

Modulation of signalling nuclear factor- κ B activation pathway by polyphenols in human intestinal Caco-2 cells

Béatrice Romier, Jacqueline Van De Walle, Alexandrine During, Yvan Larondelle and Yves-Jacques Schneider*

Biochimie Cellulaire, Nutritionnelle and Toxicologique, Institut des Sciences de la Vie and Université Catholique de Louvain, Louvain-la-Neuve B 1348, Belgium

(Received 25 May 2007 – Revised 16 November 2007 – Accepted 12 December 2007 – First published online 1 April 2008)

Recent studies support beneficial effects of polyphenols in various chronic inflammatory diseases, for example, the inflammatory bowel diseases. Inhibition of NF- κ B activation by polyphenols could explain part of their anti-inflammatory properties, but few data are available on the intestine. The purpose of the present study was thus to investigate the effects of some polyphenols on NF- κ B activation using human intestinal Caco-2 cells. Effects of standard polyphenols (50 μ mol/l) were studied on different cellular events associated with NF- κ B activation: (i) NF- κ B activity using cells transiently transfected with a NF- κ B–luciferase construct and stimulated by inflammatory agents (IL-1 β , TNF- α or lipopolysaccharides (LPS)); (ii) phosphorylation of the inhibitor of κ B (I κ B- α) analysed by Western blot; (iii) secretion of IL-8 quantified by ELISA assay. Results showed that chrysin and ellagic acid inhibited NF- κ B activity, whereas genistein and resveratrol increased it. These effects were independent of the nature of the inducer, indicating that polyphenols may modulate NF- κ B activation by acting on a common event to the cytokine- and LPS-mediated cascades. Chrysin strongly reduced (2.5-fold) IL-1 β -induced I κ B- α phosphorylation, whereas ellagic acid increased it (1.7-fold). Ellagic acid, genistein and epigallocatechin gallate reduced (4- to 8-fold) IL-1 β -induced IL-8 secretion, while resveratrol promoted (1.7-fold) the secretion. Chrysin also diminished IL-8 secretion by 1.6-fold (but $P > 0.05$). The data indicate that polyphenols can modulate the NF- κ B activation pathway in the intestine. Chrysin could block NF- κ B activation via the inhibition of I κ B- α phosphorylation. The other molecular targets of the active polyphenols are still to be identified.

Intestinal inflammation: Nuclear factor- κ B: Inhibitor of κ B pathway: Interleukin-8 production: Polyphenols: Caco-2 cells

Inflammation is a mechanism of defence developed by the body to fight infections (originated by viruses, bacteria and other pathogens) and injuries (bruising, cuts and burns). Inflammation is recognised as a type of non-specific immune response and as a component of the pathogenesis of a number of important human diseases, such as inflammatory bowel diseases (IBD). Among IBD, Crohn's disease and ulcerative colitis are two major forms commonly found in the Western world, affecting 0.5–1% of the population. Their incidence rates are high (5.6 and 10.4 new cases per 100 000 inhabitants per year, respectively for Crohn's disease and ulcerative colitis in Europe in the 1990s) and have sharply increased since the early 1950s^(1–3). One of the worst complications of IBD, especially ulcerative colitis, is the increased risk of colorectal cancer⁽⁴⁾. Current epidemiological and experimental studies support a beneficial role of dietary polyphenols in several gastrointestinal diseases, including IBD^(5,6).

Polyphenols are the most abundant antioxidants in the human diet and are found in many plant-derived products, i.e. fruits, vegetables, beverages, herbs and spices. Both their

high intake (daily intake of about 1 g polyphenols/d) and their poor intestinal absorption are responsible for high luminal concentrations of these compounds, up to 200 μ mol/l in the gastrointestinal tract^(7,8), indicating that polyphenols could contribute to digestive health. Polyphenols have indeed shown anti-inflammatory properties⁽⁹⁾ and thus could contribute, as complementary approaches to the conventional already existing therapeutic approaches (i.e. non-steroidal anti-inflammatory drugs), to the management of inflammatory bowel diseases. The idea of using natural plant products such as polyphenols to prevent intestinal inflammation is also supported by the general public to date. Therefore, it is important to better understand the molecular mechanisms and targets of polyphenols in the inflammatory process, which is, in the intestine as in other organs, regulated by cytokines and various classical mediators of inflammation.

NF- κ B activation plays a pivotal role in controlling chronic inflammatory diseases⁽¹⁰⁾. NF- κ B family proteins include several members (p50, p52, p65, RelB and cRel) and are found in the cytoplasm of most resting cells in an inactive state bound

Abbreviations: AP-1, activator protein-1; BSA, bovine serum albumin; DMSO, dimethyl sulfoxide; EGCG, epigallocatechin gallate; HRP, horseradish peroxidase; IBD, inflammatory bowel disease; I κ B, inhibitor of κ B; IEC, intestinal epithelial cells; LDH, lactate dehydrogenase; LPS, lipopolysaccharide; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium.

* **Corresponding author:** Dr Yves-Jacques Schneider, fax +32 10 47 48 95, email yjs@uclouvain.be

to inhibitory molecules of κ B (I κ B) (including I κ B- α , I κ B- β , I κ B- ϵ , I κ B- ζ , p100, p105 and Bcl-3). Upon stimulation by various substances (i.e. free radicals, pro-inflammatory cytokines such as IL-1 β and TNF- α , bacterial lipopolysaccharides (LPS), carcinogens, tumour promoters, UV radiation, etc), I κ B of the NF- κ B/I κ B heterodimer is phosphorylated via activated kinases and subsequently degraded, while the activated 'free' NF- κ B translocates to the nucleus. There, NF- κ B binds to specific DNA sequences and induces gene expression, in particular genes involved in the inflammatory response such as TNF- α , IL-1 β or IL-8 (for reviews, see Baldwin⁽¹¹⁾, Viatour *et al.*⁽¹²⁾, Chariot⁽¹³⁾ and Karin⁽¹⁴⁾). Several NF- κ B-activating cascades have been identified in relation to the nature of the stimulus, of the kinases, of the NF- κ B and I κ B proteins involved, and the subset of genes targeted. The phosphorylation of both NF- κ B and I κ B proteins mediated by kinase activities appears to play a key role in these NF- κ B-inducing pathways⁽¹³⁾. Note here that IBD are associated with a deregulation of two intracellular signalling pathways of NF- κ B activation, resulting in an aberrant NF- κ B activity⁽¹⁵⁾.

