·9	172·1
Blank/micelles/stimulus	100·0	11·7	100·0	20·2	76·1	100·0	36·7	199·7	100·0	16·4	2·8	100·0	9·5	100·0	12·6	179·4
L 10 μg/ml	111·2	7·3	137·6	18·9	104·7	101·6	36·7	217·5	95·9	26·1	2·6	86·5	10·0	104·8	12·7	187·9
L 25 μg/ml	111·5	7·9	138·6	11·2	105·4	103·3	32·1	237·9	102·9	18·2	2·8	–	–	–	–	–
BC 10 μg/ml	88·8	7·0	116·0	28·2	88·3	104·6	31·9	254	86·8	23·0	2·4	111·6	4·7	112·0	15·8	200·8
BC 25 μg/ml	86·2	4·3	108·0	43·9	82·2	112·0	37·5	376·2	114·5	18·4	3·2	107·3	10·9	88·6	11·1	159·0

*Blank, without stimuli and micelles; blank/micelles, without stimuli but with empty micelles (negative control); blank/micelles/stimulus, with stimuli and empty micelles (positive control), i.e. TNF-α plus IL1-β at 100 and 25 ng/ml (28 h) and exposed to empty micelles for 24 h; L, L (10 or 25 μg/ml), cells stimulated with the TNF-α/IL1-β mixture (28 h) exposed to micelles containing either 10 or 25 μg L/ml (24 h); BC, BC (10 or 25 μg/ml), cells stimulated with the TNF-α/IL1-β mixture (28 h) exposed to micelles containing either 10 or 25 μg BC/ml (24 h).
† *n* 12: 3 d with each six independent replicas.
‡ *n* 6: total of *n* 6 obtained from three different experiments conducted on three different days with independent duplicates.
§ *n* 4: total of *n* 4 obtained during four different days (one flask (75 cm^2), harvested proteins and finally pooled for experiment).

NF-κB and mitogen-activated protein kinase

The influence of the two different carotenoids was also investigated on intracellular signalling cascades via NF-κB and MAPK activation. TNF-α and IL-1β induced the NF-κB activity 3·2-fold (Fig. 2). Lycopene (10 μg/ml) and β-carotene (10 and 25 μg/ml) did not show any significant reduction of the NF-κB activity (Table 3). MAPK activity in unstimulated cells (B and B/M) was already high compared with the positive control, and the carotenoid treatment did not show any further significant reduction of the MAPK activity (Table 3).

Proteomic analyses

The analysis of the different treatments revealed that overall and within all pairwise comparisons, sixty-five spots were differentially regulated in their expression ($P<0·05$, expression ratio at least ±1·3-fold) and had a unique identification, belonging to fifty-two different proteins (Fig. 3 and Supplementary Table 1; http://www.journals.cambridge.org/bjn). Out of these, fifteen were differentially regulated in their expression due to the lycopene and β-carotene treatments (Table 4), compared with cells treated with empty micelles and inflammatory stimuli. The fifteen proteins were then classified with respect to their localisation and biological pathway in which they were involved, according to Gene Ontology (http://www.geneontology.org). Most of the proteins were intracellular proteins, predominantly located in the cytoplasm (67 %), predominantly involved in metabolic pathways (34 %) and in the stress response (40 %). Among the fifteen differentially expressed proteins, eleven were differentially expressed following the treatment with β-carotene at 10 μg/ml, three were differentially expressed by β-carotene at 25 μg/ml, one protein was differentially expressed in both β-carotenoid treatments and one protein was differentially expressed by lycopene (10 μg/ml). From the eleven differentially expressed proteins following the β-carotene (10 μg/ml) treatment, 82 % were down-regulated, while the three differentially expressed proteins due to the β-carotene treatment at 25 μg/ml were all up-regulated, compared with the positive control (M + S). The only protein that was differentially expressed following the lycopene (10 μg/ml) treatment was up-regulated (Table 4).

