Indicaxanthin inhibits NADPH oxidase (NOX)-1 activation and NF-κB-dependent release of inflammatory mediators and prevents the increase of epithelial permeability in IL-1β-exposed Caco-2 cells

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Abstract

Dietary redox-active/antioxidant phytochemicals may help control or mitigate the inflammatory response in chronic inflammatory bowel disease (IBD). In the present study, the anti-inflammatory activity of indicaxanthin (Ind), a pigment from the edible fruit of cactus pear (*Opuntia ficus-indica*, L.), was shown in an IBD model consisting of a human intestinal epithelial cell line (Caco-2 cells) stimulated by IL-1β, a cytokine known to play a major role in the initiation and amplification of inflammatory activity in IBD. The exposure of Caco-2 cells to IL-1β brought about the activation of NADPH oxidase (NOX-1) and the generation of reactive oxygen species (ROS) to activate intracellular signalling leading to the activation of NF-κB, with the over-expression of inflammatory enzymes and release of pro-inflammatory mediators. The co-incubation of the cells with Ind, at a nutritionally relevant concentration (5–25 μM), and IL-1β prevented the release of the pro-inflammatory cytokines IL-6 and IL-8, PGE$_2$ and NO, the formation of ROS and the loss of thiols in a dose-dependent manner. The co-incubation of the cells with Ind and IL-1β also prevented the IL-1β-induced increase of epithelial permeability. It was also shown that the activation of NOX-1 and NF-κB was prevented by Ind and the expression of COX-2 and inducible NO synthase was reduced. The uptake of Ind in Caco-2 cell monolayers appeared to be unaffected by the inflamed state of the cells. In conclusion, our findings suggest that the dietary pigment Ind may have the potential to modulate inflammatory processes at the intestinal level.

Key words: Indicaxanthin: Inflammation: Inflammatory bowel disease: *In vitro* models: Redox-active phytochemicals

The intestinal immune system is in a state of balance in which pro-inflammatory and anti-inflammatory cells and molecules are carefully regulated to promote host mucosal defence without damage to the intestinal tissue. Once this regulatory balance is disturbed, non-specific stimulation and activation can cause the overproduction and release of potent destructive immunological and inflammatory molecules[1]. The incapacity of the intestinal mucosa to control inflammatory events is the basis for the development of inflammatory bowel disease (IBD)[2].

IL-1β is a multifunctional cytokine playing a major role in both the initiation and amplification of many inflammatory conditions[3]. IL-1β is released by various cell types including monocytes–macrophages, neutrophils and endothelial cells[4], and increased concentrations have been found in the intestinal tissue of IBD patients[5–7]. A number of effects of IL-1β on intestinal epithelial cells have been shown *in vitro*, including the activation of redox-sensitive intracellular cascades leading to the increased transcriptional activity and secretion of IL-8[8], IL-6[9], PGE$_2$ and/or NO[10]. Moreover, *in vitro* and *in vivo* studies have provided evidence that the overproduction of pro-inflammatory cytokines such as TNF-α, interferon-γ and IL-1 can alter the tight junction (TJ) permeability of intestinal epithelia. This allows the permeation of inflammatory cells contributing to perpetuate the inflammatory processes in the gut mucosa[11].

Although pharmacological therapy currently plays a major role in the management of IBD, dietary compounds with immunomodulatory properties, including *n*-3 fatty acids and antioxidants, have also been shown to be effective[12–14].

Indicaxanthin (Ind), the yellow pigment characterising the edible fruit of the cactus *Opuntia ficus-indica*, is a water-soluble, nitrogen-containing compound belonging to a class of betalains, comprising phytochemicals having in common the structure of betalamic acid. Ind is the adduct of betalamic acid with proline (Fig. 1). Recent and growing interest in these substances has resulted in various studies showing that betalains can be considered as bioactive dietary compounds[15]. The cyclic amine associated with a polyene system in the Ind molecule was first considered as the reactive group conferring the molecule with reducing activity[16]. More interestingly, measurements of the redox potential and
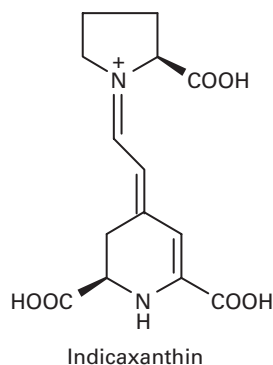

Indicaxanthin

Fig. 1. Molecular structure of indicaxanthin.

radical-scavenging activity[16,17] have suggested that Ind might behave as an effective scavenger of radicals formed in biological environments. A large amount of data has recently been published on the lipoperoxyl radical-scavenging activity and antioxidant effects of Ind in various biological contexts, ranging from LDL systems[18] to either healthy[19] or pathological[20] erythrocytes, as well as cell cultures[21,22]. From a nutritional perspective, *in vitro* studies aimed at elucidating the chemical and biochemical transformations of the pigment during the digestive and intestinal absorptive processes have provided evidence that Ind is quite stable under digestive conditions[23], is not metabolised by enterocytes and is absorbed through a paracellular mechanism characterised by a high permeability coefficient[24]. More importantly, human studies have provided evidence that Ind from a diet-consistent amount of cactus pear fruits is highly bioavailable, reaching a plasma concentration of 7 μM, with a half-life of $2 \cdot 36\,h$[25].

