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, PGE₂ 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-1 can alter the tight junction (TJ) permeability of intestinal epithelia. This allows the permeation of inflammatory cells and 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

Abbreviations: IBD, inflammatory bowel disease; IND, indicaxanthin; NOX-1, NADPH oxidase; ROS, reactive oxygen species; TJ, tight junction.

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radical-scavenging activity\textsuperscript{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\textsuperscript{18} to either healthy\textsuperscript{19} or pathological\textsuperscript{20} erythrocytes, as well as cell cultures\textsuperscript{21,22}. From a nutritional perspective, \textit{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\textsuperscript{23}, is not metabolised by enterocytes and is absorbed through a paracellular mechanism characterised by a high permeability coefficient\textsuperscript{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 2.36 h\textsuperscript{25}.

Reactive oxygen species (ROS) overproduction and cell redox imbalance play a central role in the pathophysiology of the inflammatory response\textsuperscript{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\textsuperscript{28}. Taking into account the redox chemistry and bioactivity of Ind, in the present study, we investigated the effects of this pigment in an \textit{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\textsuperscript{29}, exposed to IL-1β, known as an IBD causative pathophysiological agent\textsuperscript{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 (\textit{O. ficus-indica}) fruits (yellow cultivar). The phytochemical was separated from a methanol extract of the pulp by liquid chromatography on Sephadex G-25\textsuperscript{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\textsuperscript{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 cm\textsuperscript{2} 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% CO\textsubscript{2}. For the experiments, the cells were seeded at a density of 1·25 × 10\textsuperscript{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\textsuperscript{30} 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\textsuperscript{4} cells/cm\textsuperscript{2} 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 Ω cm\textsuperscript{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 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 PGE2 production**
The activation of the arachidonic acid cascade was estimated through the production of PGE2 after the addition of arachidonic acid. Briefly, 24 h after the treatment, the cells were washed and incubated with 10 μM-arachidonic acid in PBS for 10 min. The secretion of PGE2 in the extracellular medium was quantified (pg/ml) using a Prostaglandin E2 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 Griess reagent (equal volumes of 1 % sulphanilamide (w/v) in 5 % phosphoric acid (v/v) and 0.1 % naphtylethylene-diamine–HCl (w/v)) was added to 100 μl of Griess reagent (equal volumes of 1 % sulphamidamide (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 (GloMax 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 DCFH-DA (dichlorofluorescin diacetate). DCFH-DA 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 x 10^5 cells were analysed for each sample.

**Assay for total reduced thiols**
After the treatment, the cells were centrifuged for 5 min at 2000 g, washed twice with cold PBS containing 0.1 %-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-MgCl2, 10 mM-KCl, 0.5 mM-phenylmethylsulphonyl fluoride, 1.5 μg/ml soybean 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 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-MgCl2, 0.2 mM-EDTA, 25 % (v/v) glycerol, 0.5 mM-phenylmethylsulphonyl fluoride, 1.5 μg/ml soybean 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 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/lane) 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. AF4808; 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 g for 1 h at 4°C. The pellet, containing the membrane fraction, was suspended in a cold buffer containing...
Uptake of indicaxanthin in Caco-2 cell monolayers

Caco-2 cell monolayers were incubated (37°C, 5% CO2) 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.\(^\text{(23)}\) using an RP-18e Performance column (5 x 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).
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-1 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 cell 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. The transepithelial electrical resistance (TEER (Ω cm²)) 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.

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(23,24). 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 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(33). NO regulates blood flow, pain transmission and immune system activation(34), whereas PGE2 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, PGE2 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).
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 pretreatment 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[46], IL-8[47], COX-2[48] and iNOS[49], 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 vitro model of IBD[40,41], the anti-inflammatory effects of polyphenolic redox-active/antioxidant phytochemicals such as quercetin 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 activation 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[49]. In accordance 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 cellular 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[46,47], the observed anti-inflammatory effect may not be limited to a mere scavenging of NOX-1-derived oxidant species. Physicochemical characteristics[48,49] allow Ind to interact with and locate in the phospholipid bilayers of biomimetic membrane aqueous dispersions[46] and enter into erythrocytes both in vitro and in vivo 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 transport of the NOX activator 1 subunit and NF-κB 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.

![Diagram](https://www.cambridge.org/core/figshare/5470441.1)
mention that, differently from the great majority of phytochemicals, 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. This concept has recently put forward that epithelial permeability and TJ may be modified by dietary means. 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, 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) and considering an intestinal volume of 600 ml. 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 of the intestinal cells in our model are physiologically relevant.

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L. T. and M. A. designed the research; A. A. and C. G. carried out the experimental work; M. A. L. coordinated the work and discussed the experimental data. All authors read and approved the final manuscript.

None of the authors has any conflicts of interest.

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