Discussion

In the present study, we investigated the hypothesis that individual carotenoids might exhibit anti-inflammatory properties on cells resembling the gastrointestinal epithelium, and could constitute a preventive dietary strategy against chronic gut diseases such as UC or CD. We tested the influence of β-carotene and lycopene in artificial micelles on inflammatory-stimulated Caco-2 cell production of several inflammatory endpoints, including IL, NO, COX-2 activity, intracellular signalling pathways (NF-κB and MAPK) and proteomics. While individual carotenoids at realistic dietary concentrations did not significantly affect inflammatory markers, several proteins were differentially regulated, indicating that carotenoids could

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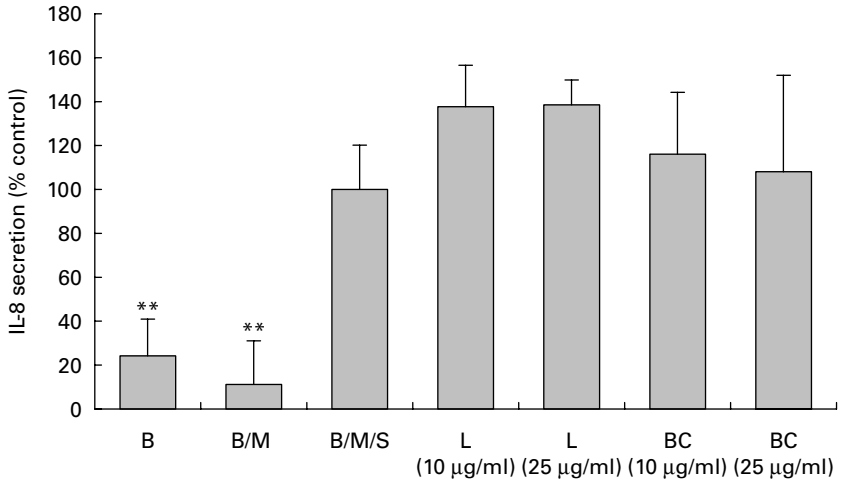

Fig. 1. Effect of carotenoids, lycopene and β-carotene, on the IL-8 secretion of Caco-2 cells stimulated for inflammation with TNF-α (100 ng/ml) and IL-1β (25 ng/ml) for 24 h. Cells were exposed to various treatments and the IL-8 secretion was measured after 28 h. Values are means from six independent replicates compared with the control (blank (B)/micelles (M)/stimulus (S) = 100 % (*n* 12 replicates)), with standard deviations represented by vertical bars. B/M, B + M; B/M/S, B + M + S; L, lycopene; BC, β-carotene. ** Mean values were significantly different by Dunnet's test ($P<0{\cdot}01$).

alter the general metabolism of these epithelial cells. To our knowledge, this is the first time that the impact of carotenoids on inflammatory markers of the gut epithelium has been studied.

Both β-carotene and lycopene are found in a wide range of frequently consumed fruits and vegetables. Concentrations tested (10 and 25 μg/ml) represented high, but still plausible concentrations that could be reached following, for example, consumption of approximately 300 g carrots, assuming a total volume of gastrointestinal liquids of 1 litre[29]. The viability studies conducted indicated that the carotenoids added did not result in adverse effects. Furthermore, micelles alone, without lycopene and β-carotene, did not result in any significant changes of the inflammatory markers studied.

Both TNF-α and IL-1β used for inflammation stimulation are activators of inflammatory pathways, and have been previously used to study the impact of cytokines on inflammation[30]. TNF-α and IL-1β in the present study resulted in inflammatory stimulation, as determined by the 4·1-fold increase in IL-8 and the 3·2-fold increase in NF-κB, albeit they did not significantly change PGE-2α, NO and MAPK. It may be that the exposure time of 24 h was not long enough to induce the production of PGE-2α, NO and to activate MAPK. NO production, for example, requires more steps than the production of IL-8. IL-8 expression follows binding of NF-κB to the promoter region of the IL-8 gene[31]. NF-κB is responsible for the expression of inducible NOS, which then catalyses the reaction of L-arginine into NO[32]. PGE-2α synthesis involves even more enzymatic steps for activation compared with, for example, IL-8 and NO. Following binding of NF-κB, COX-2 catalyses the reaction of arachidonic acid to PGG-2 and further to PGH-2. The PG synthase E then catalyses the reaction to PGE-2α[33]. Another factor might be the status of the cells. While our experiments were conducted with 1-week differentiated cells, proliferating cells could respond differently. Van de Walle *et al.*[30] showed that PGE-2 concentration was higher in proliferating cells than in differentiated cells, while NO production was detectable only in 3-week differentiated cells. However, inflammation induction following IL-8 and NF-κB was slightly lower in the present study compared with a similar earlier study[34], with a 7·5- and 5·8-fold increase, respectively. Thus, the inflammation in the present study was rather indicative of a low, chronic type.