Reactive oxygen species (ROS) overproduction and cell redox imbalance play a central role in the pathophysiology of the inflammatory response[26,27]. Although the aetiopathogenesis of IBD must be considered to be multifactorial, modulation of the cell redox environment can somewhat control the intestinal inflammatory response[13]. Taking into account the redox chemistry and bioactivity of Ind, in the present study, we investigated the effects of this pigment in an *in vitro* model of enterocytes during an acute phase of IBD. To this aim, Caco-2 cells, an established model of the human intestinal barrier[28], exposed to IL-1β, known as an IBD causative pathophysiological agent[5–7], were used and the influence of Ind on a number of inflammatory parameters, including the release of pro-inflammatory mediators and levels of inducible inflammatory enzymes, was evaluated. The effects of Ind on the imbalance of cell redox, activation of membrane NADPH oxidase 1 (NOX-1) and of NF-κB, as well as the influence on the IL-1β-induced increase of the epithelial TJ permeability, were also investigated.

Materials and methods

Reagents

Unless otherwise specified, all reagents and chemicals were obtained from Sigma Chemical Company.

Isolation of indicaxanthin

Ind was isolated from cactus pear (*O. ficus-indica*) fruits (yellow cultivar). The phytochemical was separated from a methanol extract of the pulp by liquid chromatography on Sephadex G-25[16]. Fractions containing the pigment were submitted to cryodessiccation (Freezone 4·5; Labconco). The dried material was resuspended in 1% acetic acid in water and submitted to semi-preparative HPLC using a Varian Pursuit C18 column (250 × 10 mm inner diameter; 5 μm; Varian), eluted from solvent A (1% acetic acid in water) to 20% solvent B (1% acetic acid in acetonitrile) with a 20 min linear gradient, an injection volume of 200 μl and a flow rate of 3 ml/min[20]. Spectrophotometric revelation was at 482 nm. The elution volumes relevant to the Ind peak were collected. After cryodessiccation, the samples were stored at −80°C. Dried Ind was resuspended in Dulbecco's modified Eagle's medium at a suitable concentration and filtered through a Millex HV 0·2 μm filter (Millipore) before use. The concentration of the samples was evaluated spectrophotometrically with a DU-640 Beckman spectrophotometer using a molar coefficient of 42 800 at 482 nm.

Cell culture

Human colon adenocarcinoma-derived Caco-2 cells were obtained from the American Type Culture Collection and used between passages 27 and 31. The cells were grown in 175 cm² flasks in Dulbecco's modified Eagle's medium (Gibco Life Technologies) supplemented with 10% fetal bovine serum (Gibco Life Technologies), 1% non-essential amino acids, 10 mM-HEPES, 50 units/ml of penicillin, 50 μg/ml of streptomycin and 100 μg/ml of gentamicin and were maintained at 37°C in 5% CO₂. For the experiments, the cells were seeded at a density of $1 \cdot 25 \times 10^5$ in twenty-four-well plates (Corning Costar, Inc.) in a culture medium for 21 d to obtain fully differentiated cells. The culture medium was changed thrice a week. For the measurement of intestinal epithelial TJ permeability, experiments were carried out using Transwell® inserts (polycarbonate membrane, 0·4 μm pore size and 24 mm diameter). The inserts were placed into six-well plates. Caco-2 cells were seeded at 5×10^4 cells/cm² on the membrane inserts with 1·5 ml of the medium on the apical/luminal side and 2·5 ml of the medium on the basolateral side. The culture medium was changed thrice a week throughout the 21 d of growth, and the transepithelial electrical resistance values across the cell monolayers were measured using a Millicell-ERS volt ohm meter (Millipore Corporation). Caco-2-plated filters with epithelial resistance of $400–500\,\Omega\,cm^2$ were used.

Cell treatments

Before each treatment, Caco-2 cells were made quiescent through overnight incubation in a serum-free medium. The cells were then placed in Dulbecco's modified Eagle's medium with 2% fetal bovine serum and challenged with IL-1β (25 ng/ml) at 37°C either in the absence (control) or in

the presence of Ind for the incubation times shown in the figures. Cell viability was routinely checked using the Trypan Blue Exclusion method. To evaluate the effect of the treatments on TJ permeability, IL-1β was added on the basolateral side and Ind on the apical/luminal side.

Assay for IL-6 and IL-8

The extracellular media were collected and centrifuged at $15\,000\,\boldsymbol{g}$ for 10 min. The secretion of IL-6 and IL-8 was evaluated using a sandwich ELISA method (BD Biosciences Pharmingen) in accordance with the manufacturer's protocol.

Measurement of arachidonic acid cascade activity through PGE$_2$ production

The activation of the arachidonic acid cascade was estimated through the production of PGE$_2$ after the addition of arachidonic acid. Briefly, 24 h after the treatment, the cells were washed and incubated with 10 mM-arachidonic acid in PBS for 10 min. The secretion of PGE$_2$ in the extracellular medium was quantified (pg/ml) using a Prostaglandin E$_2$ Enzyme Immunoassay Kit (Cayman Chemical Corporation, Inc.) in accordance with the manufacturer's protocol.