Several studies have focused on potential mechanisms responsible for the anti-inflammatory properties of polyphenols. The inhibitory effect of polyphenols on the NF- κ B activation pathways is one of them⁽¹⁶⁾. Such inhibition was observed for several polyphenols (quercetin, sesquiterpene lactones, quercetin, epigallocatechin gallate (EGCG), caffeic acid phenethyl ester, genistein, indole-3-carbinol and resveratrol) in various non-intestinal models^(17–24). Few data are, however, available on intestinal models. Green tea polyphenols were shown to be efficient compounds in several IBD models (i) by improving the colon health and decreasing serum markers of inflammation (including TNF- α) in induced-colitis rats⁽²⁵⁾ and mice⁽²⁶⁾ and (ii) by blocking NF- κ B activation via inhibition of I κ B phosphorylation in the rat cell line of intestinal epithelial cells (IEC)-6⁽²⁷⁾. Similarly, the flavonoid luteolin prevented NF- κ B activation by blocking I κ B kinase activity in another rat intestinal cell line, IEC-18⁽²⁸⁾.

More investigations are thus necessary to understand better by which mechanisms dietary polyphenols are involved in the inflammatory response of the gut in view of their potential use to combat IBD. The purpose of the present study was thus to investigate the potential targets of several polyphenols in the signalling NF- κ B activation pathways. In the present study, the effects of polyphenols were examined on cytokine- and LPS-induced NF- κ B activation and on IL-8 expression. Given the limitations of using human subjects for these kinds of investigations, we chose to work with a well-established intestine-like *in vitro* model, the human adenocarcinoma colon cell line Caco-2.

Experimental methods

Chemicals

Cell culture Iscove's modified Dulbecco's medium and Ham's F12 medium were purchased from Cambrex BioScience (Verriers, Belgium), non-essential amino acids and NCTC-135 medium from Invitrogen (Carlsbad, CA, USA) and other culture reagents such as L-glutamine, insulin, epidermal growth factor, linoleic acid-albumin, triiodothyronine (T₃), hydrocortisone

and ethanolamine from Sigma-Aldrich (St Louis, MO, USA). Gallic acid, ellagic acid, chrysin, naringenin, quercetin, genistein, catechin, epicatechin, resveratrol, EGCG, LPS (*Escherichia coli* O111:B4), IL-1 β , TNF- α and Igepal CA630 were obtained from Sigma-Aldrich and the proteasome inhibitor MG-132 from Calbiochem (Darmstadt, Germany). Rabbit polyclonal IgG antibodies against human phospho-I κ B- α were purchased from Cell Signaling Technology (Beverly, MA, USA), horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG antibodies from Dako (Glostrup, Denmark) and mouse monoclonal IgG1 antibodies against human β -actin from Sigma-Aldrich.

Cell culture

Caco-2 cells obtained from the American Type Culture Collection (Rockville, MD, USA) (passages 32 to 100) were routinely grown in a serum-free medium (mixture of Iscove's modified Dulbecco's medium, Ham's F12 and NCTC 135 media, 5:5:1, by vol.)⁽²⁹⁾ supplemented with 1% (v/v) non-essential amino acids, 2 mM-L-glutamine, insulin (1 μ g/ml), epidermal growth factor (1 ng/ml), albumin complexed to linoleic acid (10 μ g/ml), 2 nM-triiodothyronine, 100 nM-hydrocortisone, 0.06 mM-ethanolamine and NaHCO₃ (3 g/l). The seeding density was 2.85×10^4 cells/cm² with a 7 d passage frequency. Cells were incubated at 37°C in a humidified atmosphere of air-carbon dioxide (95:5, v/v).

Determination of cell viability

Caco-2 cells were plated at a density of 3×10^4 cells/cm² in ninety-six-well plates (Nunc A/S, Roskilde, Denmark). After 48 h, the cells were exposed to the different polyphenols at 50 μ M during a 24 h growth period, which was followed by cell proliferation and cytotoxicity assays. The inhibitory effect of the different polyphenols and their carrier vehicles (dimethyl sulfoxide (DMSO) and ethanol) on cell proliferation was determined by using a colorimetric method (CellTiter 96 AQueous One Solution Cell Proliferation assay; Promega Corp., Madison, WI, USA). This assay is based on the reduction of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS; inner salt) into formazan, which is directly proportional to the number of living cells in culture. The formazan production was recorded at 490 nm using a ninety-six-well plate reader. The percentage of inhibition of cell proliferation was calculated using the non-treated cells as control (100% proliferation). In a parallel experiment, the cytotoxic effect of polyphenols was determined by a colorimetric assay (Cytotoxicity detection kit (LDH); Roche Diagnostics GmbH, Mannheim, Germany), which is based on the measurement of the lactate dehydrogenase (LDH) activity released from damaged cells into the culture medium. This assay is thus performed on cell culture media and measures the reduction of MTS into formazan by NADH produced during the LDH-catalysed oxidation of lactate to pyruvate. Here, the formazan production, directly proportional to the LDH activity present in the cell culture medium, is monitored at 490 nm. The percentage of cytotoxicity was calculated using cells treated with Triton X-100 (1 g/l) as control (100% released LDH activity).

Plasmid preparation

The NF- κ B-dependent luciferase reporter plasmid containing the gene for luciferase under the transcriptional regulation of four copies of the NF- κ B response element (TGGGGATTC-CCCA) was a generous gift of Dr T. Mitchell (Madison, WI, USA). The number of copies of the plasmid was amplified in *E. coli* using the Subcloning Efficiency DH5 α Competent Cells kit (Invitrogen) and then the plasmid copies were purified according to the protocol of the QIAGEN Plasmid Purification Mega kit (Qiagen Inc., Venlo, The Netherlands).