IL-8 is a chemokine responsible for the chemotaxis of neutrophiles and their degranulation[35]. We did not observe any significant changes of this marker following carotenoid exposure, neither of the regulation of NF-κB, being in line with the theory that IL-8 is regulated via NF-κB[36]. As with IL-8, PGE-2α is likewise a major mediator of intestinal inflammation[37], resulting from COX-2 activity and arachidonic acid. While in a previous study[38], a similar concentration of arachidonic acid resulted in a 5·6-fold increase of PGE-2, in the present study, PGE-2α production remained low during all experiments, suggesting that carotenoids were not able to modify the activity of COX-2 significantly.

NO plays a central role in human IBD[39]. In the present study, no significant change in NO concentration following carotenoid exposure was observed. Contrarily, Romier-Crouzet *et al.*[38] showed an increase in NO production after inflammatory stimuli by 1·9-fold; however, stimulation was done for 48 h and with a mixture of interferon-γ, IL-1β, TNF-α and lipopolysaccharide.

We also investigated the effect of carotenoids on the NF-κB cascade. NF-κB has many target genes, playing an important role in inflammation. In the present study we determined inducible NOS through NO production, COX-2 through PGE-2α production and the IL-8 gene through the IL-8 production. All compounds have been reported to be impacted via the NF-κB pathway[16,32,36], albeit they may also be regulated by NF-κB-independent pathways[40]. In this study, the stimulation of Caco-2 cells with TNF-α and IL-1β resulted in

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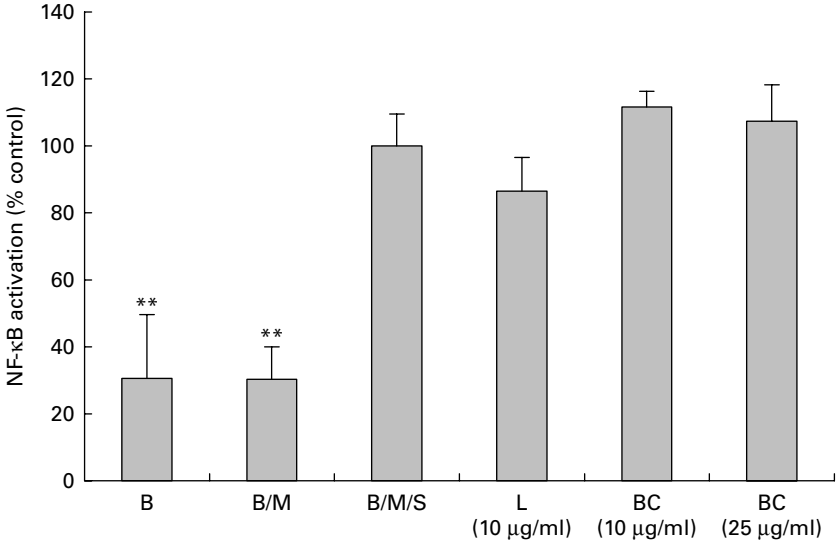

Fig. 2. Effect of the carotenoids on the NF-κB activation of Caco-2 cells stimulated for inflammation with TNF-α (100 ng/ml) and IL-1β (25 ng/ml) for 24 h. The cells were exposed to various treatments and the NF-κB activation was measured after 28 h. Values are means from four replicates of each sample compared with the control (blank (B)/micelles (M)/stimulus (S) = 100 %), with standard deviations represented by vertical bars. B/M, B + M; B/M/S, B + M + S; L, lycopene; BC, β-carotene. Mean values were significanly different by Dunnet's test ($P<0.01$).

a significant increase in NF-κB compared with the unstimulated B, indicating activation of NF-κB-dependent pathways, as shown by the increased IL-8 production in the present study.

The MAPK cascades are also involved in the production of inflammatory mediators, three are well-characterised in mammals: extracellular signal-regulated kinases (ERK1/ERK2); c-Jun N-terminal kinases (JNK1, JNK2 and JNK3); p38 kinases[41]. The target genes of the p38 kinases are mostly cytokines, and thus play a crucial role in inflammatory responses[41]. As the immunoassay kit used during our study detected the total produced p38MAPK (independently of phosphorylation state), we already observed high concentrations of p38 in unstimulated cells, but no further impact following the inflammation trials.