Assay for nitric oxide

The production of NO in Caco-2 cell monolayers stimulated by IL-1β (25 ng/ml) for 24 h was evaluated. NO, present in the medium as nitrite, was assayed according to the Griess reaction. Briefly, 100 μl of the cell culture medium were mixed with 100 μl of Griess reagent (equal volumes of 1 % sulphanilamide (w/v) in 5 % phosphoric acid (v/v) and 0·1 % naphtylethylene-diamine–HCl (w/v)) and incubated at room temperature for 10 min and then absorbance was measured at 550 nm using a microplate reader (GloMaxÂ®-Multi Microplate Reader; Promega Corporation). Fresh culture medium was used as the blank. The amount of nitrite in the samples was evaluated by referring to a sodium nitrite serial dilution standard curve.

Detection of intracellular reactive oxygen species

The intracellular levels of ROS were assessed by measuring the fluorescence resulting from the intracellular oxidation of 2′,7′-dichlorofluorescin diacetate. 2′,7′-Dichlorofluorescin diacetate in PBS (10 μM) was added to the medium 30 min before ending the treatment of the cells to label intracellular ROS. The medium was then removed, and the cells were washed with PBS, resuspended in the same buffer and immediately subjected to fluorescence-activated cell sorting analysis with an Epics XL™ flow cytometer, using the Expo32 software (Beckman Coulter). At least 1×10^4 cells were analysed for each sample.

Assay for total reduced thiols

After the treatment, the cells were centrifuged for 5 min at $2000\,\boldsymbol{g}$, washed twice with cold PBS containing 0·1 M-butylated hydroxytoluene and ruptured by sonication. Cell lysates were mixed with 10 % SDS and 30 μM-5,5′-dithiobis (2-nitrobenzoic acid) and incubated at 30°C for 30 min. The levels of total reduced thiols, including protein thiols and glutathione, were measured spectrophotometrically at 412 nm.

Western blot analysis

Caco-2 cells were rinsed twice with ice-cold PBS and harvested by scraping into 200 μl/well ice-cold hypotonic lysis buffer (10 mM-HEPES, 1·5 mM-MgCl$_2$, 10 mM-KCl, 0·5 mM-phenylmethylsulphonyl fluoride, 1·5 μg/ml soyabean trypsin inhibitor, 7 μg/ml pepstatin A, 5 μg/ml leupeptin, 0·1 mM-benzamidine and 0·5 mM-dithiothreitol). After 15 min of incubation on ice, the lysates were centrifuged at $13\,000\,\boldsymbol{g}$ for 10 min, and the supernatants (cytosolic fraction) were immediately portioned and stored at −80°C up to 2 weeks. The nuclear pellet was resuspended in 60 μl of a high-salt extraction buffer (20 mM-HEPES, pH 7·9, 420 mM-NaCl, 1·5 mM-MgCl$_2$, 0·2 mM-EDTA, 25 % (v/v) glycerol, 0·5 mM-phenylmethylsulphonyl fluoride, 1·5 μg/ml soyabean trypsin inhibitor, 7 μg/ml pepstatin A, 5 μg/ml leupeptin, 0·1 mM-benzamidine and 0·5 mM-dithiothreitol) and incubated with shaking at 4°C for 30 min. The nuclear extract was then centrifuged for 15 min at $13\,000\,\boldsymbol{g}$, and the supernatant was portioned and stored at −80°C up to 2 weeks. The protein concentration of each sample was determined using a Bradford protein assay reagent (Bio-Rad). Protein samples (80 μg/line) were separated on 12 % SDS–PAGE gel and transferred onto nitrocellulose membranes. The immunoblots were incubated overnight at 4°C with a blocking solution (5 % skimmed milk), followed by incubation with human anti-IκB-α monoclonal antibodies (clone 417208, catalogue no. MAB4299; R&D Systems) or human anti-phospho-IκB-α polyclonal antibodies (S32/S36, catalogue no. AF4809; R&D Systems), human anti-NF-κB p65 monoclonal antibodies (clone 532301, catalogue no. MAB 5078; R&D Systems) and human anti-poly(ADP-ribose) polymerase monoclonal antibodies (clone D-1, catalogue no. SC-365315; Santa Cruz Biotechnology) for 1 h at room temperature. The blots were washed twice with Tween 20/Tris-buffered saline and incubated with a 1:2000 dilution of rabbit anti-human horseradish peroxidase-conjugated anti-IgG antibodies (Dako Denmark) for 1 h at room temperature. The blots were again washed five times with Tween 20/Tris-buffered saline and then developed by enhanced chemiluminescence (Amersham Life Science). Immunoreactions were also performed using β-actin antibodies as the loading controls. The immunoreactive bands were densitometrically analysed using an Image Tool program (Image J 1·455; National Institutes of Health).

Evaluation of activated NADPH oxidase complex

The activity of NOX-1 was evaluated through Western blot analysis of the cytosolic subunit NOX activator 1 assembled in the membrane NOX-1 enzyme complex. To this aim, the cytosolic fractions of Caco-2 cells (see above) were centrifuged at $100\,000\,\boldsymbol{g}$ for 1 h at 4°C. The pellet, containing the membrane fraction, was suspended in a cold buffer containing

10 mm-Tris−HCl, pH 7·4, 1 mm-EDTA and 0·5 % Triton X-100 (v/v) for 20 min. The membrane proteins (200 μg) were incubated overnight at 4°C with rabbit anti-NOX activator 1 polyclonal antibodies (ab103007; Abcam). Protein A-coupled agarose beads were used for immune complex purification. After 2 h of incubation, the beads were washed thrice in a radioimmunoprecipitation assay buffer, denatured in boiling Laemmli buffer and used for Western blot analysis. The antibodies employed for immunodetection were rabbit anti-NOX-1 polyclonal primary antibodies (ab55831; Abcam) (1:250 dilution) and mouse anti-rabbit horseradish peroxidase-conjugated secondary antibodies (1:2500 dilution). The immunoreactive bands were subjected to a densitometric analysis as described previously.