Transient transfection and luciferase assays

Caco-2 cells seeded at 2×10^4 cells/cm² in twenty-four-well plates were cultured with the cell culture medium described above. Subconfluent cells (30–40% confluency) were transfected with 1 μ g of NF- κ B-dependent luciferase reporter plasmid preparation and 2 μ l of the cationic polymer transfection reagent jetPEI[™] (Lucron Bioproducts, De Pinte, Belgium). At 24 h post-transfection, the proliferating cells were pretreated with polyphenols at 50 μ mol/l for 4 h⁽³⁰⁾, and further incubated with a NF- κ B activator, either IL-1 β (25 ng/ml), TNF- α (50 ng/ml) or LPS (10 μ g/ml), for 24 h (in the presence of the polyphenol). Luciferase activity was then measured by using the Luciferase Reporter-Gene Assay (Promega Corp.) and a plate-reading luminometer. The effect of polyphenols on NF- κ B induction (estimated through the luciferase activity) was expressed as relative light units/ μ g protein and was calculated using cells treated with IL-1 β , TNF- α or LPS only, as positive controls (100% NF- κ B induction). The protein concentration of the cell homogenates was determined by using the Bicinchoninic Acid Protein assay kit (Sigma-Aldrich) based on the Lowry procedure⁽³¹⁾.

Western blot analysis of phospho-inhibitor of κ B- α

Cells were grown until confluency in six-well plates and then incubated with the different polyphenols at 50 μ mol/l for 4 h before the addition of IL-1 β at 25 ng/ml for 30 min. When indicated, the proteasome inhibitor MG-132 was added at 20 μ mol/l for 30 min before the IL-1 β treatment. Cells were washed in an ice-cold phosphate buffer (137 mM-NaCl, 2.68 mM-KCl, 1.14 mM-KH₂PO₄ and 8 mM-Na₂HPO₄; pH 7.2) and suspended in lysis buffer (phosphate buffer containing 1% (w/v) Igepal CA630, sodium deoxycholate (5 g/l) and sodium dodecylsulfate (1 g/l), supplemented with 0.2 mM-sodium vanadate, 50 mM-sodium fluoride and 1% (v/v) of a protease inhibitor cocktail used for tissue culture media (Sigma-Aldrich)). The lysate was centrifuged at 12 000 g for 10 min at 4°C. Protein concentration of the resultant supernatant fraction was then determined according to the bicinchoninic assay (Sigma-Aldrich) and 20 μ g protein were used for Western-blot analysis. Proteins were separated by SDS/PAGE electrophoresis on an 11% (w/v) acrylamide gel and transferred onto a hybond-P polyvinylidene difluoride membrane (Amersham Biosciences, Piscataway, NJ, USA). After blocking with bovine serum albumin (BSA) at 7% (w/v) in the Tris-buffered saline (137 mM-NaCl, 20 mM-Tris, pH 7.6) plus Tween 20 at 0.05% (v/v) (TBST) solution, the membrane was incubated overnight at

4°C with the primary antibody against total or phospho-I κ B- α at a 1:1000 dilution in TBST solution containing 0.5% (w/v) BSA. The membrane was washed three times with the TBST solution and incubated with the secondary HRP-labelled antibody at a 1:2000 dilution in TBST solution containing 0.5% (w/v) BSA for 1 h. After three washings with the TBST solution, the bound HRP-conjugated antibody was detected by chemiluminescence at a maximum emission of 430 nm according to the ECL Plus[™] Western Blotting Detection kit (Amersham Biosciences). The resulting light was detected on an autoradiography film. The membrane was then stripped and reprobed as described above, using non-fat dried milk and the primary antibody against β -actin instead of BSA and the antibody against total or phospho-I κ B- α , respectively.

Determination of interleukin-8 secretion

Confluent cells grown on six-well plates were first incubated with or without polyphenols at 50 μ mol/l for 4 h, and then stimulated by a 48 h exposure with IL-1 β at 25 ng/ml in the presence of the same polyphenols. After incubation, culture media were collected and processed for IL-8 quantification by the sandwich ELISA method according to the manufacturer's instructions (Human IL-8 ELISA Kit II; BD Biosciences Pharmingen, San Diego, CA, USA). Briefly, 100 μ l of the culture media were added into the wells of a ninety-six-well microplate precoated with a monoclonal IL-8 antibody, and incubated at room temperature for 2 h. The plate was then washed and a biotinylated anti-human IL-8 HRP-conjugated antibody was added to each well. After 2 h incubation, wells were washed and 100 μ l of a tetramethylbenzidine solution was added. After 30 min, the reaction was stopped by the addition of 50 μ l of a 1 M-phosphoric acid solution and the absorbance was read at 450 nm using a ninety-six-well plate reader. Values were determined in pg IL-8/ μ g cellular protein by using a standard curve made with a recombinant human IL-8 standard provided with the kit. Effects of polyphenols on IL-8 secretion were expressed as percentage of the positive control (cells stimulated by IL-1 β in the absence of polyphenols). Protein concentration in cells was determined according to the bicinchoninic assay (Sigma-Aldrich).

Statistical analysis

All data are expressed as mean values with their standard errors. Data were tested for homogeneity of variances by Bartlett's test. When homogeneous variances were confirmed, the data were analysed by one-way ANOVA coupled with the *post hoc* Fisher's least significant difference test to identify means with significant differences. The values underwent log-transformation before the tests, if necessary (as indicated in the figure legends). When heterogeneous variances were detected, the data were analysed by using the non-parametric Kruskal–Wallis test and significant differences of means were evaluated by the *post hoc* Scheffé test. All statistical analyses were performed using StatView software (version 5.0; SAS Institute, Cary, NC, USA). *P* values <0.05 were considered significant.

Results

Effects of polyphenols on cell proliferation

The influence of polyphenols on Caco-2 cell proliferation and metabolism was evaluated by measuring the mitochondrial succinate dehydrogenase activity (MTS test) in proliferating Caco-2 cells incubated for 24 h in the absence or presence of standard polyphenols. The working concentration for the different polyphenols was fixed at 50 μ mol/l, which fits into the range of the physiological concentrations that may be found in the human gut. Vehicles used for polyphenol delivery to cells were also tested alone: 0.5% DMSO, used for most of the standards, and 0.4% ethanol for genistein. Results are expressed as percentage of control (non-treated cells) and data are shown in Table 1. Neither DMSO nor ethanol had a significant effect on the cell proliferation. Among the polyphenols tested, only gallic acid and EGCG tended to reduce the cell proliferation, but these effects were not significantly different from that of DMSO. Note here that quercetin, catechin and epicatechin increased slightly the cell proliferation compared with DMSO ($P < 0.05$), but these effects were not significantly different from control cells.