Contrarily to earlier studies suggesting anti-inflammatory properties of β-carotene and lycopene in cellular trials as measured by NO, PGE-2, inducible NOS and COX-2[14,42], in the present study, the two carotenoids did not affect NO, IL-8 and COX-2 activity, further confirmed by NF-κB and

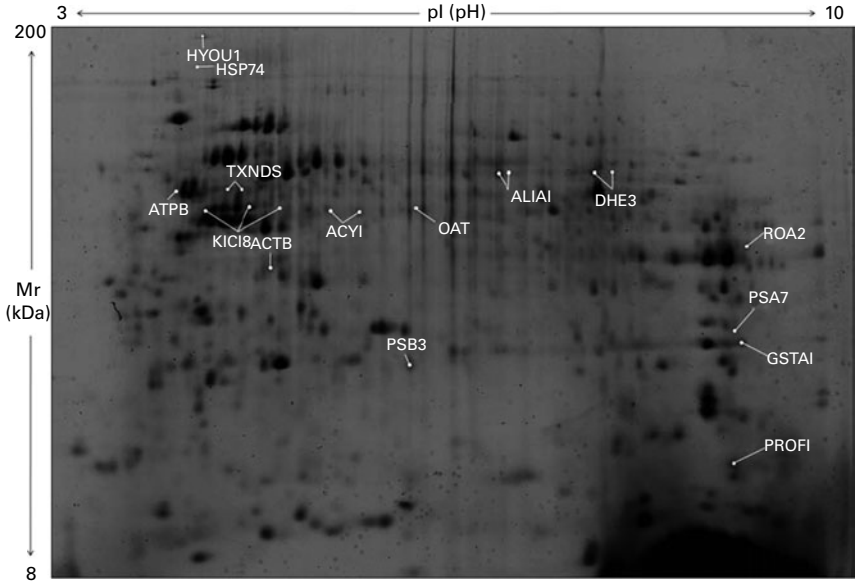

Fig. 3. Differentially expressed protein spots identified following two-dimensional differential in-gel electrophoresis (2D-DIGE) analysis. The 2D-DIGE analysis was done on the whole protein extract from Caco-2 cells which were treated with empty micelles and inflammation stimulus for 24 h (TNF-α (100 ng/ml)/IL-1β (25 ng/ml)), treated with lycopene (10 μg/ml) or β-carotene (10 and 25 μg/ml) for 24 h plus the inflammation stimulus for 28 h. Proteins were separated in the first dimension on a non-linear pH gradient and in the second dimension on a SDS-PAGE gel. Comparisons were made for each treatment against blank + micelles + inflammation stimulus (control).

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Table 4. Up- and down-regulation of the fifteen differentially expressed proteins following carotenoid exposure compared with the inflammatory-stimulated blank (positive control, blank + micelles (M) + Stimulus (S))*

					Blank + M + S		
Spot no.	*T* test	Fold change†	Accession no.‡	Protein name	L (10 μg/ml)	BC (10 μg/ml)	BC (25 μg/ml)
104	0·021	−1·78	P34932	Heat shock 70 kDa protein 4	–	Up-regulated	–
665	0·044	1·82	P00352	Retinal dehydrogenase 1	–	Down-regulated	–
695	0·036	1·68	P00367	Glutamate dehydrogenase 1 mitochondrial	–	Down-regulated	–
748	0·002	−2·09	Q8NBS9	Thioredoxin domain-containing protein 5	–	–	Up-regulated
761	0·027	−1·63	P06576	ATP synthase subunit β mitochondrial	–	–	Up-regulated
842	0·026	−1·74	P05783	Keratin, type I cytoskeletal 18	–	–	Up-regulated
875	0·029	1·67	P04181	Ornithine aminotransferase mitochondrial	–	Down-regulated	–
879	0·030	1·69	O75874	Isocitrate dehydrogenase (NADP) cytoplasmic	–	Down-regulated	–
1149	0·024	−1·64	P22626	Heterogeneous nuclear ribonucleoproteins A2/B1	–	Up-regulated	–
1156	0·047	1·52	P60709	Actin cytoplasmic 1	–	Down-regulated	–
1533	0·037	1·95	O14818	Proteasome subunit α type-7	–	Down-regulated	–
1580	0·008	1·93	P08263	Glutathione *S*-transferase A1	–	Down-regulated	–
1671	0·028	1·48	P49720	Proteasome subunit β type-3	–	Down-regulated	–
1931	0·035	−1·68	P07737	Profilin-1	Up-regulated	–	–
2765	0·001	1·94	Q9Y4L1	Hypoxia up-regulated protein	–	Down-regulated	–