Uptake of indicaxanthin in Caco-2 cell monolayers

Caco-2 cell monolayers were incubated (37°C, 5 % CO_2) with 25 μm-Ind. The incubation medium was removed at time intervals, and the cells were washed with sodium taurocholate and extracted with methanol for the HPLC measurement of the pigment. HPLC was performed according to the method of Tesoriere *et al.*[23] using an RP-18e Performance column (100 × 4·6 mm; Merck), equipped with an RP-18e Chromolith guard cartridge (5 × 4·6 mm; Merck), eluted from solvent A (1 % acetic acid in water) to 20 % solvent B (1 % acetic acid in acetonitrile) with a 20 min linear gradient at a flow rate of 1 ml/min. Spectrophotometric detection of Ind (retention time 9·3 min) was carried out at 482 nm. Quantification was done by referring to standard curves constructed with 5−100 ng of the pigment and by relating its amount to the peak area.

Statistical analysis

Results are given as means and standard deviations. Unless specified, three independent observations were carried out for each experiment replicated thrice. Calculations and graphs were obtained using the INSTAT-3 statistical software (GraphPad Software, Inc.) with a test for normality followed by ANOVA, with Bonferroni's correction for multiple comparisons. In all cases, significance was accepted if the null hypothesis was rejected at the $P < 0.05$ level.

Results

Effects of indicaxanthin on IL-1β-induced secretion of inflammatory mediators and increase of tight junction permeability

The eventual cytotoxic effect of Ind was preliminarily checked by assaying the extracellular activity of lactate dehydrogenase released from Caco-2 cells. No significant activity of lactate dehydrogenase was detected in the culture media after 24 h of incubation of the cells with amounts of Ind ranging from 25 to 100 μm (data not shown). The incubation of Caco-2 cells with 25 ng/ml of IL-1β, for 24 h, led to an increased secretion of IL-6 and IL-8, to the extent of 10-fold and 24·8-fold, respectively, compared with the cells incubated

in the absence of the cytokine (Fig. 2). The co-incubation of the cells with Ind at amounts ranging from 5 to 25 μm resulted in a concentration-dependent inhibition of the release of both pro-inflammatory cytokines. Treatment with 25 μm-Ind reduced the levels of IL-6 and IL-8 in the inflamed cells by 75 and 65 %, respectively (Fig. 2).

PGE2 and NO are the effectors of an inflammatory response; however, basal levels of PGE2 (0·31 (sd 0·05) ng/mg protein) and NO (3·0 (sd 0·4) μm) were observed in the colon epithelial cells due to the activities of COX-1 and constitutive NOS, respectively, even in the absence of inflammatory stimulation
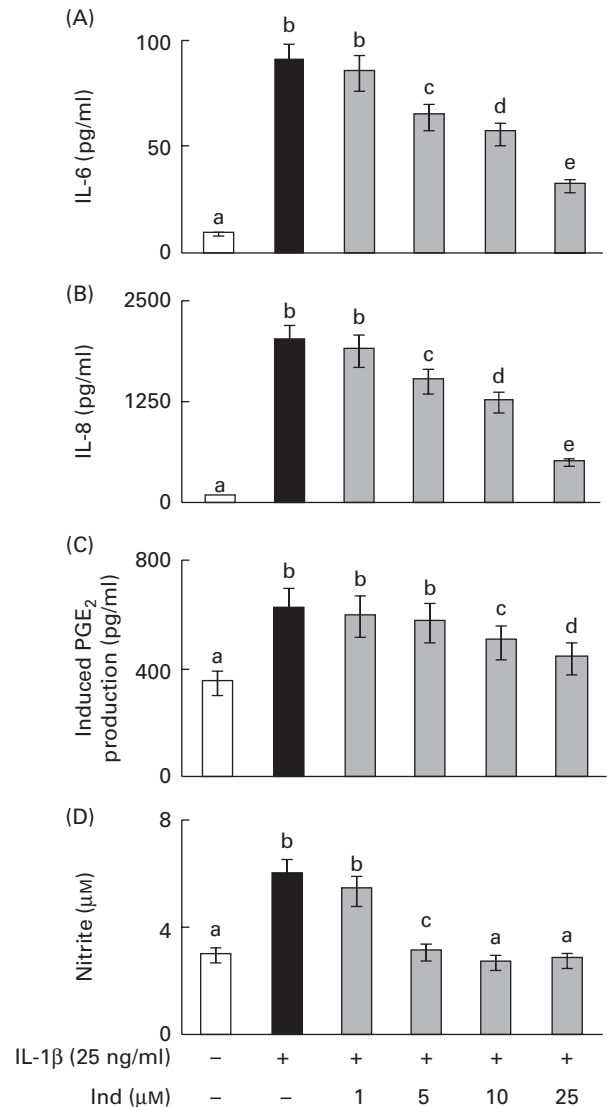

Fig. 2. Effect of indicaxanthin (Ind) on the release of inflammatory mediators by IL-1β-treated Caco-2 cell monolayers. Caco-2 cell monolayers were incubated with IL-1β either in the absence or in the presence of Ind. The release of (A) IL-6, (B) IL-8, (C) PGE2 and (D) nitric oxide into the medium was measured after 24 h of incubation. The levels of IL-6 and IL-8 were evaluated using ELISA. The activation of the arachidonic acid cascade was determined as the amount of PGE2 secreted after incubation for an additional 10 min with arachidonic acid and is referred to as the induced PGE2 production. Nitric oxide present in the medium as nitrite was assayed using the Griess colorimetric method. Values are means of three experiments carried out in triplicate, with standard deviations represented by vertical bars. [a,b,c,d,e] Mean values with unlike letters were significantly different ($P < 0.05$; Bonferroni's test).