Cytotoxic effects of polyphenols on Caco-2 cells

For each set of experiments, the cytotoxic effect of polyphenols was checked by assaying the extracellular LDH activity release from damaged cells. As illustrated by Table 1 for one typical experiment, no significant LDH activity was detected in the cell culture media after incubation of cells with the different polyphenols at 50 μ mol/l (less than 3% LDH activity, compared with the positive control (Triton-X100 at 0.1%)). Only genistein treatment showed a small

relative cytotoxicity when compared with the corresponding negative control (ethanol at 0.4%) ($P < 0.05$). Other polyphenol treatments did not have any cytotoxic effect that was significantly different in comparison with DMSO (0.5%) treatment.

Effects of polyphenols on the induced-nuclear factor- κ B activation

Caco-2 cells were transiently transfected with a reporter plasmid containing the luciferase gene under the transcriptional regulation of four NF- κ B response elements. Cells were then pretreated or not with the polyphenols at 50 μ mol/l for 4 h, and then incubated with one NF- κ B activator, either IL-1 β (25 ng/ml), TNF- α (50 ng/ml) or LPS (10 μ g/ml) for 24 h. The effects of polyphenols on NF- κ B induction (estimated through the luciferase activity) were expressed in relative terms (percentage of activity in cells treated with the inducer only) and data are presented in Fig. 1. IL-1 β , TNF- α and LPS alone increased NF- κ B activity by 3.9-, 1.9- and 3.0-fold, respectively, when compared with the control cells (non-treated cells). Effects of DMSO and ethanol (vehicles of polyphenols) were measured on NF- κ B activity; no significant differences from the control cells were observed. For the three pro-inflammatory molecules (IL-1 β , TNF- α and LPS), treatment with ellagic acid and chrysin decreased NF- κ B activation by about 2.0-fold (1.6–2.4) and by about 2.8-fold (2.3–2.9), respectively. Ellagic acid effects were significant only with LPS stimulation, while chrysin effects were significant with the three stimuli ($P < 0.05$). In contrast to chrysin and ellagic acid, genistein and resveratrol led to a significant increase in NF- κ B activation by about 2.7-fold (2.4–3.0) and by about 2.1-fold (1.7–2.4), respectively ($P < 0.05$). Treatment with gallic acid, quercetin, naringenin, catechin and

Table 1. Effect of polyphenols on cell proliferation (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium (MTS) test) and cytotoxicity (lactate dehydrogenase activity) in Caco-2 cells (Mean values with their standard errors from three independent experiments)

Polyphenol	Subclass	Treatment	Cell proliferation (% control)		Cytotoxicity (%)	
			Mean	SEM	Mean	SEM
		Control*	100.0 ^{b,c}	3.2	0.000	0.000
		Ethanol (0.4%)†	90.7 ^{a,b}	6.4	0.000 ^a	0.311
		Dimethyl sulfoxide (0.5%)†	91.7 ^{a,b}	3.9	0.000 ^a	0.247
		Triton X-100 (0.1%)‡	–	–	100 ^c	1.040
Phenolics		Ellagic acid§	90.6 ^{a,b}	2.1	0.000 ^{a,b}	0.930
		Gallic acid	79.9 ^a	4.0	0.000 ^a	0.033
Flavonoids	Flavonols	Quercetin	113.3 ^c	6.7	0.000 ^{a,b}	0.934
		Chrysin	101.1 ^{b,c}	3.8	0.407 ^{a,b}	0.107
	Isoflavones	Genistein	95.5 ^b	3.2	2.860 ^b	0.358
		Naringenin	87.3 ^{a,b}	2.9	0.271 ^{a,b}	0.368
	Flavanols	Catechin	111.4 ^c	4.5	0.530 ^{a,b}	0.065
		Epicatechin	111.4 ^c	4.7	0.555 ^{a,b}	0.216
Stilbenes		EGCG	79.2 ^a	2.5	0.000 ^{a,b}	0.101
		Resveratrol	86.6 ^{a,b}	8.3	0.246 ^{a,b}	0.685

EGCG, epigallocatechin gallate.

^{a,b,c} Mean values in a column with unlike superscript letters are significantly different ($P < 0.05$).

* The control condition corresponds to non-treated cells.

† All the polyphenol standards were delivered to cells in 0.5% (v/v) dimethyl sulfoxide, except genistein, which was solubilised in 0.4% (v/v) ethanol.

‡ For lactate dehydrogenase measurement (or cytotoxicity), Triton X-100 at 0.1% (v/v) was used as the positive control.

§ Each polyphenol standard was at the final concentration of 50 μ mol/l in the cell culture medium.