L, lycopene; BC, β-carotene.
*Among the eleven differentially expressed proteins during the BC (10 μg/ml) treatment, 73 % were down-regulated compared with the positive control. Among the three differentially expressed proteins during the BC (25 μg/ml) treatment, all were up-regulated. For the L (10 μg/ml) treatment, only one protein was differentially expressed and was up-regulated.
†Fold change: protein expression compared with the control (mean expression control/mean expression treatment).
‡Swissprot accession number.

MAPK. The reasons remain speculative. It has been argued that dietary antioxidants, including β-carotene and lycopene, possess biphasic effects, with higher concentrations resulting even in negative, i.e. pro-oxidative, effects[43].

Despite no effects on selected markers, proteomic analysis revealed that a total of fifteen proteins were differentially expressed following the carotenoid treatments. These proteins were predominately involved in metabolic pathways. About two-thirds of these differentially expressed proteins were present in the cytosol. Lycopene appeared to have a lower impact compared with β-carotene, as only one protein (profilin-1) was differentially expressed. One explanation could be the low cellular uptake as opposed to β-carotene[44], also observed in the present study. Profilin-1 is an actin-binding protein, involved in the regulation of the cytoskeleton assembly. It is also able to bind cell membrane components such as phosphatidyl-inositol-4,5-bisphosphate and to regulate the formation of inositol triphosphate, thus acting as a signalling protein[45].

At intermediate concentrations (10 μg/ml), β-carotene resulted rather in a down-regulation of differentially expressed proteins. In contrast, changes induced by β-carotene at 25 μg/ml were limited to only three proteins, all of which were up-regulated. These included mitochondrial ATP synthase subunit β, part of the catalytic domain of the ATP synthase and responsible for the formation of ATP from ADP and inorganic phosphate; cytoskeletal keratin type I constituting a component of the intermediate filaments exerting vital structural functions in eukaryotic cells and also involved in regulatory processes, including apoptosis modulation[46]; and thioredoxin domain-containing protein 5, involved in the resistance to nitrosative stress[47].

Most proteins down-regulated by β-carotene at 10 μg/ml are potentially involved in inflammation. For example, glutathione transferase participates in the elimination of xenobiotics, and glutathione is a major endogenous antioxidant, important for controlling pro-inflammatory processes[48]. The reduced activity of these enzymes observed may be representative of a decreased cellular stress following carotenoid exposure. Hypoxia up-regulated protein 1 has an important role in response to hypoxia-induced stress, modulating apoptosis. Furthermore, up-regulated were heterogeneous nuclear ribonucleoproteins A2/B1 and heat shock protein 70 kDa protein 4, the former being involved in mRNA maturation and processing, the latter in folding and assembly of proteins, as well as in stress response.

Caco-2 cells have been frequently employed in various studies; however, it is also important to point out the limitations of this model. While Caco-2 cells resemble the human intestinal epithelium, they are of neoplastic origin and might therefore differ in features from the natural intestine. Caco-2 cells further are monolayers mimicking only enterocytes, therefore lacking, for example, the mucus layer. Nevertheless, Caco-2 cells express various transporter and efflux proteins and have been frequently used for investigating bioavailability aspects of various compounds[49,50] and also for stimulating intestinal inflammation[30,38]. In addition, Caco-2 cells express various transporter proteins

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and efflux proteins. Finally, it cannot be excluded that the artificial system chosen here for carotenoid emulsification could have compromised the uptake of carotenoids and therefore their influence on Caco-2 cells, as artificial micelles could differ from micelles formed during the normal gastrointestinal digestion, for example in terms of size and release kinetics of carotenoids.

In summary, β-carotene and lycopene were not able to show significant effects on selected inflammatory mediators in stimulated Caco-2 cells, albeit the proteomic results indicate that several pathways related to inflammation, such as antioxidative enzymes, were involved. More studies in this domain, examining different carotenoids, and perhaps mixtures representing more natural conditions, are warranted.

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