(Fig. 2). Under inflammatory stimulation, a sharp increase in the levels of PGE$_2$ and NO, by the activity of the inducible COX-2 and iNOS, is expected. Accordingly, the activation of Caco-2 cell monolayers with IL-1β for 24 h brought about a 5·2-fold and a 2-fold increase in the levels of PGE$_2$ and NO levels, respectively. The co-treatment of the cells with Ind inhibited the production of PGE$_2$ and NO in a dose-dependent manner. The release of PG was inhibited by 70 % in the presence of 25 μM pigment, whereas the IL-1β-dependent NO formation was totally prevented by 10 μM-Ind (Fig. 2). The incubation of Caco-2 cell monolayers with Ind (25 μM) for 24 h did not significantly modify the basal release of any of the considered inflammatory mediators (not shown). Amounts of Ind as low as 1·0 μM were not effective in decreasing the levels of any of the measured inflammatory mediators (Fig. 2(A)–(D)).

The effect of IL-1β on the TJ permeability of filter-grown Caco-2 monolayers was evaluated by measuring the transepithelial electrical resistance with respect to the untreated cells. A drop in the transepithelial electrical resistance of Caco-2 cells by about 30 % was evident after 24 h of treatment with IL-1β, which was prevented by co-incubating the cells with 25 μM-Ind (Fig. 3).

Effect of indicaxanthin on the IL-1β-induced cell redox imbalance and NADPH oxidase activation

ROS are considered as intracellular second messengers of the signalling pathway associated with the Toll/IL-1β receptor[29]. The levels of intracellular ROS were monitored from 30 min to 3 h in Caco-2 cell monolayers incubated with IL-1β, either in the absence or in the presence of Ind. Cytofluorimetric analysis with 2′,7′-dichlorofluorescin diacetate showed a rapid production of ROS, with a significant peak after 1 h of treatment and a plateau in the following 2 h (Fig. 4(A)). As a consequence of the production of ROS, the levels of cell total thiols progressively decreased (Fig. 4(B)). The co-incubation of the cells with IL-1β and Ind resulted in the inhibition of both the production of ROS and the loss of thiols, an effect dependent on the concentration of Ind (Fig. 4(A) and (B)).
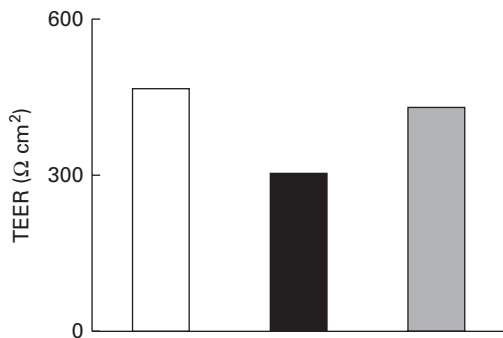

Fig. 3. Effect of indicaxanthin (Ind) on IL-1β-induced decrease of tight junction permeability in Caco-2 cells. Caco-2 cells grown in Transwells were incubated for 24 h with IL-1β (25 ng/ml) either in the absence or in the presence of Ind (25 μM). Control, cells treated with the vehicle. Transepithelial electrical resistance (TEER) values across the cell monolayers were measured using a volt ohm meter as reported in the Materials and methods section. Data are the mean of two experiments carried out in duplicate. □, Control; ■, IL-1β; ▨, IL-1β + Ind.
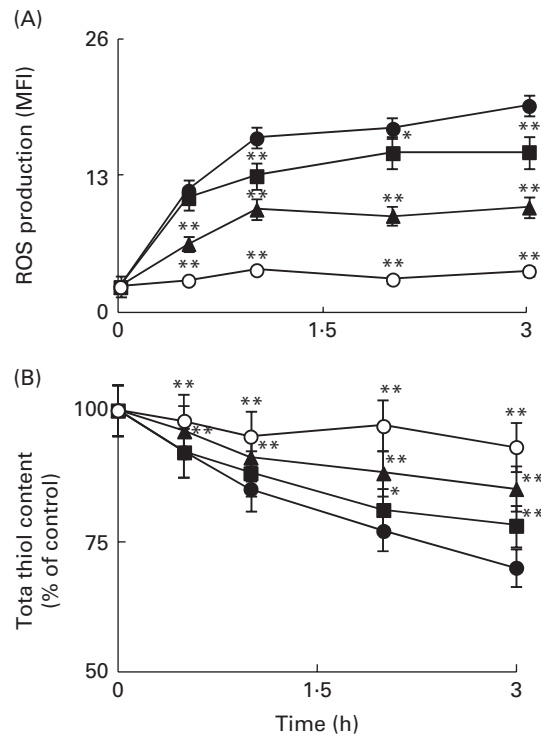