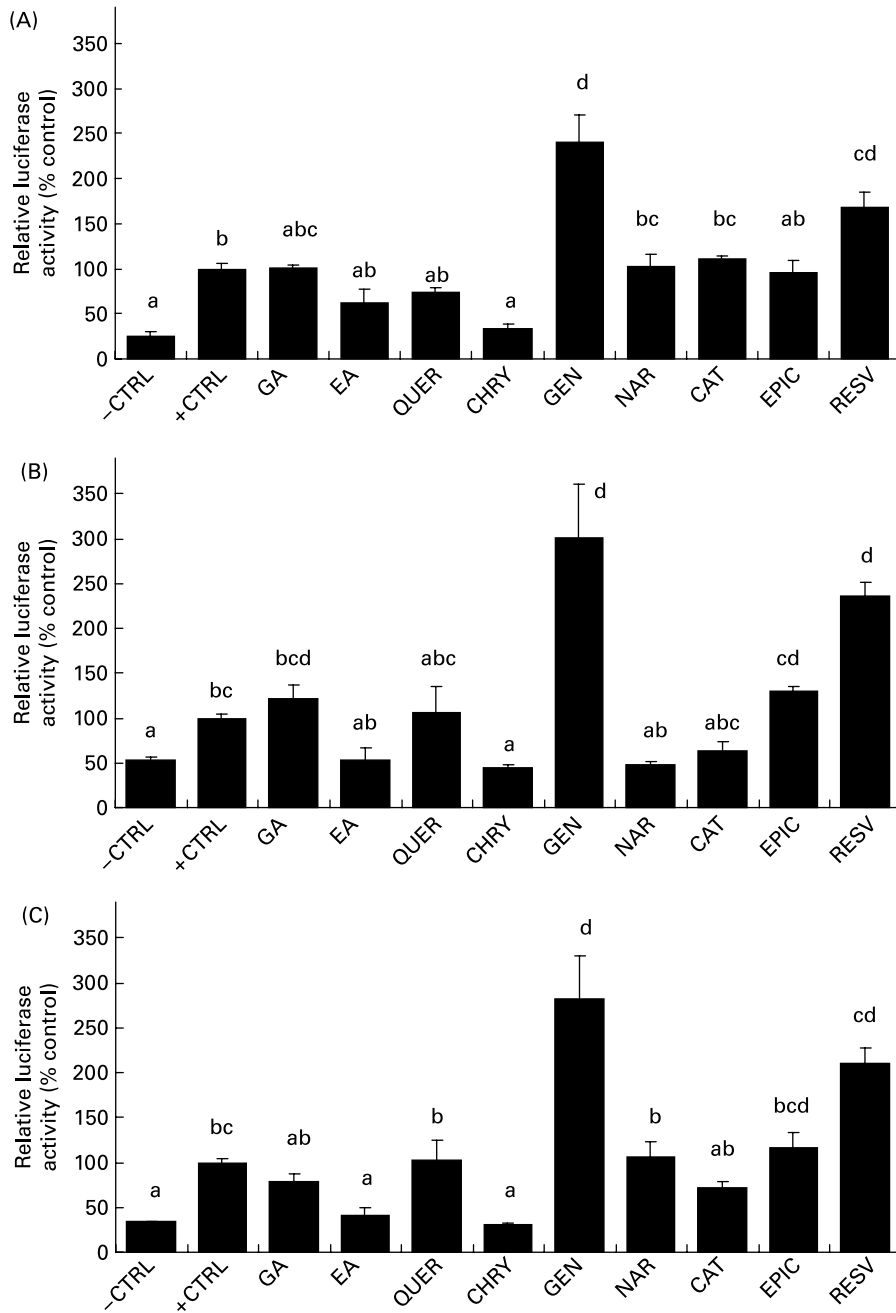


Fig. 1. Effects of polyphenols on NF-κB induction by (A) IL-1β, (B) TNF-α or (C) lipopolysaccharides in Caco-2 cells at confluency. The effect of polyphenols on NF-κB induction (evaluated through the luciferase activity) was expressed as relative light units/μg cell protein and was calculated using cells treated with IL-1β, TNF-α or LPS only, as positive controls (+CTRL) (100% NF-κB induction). Negative controls (-CTRL) corresponded to cells incubated with neither NF-κB inducer nor polyphenol. GA, gallic acid; EA, ellagic acid; QUER, quercetin; CHRY, chrysin; GEN, genistein; NAR, naringenin; CAT, catechin; EPIC, epicatechin; RESV, resveratrol. Values are means of three or four independent experiments (A and C) and of four to six assays from two or three independent experiments (B), with standard errors represented by vertical bars. Data were compared among groups (Scheffé's test). ^{a-d} Mean values with unlike letters are significantly different (*P* < 0.05).

epicatechin did not show any significant effect on NF-κB activation induced by either IL-1β, TNF-α or LPS.

The effects of the concentration of four polyphenols on NF-κB induction by IL-1β were also examined. Caco-2 cells were pretreated for 4 h with different concentrations (5, 25, 50 and 100 μmol/l) of either chrysin, ellagic acid, genistein or resveratrol and then stimulated with IL-1β for

24 h (data from two independent experiments). Genistein and resveratrol increased NF-κB activation in a dose-dependent manner; an effect was observed from 5 μmol/l for the two polyphenols. Chrysin and ellagic acid decreased NF-κB activation also in a dose-dependent manner and the effects occurred from 25 μmol/l for chrysin and from 50 μmol/l for ellagic acid (data not shown).

Effects of polyphenols on interleukin-1β-induced inhibitor of κB-α phosphorylation

The translocation of NF-κB to the nucleus and its action on inflammatory genes is preceded by the phosphorylation, ubiquitination and proteasome degradation of IκB-α. In order to determine if some of the polyphenols tested had an effect on IκB-α phosphorylation and consequently its degradation, we examined the expression level of phosphorylated IκB-α by Western blot. In this experiment, cells were pre-treated with polyphenols (50 μmol/l) and then incubated with the proteasome inhibitor MG-132 (50 μmol/l) for 30 min before their stimulation with IL-1β (25 ng/ml) for 30 min, still in the presence of polyphenols. After incubation, cells were lysed and assayed by Western blot using an antibody against serine-phosphorylated IκB-α (see Fig. 2). The data indicate that the treatment with IL-1β alone resulted in an increase by 2.6-fold of the level of phosphorylated IκB-α and was not influenced by the presence of vehicles (DMSO

or ethanol). Chrysin significantly decreased (2.5-fold) the IκB-α phosphorylation induced by IL-1β ($P < 0.05$), whereas ellagic acid further increased IκB-α phosphorylation by 1.7-fold ($P < 0.05$). No significant effect was observed for the other polyphenols (Fig. 2 (A)).

To determine whether the effects of chrysin and ellagic acid were followed by an effect on total IκB-α, the cytoplasmic level of IκB-α proteins was examined by Western blot. Results are expressed as the phospho:total IκB-α ratio. DMSO and ethanol had no effect on the level of the ratio. The phospho:total IκB-α ratio was significantly elevated for ellagic acid, quercetin, epicatechin and resveratrol treatments, when compared with the control cells (i.e. non-treated cells or cells treated with the vehicles or cells incubated with IL-1β), indicating that these four polyphenols increased the phosphorylated form of IκB-α. In contrast, the phospho:total IκB-α ratio was significantly reduced for chrysin treatment, when compared with the other polyphenol treatments and cells treated with IL-1β, suggesting that chrysin decreased

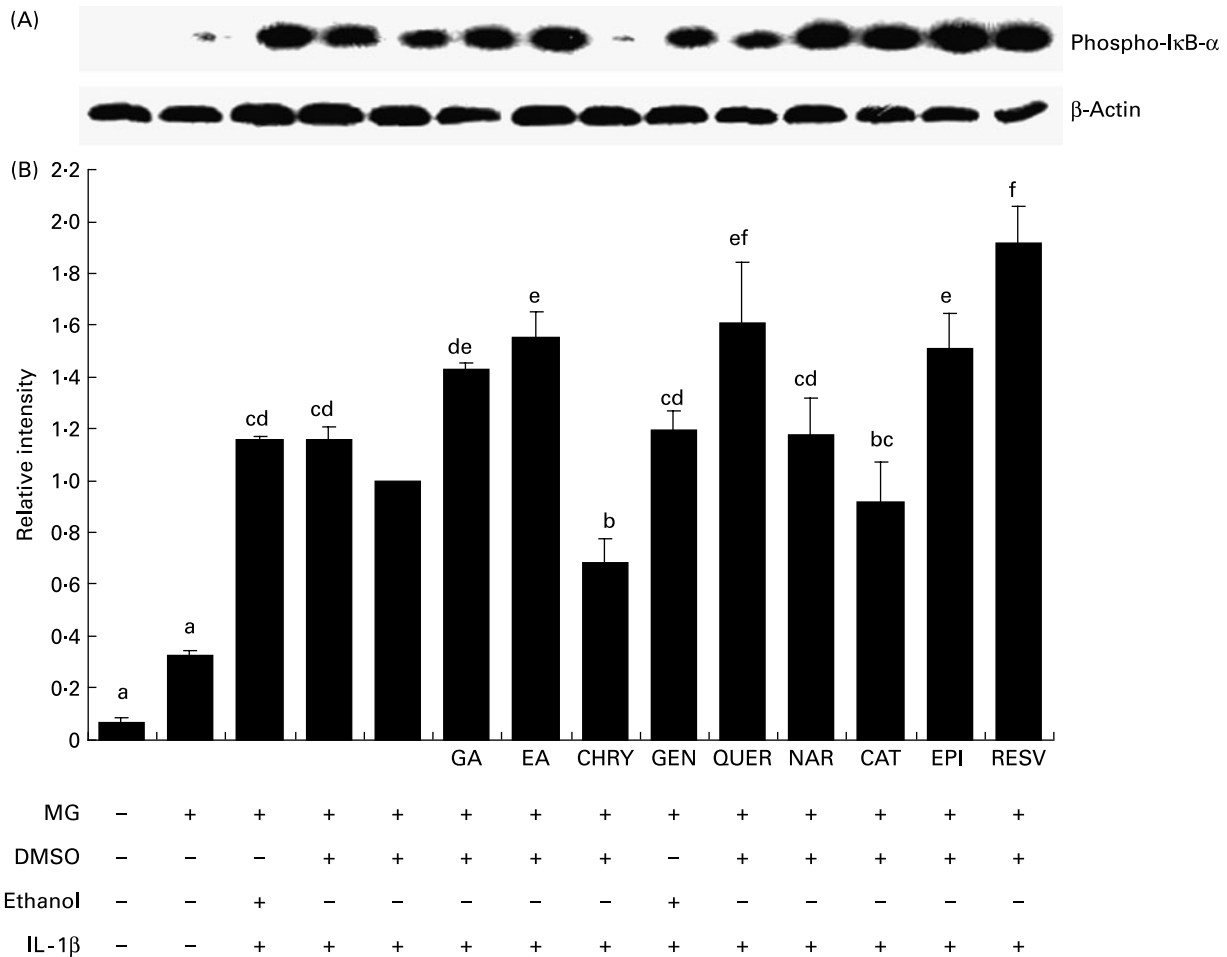


Fig. 2. Effects of polyphenols on the phosphorylation of IκB-α in IL-1β-treated Caco-2 cells. Cells at confluency were incubated with the different polyphenols at 50 μmol/l solubilised in dimethyl sulfoxide (DMSO; 0.5%) (except genistein, in ethanol; 0.4%) for 4 h before the addition of IL-1β at 25 ng/ml for 30 min, still in the presence of the polyphenols. When indicated, the proteasome inhibitor MG-132 (MG) was added at 50 μmol/l for 30 min before IL-1β treatment. After incubation, the cells were lysed and cell lysates (20 μg protein each) were analysed by Western blot to visualise the β-actin, total and phospho-IκB proteins using respective specific antibodies (for details, see Experimental methods). (A) Immunoblots of phospho-IκB-α and of β-actin (after stripping) from one representative experiment (20 μg total protein/well). (B) Phospho-IκB:total-IκB ratio band intensities quantified by densitometry and expressed in relative terms with regard to the positive control (cells treated with IL-1β alone). GA, gallic acid; EA, ellagic acid; CHRY, chrysin; GEN, genistein; QUER, quercetin; NAR, naringenin; CAT, catechin; EPI, epicatechin; RESV, resveratrol. Values are means of four experiments, with standard errors represented by vertical bars, and were log-transformed for statistical analyses (Fisher's test). ^{a-d} Mean values with unlike letters are significantly different ($P < 0.05$).

the phosphorylated form of I κ B- α . The other polyphenols did not have any effect on the phospho:total I κ B- α ratio (Fig. 2 (B)).

Effects of polyphenols on interleukin-1 β -induced secretion of interleukin-8

We next examined if the polyphenol effects on NF- κ B activation were followed by an effect on IL-8 production. In addition to chrysin, ellagic acid, genistein and resveratrol, we also tested EGCG since it had been reported to be a potent inhibitor of IL-8 production. After a 4 h polyphenol pretreatment (50 μ mol/l), the Caco-2 cells were treated with IL-1 β (25 ng/ml) still in the presence of polyphenols, and the amount of IL-8 released in the medium after 48 h was quantified by using an ELISA assay specific to human IL-8 (Fig. 3). When the cells were treated with IL-1 β , IL-8 secretion was increased by about 7.5-fold compared with non-treated cells. Ellagic acid, genistein and EGCG decreased IL-8 secretion by 5.9-, 4.4- and 8.5-fold, respectively, when compared with IL-1 β -treated cells ($P < 0.05$). Chrysin also reduced IL-8 secretion by 1.6-fold (NS; $P > 0.05$). In contrast, resveratrol increased significantly IL-8 secretion by 1.7-fold ($P < 0.05$).