Fig. 4. Effect of indicaxanthin (Ind) on oxidative stress induced by IL-1β in Caco-2 cells. (A) Reactive oxygen species (ROS) production. (B) Thiol depletion. Caco-2 cells were treated with IL-1β (25 ng/ml) either alone or in combination with Ind at different time intervals. Control, cells treated with the vehicle. The levels of cellular ROS and total thiols were assayed using flow cytometry (2′,7′-dichlorofluorescin diacetate staining) and by the 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) reaction, respectively, as reported in the Materials and methods section. Values are means of three separate experiments carried out in triplicate, with standard deviations represented by vertical bars. Mean value was significantly different from that of the relevant control: * $P<0.05$, ** $P<0.01$ (Student's t test). MFI, mean fluorescence intensity. ●, Control; ■, 5 μM-Ind; ▲, 10 μM-Ind; ○, 25 μM-Ind.

The membrane NOX-1 has been identified as a source of ROS in the gastrointestinal tract. Under inflammatory stimulation, the activation of NOX-1 requires the assembly of cytosolic subunits (NADPH oxidase organiser 1 (NOXO1), NOX activator 1 and Ras-related C3 botulinium toxin substrate 1 (Rac1)) with the membrane-bound functional heterodimer (NOX-1 and p22$_{phox}$)[30]. The activation of the membrane NOX-1 complex in Caco-2 cells, after 30 min of incubation with either IL-1β or IL-1β and Ind, was evaluated by Western blot analysis of the cytosolic subunit NOXA-1 assembled in the membrane enzyme complex (Fig. 5). With respect to the control cells, the densitometric analysis of IL-1β-activated cells showed the appearance of NOX-1-activated complex. When Caco-2 cells were co-incubated with IL-1β and 25 μM-Ind, the activation of the NOX-1 complex was prevented (Fig. 5).

Effect of indicaxanthin on IL-1β-induced cyclo-oxygenase-2 and inducible nitric oxide synthase expression and NF-κB activation

The expression of COX-2 and iNOS was assessed by Western blot analysis in IL-1β-stimulated Caco-2 cells and in cells

treated simultaneously with IL-1β and Ind. While COX-2 and iNOS were undetectable in the control cells, both enzymes were noticeably induced in cells treated with IL-1β for 24 h. Ind significantly reduced the expression of both COX-2 and iNOS; the higher the concentration, the lower the enzyme level (Fig. 6).

The transcriptional activation of pro-inflammatory genes is regulated by NF-κB. IL-1β induces the activation of the NF-κB transcription factor by phosphorylation of its inhibitor IκB-α, which is then degraded by the proteasome, allowing the translocation of NF-κB into the nucleus[31]. A Western blot analysis of Caco-2 cells treated with IL-1β for 12 h showed a net decrease in the levels of cytosolic IκB-α with an increase in the levels of its phosphorylated form (phospho-IκB-α), accompanied by the nuclear translocation of the NF-κB p65 subunit. The co-incubation of the cells with Ind and IL-1β inhibited the activation of NF-κB in a dose-dependent manner (Fig. 7).

Uptake of indicaxanthin in Caco-2 cell monolayers

HPLC measurements of cell extracts were carried out to explore the uptake of Ind (25 μM) in IL-1β-treated cells when compared with control Caco-2 cells. Under both conditions, a quite comparable ($P > 0.05$, Student's t test) concentration-dependent uptake was observed, reaching equilibrium within 20 min of incubation (Fig. 8).

Discussion

Ind, a dietary pigment that characterises the fruit of cactus pear[15], has recently attracted attention for a number of biological activities based on *in vitro* data, ranging from cytoprotective effects[19,20] to the modulation of intracellular redox-dependent signalling[21,22], and for its high bioavailability in humans[25]. In the present study, we provide evidence of a remarkable anti-inflammatory effect of the molecule in an *in vitro* model of intestinal inflammation consisting of IL-1β-activated Caco-2 cell monolayers. The effect was found to be associated with the inhibition of the production of NOX-1 and ROS and the activation of NF-κB and downstream events, such as the release of inflammatory mediators
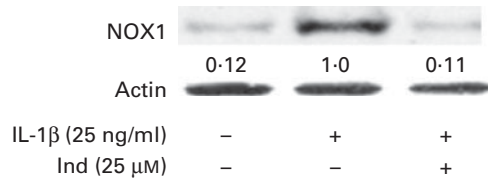
Fig. 6. Effect of indicaxanthin (Ind) on the IL-1β-induced increase of inducible nitric oxide synthase (iNOS) and cyclo-oxygenase-2 (COX-2) expression in Caco-2 cells. Caco-2 cells were incubated for 24 h with IL-1β either in the absence or in the presence of Ind. Cell lysates were subjected to Western blot analysis with the indicated antibodies as reported in the Materials and methods section. The numbers below each line represent the relative expression level normalised to actin, as determined on the basis of the intensity of the band. Representative image of three experiments with similar results.
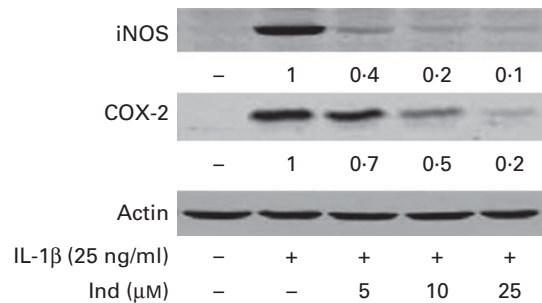
and the over-expression of COX-2 and iNOS. Finally, the epithelial permeability appeared to be preserved.