Discussion

In the present study, we tested the hypothesis that the anti-inflammatory properties of several polyphenols could be mediated through their modulation of the NF- κ B activation

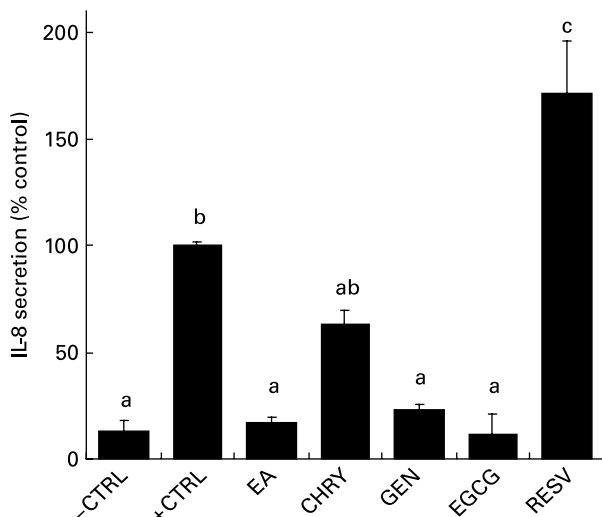


Fig. 3. Effects of polyphenols on IL-8 secretion in IL-1 β -treated Caco-2 cells. Confluent cells were first incubated with or without polyphenols at 50 μ mol/l for 4 h, and then stimulated by a 48 h exposure with IL-1 β at 25 ng/ml, still in the presence of the polyphenols. After incubation, culture media were collected and processed for IL-8 quantification as described in Experimental methods. Values were determined in pg IL-8/ μ g cellular protein. Effects of polyphenols on IL-8 secretion were expressed as percentages of the positive control (+CTRL) (cells stimulated by IL-1 β in the absence of polyphenol). -CTRL, negative control; EA, ellagic acid; CHRY, chrysin; GEN, genistein; EGCG, epigallocatechin gallate; RESV, resveratrol. Values are means of three to five independent experiments, with standard errors represented by vertical bars. Data were compared among groups (Scheffé's test). ^{a-c} Mean values with unlike letters are significantly different ($P < 0.05$).

pathways in the intestine. For this purpose, the potential effects of polyphenols on the signalling NF- κ B activation cascade were investigated on different associated cellular events: (i) the phosphorylation of I κ B that plays a key role in NF- κ B activation; (ii) the degree of NF- κ B induction initiated by either one of the three following stimuli – two cytokines (IL-1 β and TNF- α) and by LPS; (iii) the secretion of the pro-inflammatory cytokine IL-8, whose expression is up-regulated by NF- κ B. These different mechanisms were investigated in the human intestinal cell line Caco-2 at the confluency stage.

Several polyphenols were tested: two phenolic acids (ellagic and gallic acids), seven flavonoids (quercetin, chrysin, genistein, naringenin, catechin, epicatechin and EGCG) and one stilbene (resveratrol). These polyphenols are found in a wide range of common food products of the human diet⁽⁸⁾. For instance, chrysin is a natural flavonoid largely present in honey, ellagic acid in berries, genistein in soya-derived products, and resveratrol in grape skin and red wine. The different polyphenols tested at a final concentration of 50 μ mol/l for 24 h did not show any cytotoxic effect on Caco-2 cells and did not affect the cell proliferation.

Dietary polyphenols have shown anti-inflammatory properties⁽⁹⁾ and their interaction with the NF- κ B activation pathways was presented as one potential mechanism. In confluent Caco-2 cells transfected with the NF- κ B-luciferase vector (in the absence of polyphenol), NF- κ B-luciferase activity was induced by the three stimuli according to the following decreasing order: IL-1 β > LPS > TNF- α . It was indeed reported that the intestinal cell lines Caco-2 and IEC are less responsive to TNF- α than to IL-1 β ^(32,33). In agreement with a cohort of studies reporting that polyphenols act as NF- κ B inhibitors *in vitro*^(17–24,27,28), our data showed that chrysin and ellagic acid suppressed the NF- κ B induction mediated by IL-1 β , TNF- α or LPS. Polyphenol effects on NF- κ B induction appear to be dependent on the cell type as illustrated by chrysin. For instance, chrysin (3 μ mol/l) suppressed TNF- α -mediated NF- κ B induction in respiratory epithelial A549 cells⁽³⁴⁾, while it increased NF- κ B binding activity and NF- κ B induction in LPS-stimulated mice Raw264.7 macrophages⁽³⁵⁾. In the present study, genistein and resveratrol also promoted NF- κ B induction initiated by either one of the stimuli (IL-1 β , TNF- α or LPS) in Caco-2 cells. However, these two polyphenols were reported to block NF- κ B induction in various non-colon cell models, i.e. in rat alveolar macrophages and human pancreatic PC3 cells for genistein^(22,36), and in human monocytes (THP-1), macrophages (U-937), lymphoid (Jurkat) and epithelial cell (HeLa and H4) lines for resveratrol^(24,37,38). In agreement with our data, Jeong *et al.*⁽³⁹⁾ reported that resveratrol promoted NF- κ B activation in the human intestinal HT-29 cells stably transfected with a NF- κ B-luciferase construct. Taken together, the results suggest that NF- κ B responses to polyphenols in human intestinal cells are distinct from other cell types, probably via programmed sets of signals that are different in intestinal cells from in other cells. The permanent exposure of the intestinal cells to high levels of polyphenols for decades may have modified their genetic print, compared with cells present in blood and other organs that are exposed only to small polyphenol levels. Finally, in our experimental conditions, the other polyphenols tested (gallic acid, quercetin, naringenin,

catechin and epicatechin) did not have any effect on NF- κ B induction and no structure–activity relationship could be established among the various polyphenols studied.

Interestingly, the modulatory effects of all four active polyphenols (chrysin, ellagic acid, genistein and resveratrol) on NF- κ B–luciferase activity were independent of the nature of the inducer (IL-1 β , TNF- α and LPS). In a similar way, Manna *et al.*⁽³⁸⁾ observed in a study with myeloid (U-937), lymphoid (Jurkat) and epithelial (HeLa and H4) cells that resveratrol blocked the NF- κ B activation induced by five different stimuli (phorbol ester, H₂O₂, LPS, okadaic acid and ceramide) that have different signal transduction pathways. Note here that the pro-inflammatory cytokines (IL-1 β and TNF- α) and LPS trigger two distinct NF- κ B signalling cascades via the recognition of different transmembrane ‘receptor’-associated domains (the TNF receptor 1 (TNFR1) complex and the Toll-like receptor 4 (TRL4) complex, respectively for cytokines and LPS)⁽¹²⁾. Thus, the present data suggest that the active polyphenols may modulate the NF- κ B activation pathway by acting on an event common to the cytokine- and LPS-mediated cascades.

One common intracellular mechanism in response to the stimuli IL-1 β , TNF- α and LPS is the rapid phosphorylation on two N-terminal serine residues of I κ B- α mediated by an I κ B-kinase complex. This event is quickly followed by the ubiquitination and proteasome degradation of I κ B, resulting in NF- κ B activation and nuclear translocation^(11,12). Therefore, in the next step, effects of polyphenols on I κ B- α phosphorylation were studied using the inducer IL-1 β , since it had shown the strongest signal transduction to NF- κ B in Caco-2 cells. Several reports have mentioned that polyphenols (EGCG, luteolin, resveratrol and curcumin) are inhibitors of NF- κ B activation by blocking I κ B- α phosphorylation and degradation^(25,28,37,39,40). In accordance with these previous studies and consistent with the inhibition of NF- κ B activation observed here, there was a significant inhibition of the IL-1 β -induced I κ B- α phosphorylation by chrysin. However, ellagic acid increased I κ B- α phosphorylation, whereas this agent showed decreased activation of NF- κ B–luciferase activity. Similar opposite processes were previously mentioned for resveratrol and EGCG in HT29 cells⁽³⁹⁾. The dichotomy between NF- κ B–luciferase activity and I κ B- α phosphorylation induced by ellagic acid suggests that there might be other mechanism(s) involved in the NF- κ B transcription activation that might be independent of I κ B- α phosphorylation. Supportive to this idea, resveratrol and genistein increased NF- κ B–luciferase activity without affecting I κ B- α phosphorylation in Caco-2 cells.

There is increasing evidence that locally produced pro-inflammatory cytokines, such as IL-1, IL-6 and TNF- α , might be involved in normal physiological processes as well as in regulating mucosal immune responses and thus IBD⁽³²⁾. Human colon epithelial cell lines (T84, Caco-2, SW620 and HT29) expressed mRNA for IL-8, but did not express mRNA for IL-2, IL-4, IL-5, IL-6 or interferon γ ⁽⁴¹⁾. In addition, these intestinal cells secreted IL-8 constitutively or after stimulation with the agonists IL-1 β , TNF- α and LPS⁽⁴¹⁾. In the present study, confluent Caco-2 cells intrinsically produced IL-8, the level of which was dramatically increased (10-fold) by IL-1 β . Only few reports have mentioned effects of polyphenols on IL-8 secretion. Green tea

polyphenols, in particular EGCG, and genistein were reported as potent inhibitors of IL-8 production in various non-intestinal cells after stimulation by pro-inflammatory cytokines^(42–44). Similarly, we report here that EGCG and genistein, but also ellagic acid, inhibited IL-8 secretion by IL-1 β -stimulated Caco-2 cells. In addition, chrysin tended to show the same effect, although not in a significant manner. By contrast, resveratrol had a clear opposite effect.

IL-8 is a cytokine with neutrophil chemotactic and activation properties that has been found in previous studies to be produced at an increased rate in cytokine-stimulated enterocytes⁽⁴⁵⁾. The promoter of the IL-8 gene contains binding sites for NF- κ B⁽⁴⁶⁾, activator protein-1 (AP-1) and the redox-responsive transcription factors^(47,48) that are essential to regulate IL-8 expression. NF- κ B binding to the IL-8 gene promoter was reported to increase IL-8 production. The active polyphenols may thus act on IL-8 secretion through this route. In accordance with that hypothesis, the results obtained in the present study suggest that chrysin, ellagic acid and resveratrol could control IL-8 secretion via their modulatory effects on the NF- κ B transcriptional activity in stimulated Caco-2 cells. However, in the present study, genistein blocked IL-8 secretion induced by IL-1 β , while it increased NF- κ B activation, suggesting that this agent could affect IL-8 production via a pathway independent of NF- κ B induction, possibly via AP-1 induction. Recently, it was reported that hyperforin (a herbal antidepressant) induction of IL-8 expression in the cell line IEC occurred through an AP-1-dependent, but NF- κ B-independent mechanism⁽⁴⁹⁾. Activation of AP-1 is known to be downstream of the mitogen-activated protein kinase signalling pathway and extracellular signal-regulated kinase (ERK) 1/2 signalling has been shown to control IL-8 production in many cell types including IEC^(50,51). One may thus suspect that some polyphenols do control IL-8 production through the ERK/AP-1 signalling pathway. In preliminary experiments, for which confluent Caco-2 cells were stimulated with IL-1 β , we indeed observed that ellagic acid and EGCG decreased the phosphorylated active form of ERK1/2, whereas resveratrol seemed to increase it. Chrysin had no effect on ERK1/2 activation, but those results need to be confirmed by further investigations.

The present study is a screening of the putative polyphenols affecting the NF- κ B pathway in confluent Caco-2 cells. Data indicate that some of the polyphenols tested were able to modulate this pathway. Chrysin appears to be a particularly potent inhibitor of the signalling NF- κ B activation cascade by acting on different associated cellular events: (i) by reducing the phosphorylated level of I κ B; (ii) by inhibiting the NF- κ B induction initiated by the cytokines and LPS; (iii) by reducing the secretion of the pro-inflammatory cytokine IL-8. For ellagic acid, genistein and resveratrol, those three cellular events are not correlated. Ellagic acid decreased NF- κ B activation and IL-8 secretion but not I κ B- α phosphorylation, resveratrol increased NF- κ B activation and IL-8 secretion and had no effect on I κ B- α phosphorylation and, finally, genistein inhibited NF- κ B activation but decreased IL-8 secretion.

The inhibition of intestinal inflammation by natural plant products such as polyphenols through a modulation of NF- κ B signalling pathway is of great interest to select effective anti-inflammatory compounds for nutritional prevention.

Acknowledgements

There is no conflict of interest. The present study was supported by a grant from the Walloon government (DGTRE) and the Fondation Louvain (Université Catholique de Louvain); J. V. D. W. is a fellow of the Formation à la Recherche dans l'Industrie et dans l'Agriculture (FRIA). Contribution to the manuscript: B. R., experimental work and writing; J. V. D. W., experimental work; A. D., writing; Y. L. and Y.-J. S., heads of laboratory. The study was presented in part at the 23rd International Conference on Polyphenols, Winnipeg, Manitoba, Canada in August 2006⁽⁵²⁾.

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