Inflammatory mediators such as IL-8, IL-6, PG and NO are constitutively produced by intestinal cells as a part of the physiological immune response of the mucosa. The IL-8 chemokine directs the migration of polymorphonuclear leucocytes, monocytes and macrophages towards the site of inflammation, and the inflammatory IL-6 cytokine stimulates the chemotaxis of neutrophils and is associated with necrosis in the colon, which in turn leads to tissue destruction[3,6]. NO regulates blood flow, pain transmission and immune system activation[32], whereas PGE$_2$ stimulates mucus and bicarbonate secretion, elevates mucosal blood flow, regulates leucocyte recruitment, and mediates fever and pain[4,33]. IL-1β, a key mediator of cell communication within the inflammatory intestinal area, stimulates the synthesis and secretion of all these factors[5–7] and then allows recruitment to the intestinal mucosa and activation of other immune cells such as neutrophils, which promote a self-sustaining inflammatory loop. In our model, the activation of Caco-2 cells by IL-1β resulted in a dramatic increase in the secretion of IL-6, IL-8, PGE$_2$ and NO, an effect remarkably inhibited by co-treatment with Ind, with a concurrent inhibition of the expression of COX-2 and iNOS. As an apparent contrast with our findings, other authors have reported that the release of NO from Caco-2 cells is not modified by IL-1β treatment[34]. Differences including cell phenotype, passage number and seeding can account for this discrepancy[35].

The NF-κB transcription factor is crucial for a number of cell processes including inflammation, proliferation and apoptosis and plays a pivotal role in chronic inflammatory diseases[36]. In resting cells, the most abundant form of NF-κB is the cytosolic inactive p65/p50 heterotrimer, with the p65 subunit, containing the transcriptional activation domain, bound to the IκB inhibitory protein. A number of extracellular stimuli, including inflammatory cytokines, activate signalling pathways that converge on the phosphorylation and inactivation of IκB, followed by nuclear translocation of the p65/p50 dimer and its binding to specific response elements in the DNA[37].

Fig. 5. Effect of indicaxanthin (Ind) on the activation of NADPH oxidase 1 (NOX-1). Caco-2 cells were treated with IL-1β (25 ng/ml) either alone or in combination with Ind for 15 min. The activation of NOX-1 was detected by Western blot analysis. After immunoprecipitation with NOXA1 antibodies, cell membrane proteins were separated by SDS–PAGE and immunoblotted with membrane enzyme component NOX-1 as reported in the Materials and methods section. The numbers below each line represent the relative expression level normalised to actin, as determined on the basis of the intensity of the band. Representative image of three experiments with similar results.

(A)

Iκ-Bα	1	0·2	0·4	0·7	1·0
Phospho-Iκ-B	–	1	0·8	0·4	0·2
Actin					
IL-1β (25 ng/ml)	–	+	+	+	+
Ind (μM)	–	–	5	10	25

(B)

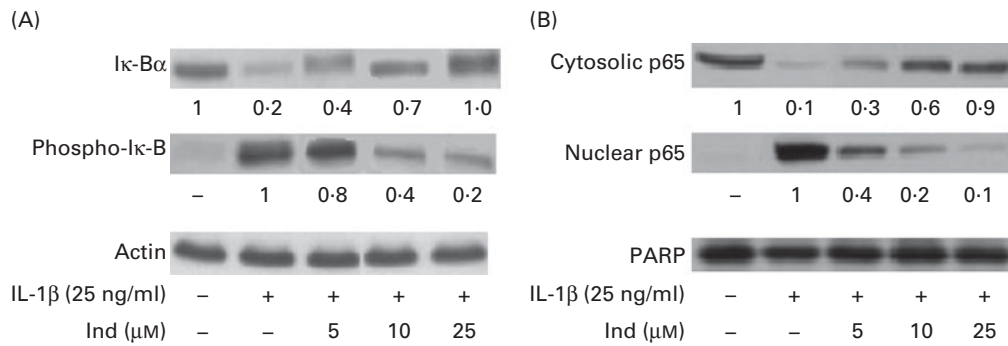| Cytosolic p65 | 1 | 0·1 | 0·3 | 0·6 | 0·9 |
Nuclear p65	–	1	0·4	0·2	0·1
PARP					
IL-1β (25 ng/ml)	–	+	+	+	+
Ind (μM)	–	–	5	10	25

Fig. 7. Effect of indicaxanthin (Ind) on IL-1β-induced (A) Iκ-B phosphorylation and (B) p65 nuclear translocation in Caco-2 cells. Caco-2 cells were incubated for 12 h with IL-1β either in the absence or in the presence of Ind. Control, cells treated with the vehicle. Cellular and nuclear lysates were subjected to Western blot analysis with the indicated antibodies as reported in the Materials and methods section. The numbers below each line represent the relative expression level normalised to actin (cytosolic protein) or poly(ADP-ribose) polymerase (nuclear proteins) as determined on the basis of the intensity of the band. Representative image of three experiments with similar results.

In the present study, the remarkable increase in the levels of the nuclear p65 subunit consequent to the stimulation of IL-1β was inhibited by Ind pre-treatment of Caco-2 cells. Considering that NF-κB, in synergy with other transcriptional activators, coordinates the gene expression of pro-inflammatory enzymes and cytokines, including IL-6[38], IL-8[39], COX-2[33] and iNOS[31], these findings suggest that the anti-inflammatory activity of Ind is, at least in part, mediated by the inhibition of the NF-κB activation pathway in our cell model. Consistent with these conclusions, in an in vivo model of IBD[40,41], the anti-inflammatory effects of polyphenolic redox-active/antioxidant phytochemicals such as quercitrin and curcumin have been shown to be mediated through the suppression of iNOS and COX-2 and the down-regulation of NF-κB signalling.

Signal transduction and inflammatory response induced by IL-1β have been reported to be related to the activity of NOX-1 in a number of cells. NOX-1, a member of the NOX-1 family, is highly expressed in colon tissue, as well as in Caco-2 cells[42,43], and ROS produced by this enzyme have been suggested to be second messengers in the immune response of these cells[42,44], therefore being potentially involved in the pathogenesis of IBD[45]. In accordance
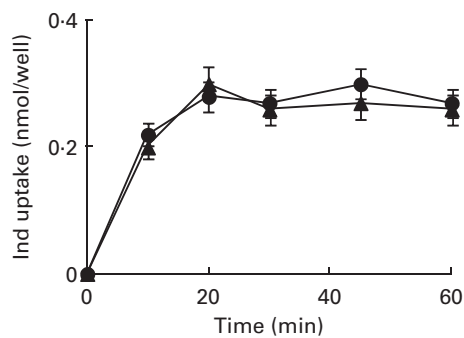

Fig. 8. Uptake of indicaxanthin (Ind) in Caco-2 cells. Caco-2 cell monolayers were incubated with 25 μM-Ind either in the absence or in the presence of IL-1β (25 ng/ml) for the indicated times. The gathered cells were extracted for the HPLC analysis of Ind as reported in the Materials and methods section. Values are means of three separate experiments carried out in triplicate, with standard deviations represented by vertical bars. —●—, Ind; —▲—, Ind + IL-1β.

with the expected inflammatory sequence of events, we report that the stimulation of Caco-2 cells with IL-1β results in a very rapid ROS production with thiol loss, concomitant with the transport of the NOX activator 1 subunit and activation of NOX-1 at the plasma membrane. Ind inhibited the IL-1β-induced ROS generation and maintained the cell redox balance. Moreover, the activated NOX-1 complex was not detected in the presence of the pigment. Then, although Ind is an effective radical scavenger[16,17], the observed anti-inflammatory effect may not be limited to a mere scavenging of NOX-1-derived oxidant species. Physicochemical characteristics[24] allow Ind to interact with and locate in the phospholipid bilayers of biomimetic membrane aqueous dispersions[46] and enter into erythrocytes both in vivo and in vitro and, as observed in the present study, in Caco-2 cells under either normal conditions or an inflammatory stimulus. In this context, the activity of Ind could be hypothesised to be at the cell membrane level, e.g. interference with the binding of IL-1β with its cell surface receptor and/or the ensuing recruiting of effectors leading to the activation of NF-κB.

A leaky epithelial barrier is a hallmark of many diseased states and, in particular, of those affecting the intestine such as IBD, resulting in aberrant exposure of the bloodstream to luminal substances and pathogens, toxins and allergens. This perturbation is attributed to the effect of cytokines on the structure and function of TJ[47,48]. It has been reported that IL-1β induces an increase of intestinal epithelial TJ permeability both in vivo[49] and in Caco-2 cell monolayers[11], as a result of the activation of NF-κB-dependent pathways. Under the conditions of the present study, Ind inhibited the activation of NF-κB and significantly corrected the increase of permeability caused by the inflammatory stimulus. Although there is active research on the anti-inflammatory activity of putative nutraceuticals at the intestinal level[50,51], such a protective effect on TJ permeability has not been reported. Polyphenols such as epigallocatechin-3-gallate and genistein, reported to inhibit the production of inflammatory mediators, have been shown to not attenuate the loss of epithelium integrity in inflamed Caco-2 monolayers[52]. Although pathways downstream the activation of NF-κB may have been inhibited or modulated by Ind, it may be interesting to

mention that, differently from the great majority of phyto-chemicals, Ind does not undergo metabolism in the mucosal cells and can cross the intestinal epithelial barrier through the paracellular route, thus interacting with the TJ[24]. This concept has recently put forward that epithelial permeability and TJ may be modified by dietary means[53]. The specific activity of Ind at the level of the complex structure of intestinal TJ, in both normal and diseases states, deserves to be investigated.

The modulation of redox-sensitive signalling pathways that support inflammatory processes by dietary redox-active phytochemicals in the intestinal mucosa may be of particular interest, the region in which these compounds can attain relatively high concentrations. In this context, Ind has been shown to be quite stable under digestive conditions[23,24], with only a 30 % loss at the gastric level. This would allow this phytochemical to reach even a 100 μM concentration in the small intestine, when ingested with dietary amounts of cactus pear fruit pulp (200 g, 24 mg Ind)[23,25] and considering an intestinal volume of 600 ml[54]. On the basis of this, Ind concentrations effective in modulating the immune response of the intestinal cells in our model are physiologically relevant. Given the interest in the identification of natural compounds for the prevention and/or progression of inflammatory states and inflammation-associated diseases[55], our data concerning a dietary highly bioavailable compound may offer an interesting perspective for a multimodal approach to IBD therapy.

Acknowledgements

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