Cocoa polyphenols prevent inflammation in the colon of azoxymethane-treated rats and in TNF-α-stimulated Caco-2 cells

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Abstract

Numerous lines of evidence support a relationship between intestinal inflammation and cancer. Therefore, much attention has recently been focused on the identification of natural compounds with anti-inflammatory activities as a strategy to suppress the early stages of colorectal cancer. Because cocoa is a rich source of bioactive compounds, the present study investigated its anti-inflammatory properties in a rat model of azoxymethane (AOM)-induced colon carcinogenesis and in TNF-α-stimulated Caco-2 cells. A total of forty male rats were fed with control or cocoa-enriched diets (12 %) during 8 weeks and injected with saline or AOM (20 mg/kg body weight) during the third and fourth week (*n* 10 rats/group). At the end of the experiment, colon samples were evaluated for markers of inflammation. The anti-inflammatory activity of a cocoa polyphenolic extract (10 µg/ml) was examined in TNF-α-stimulated Caco-2 cells, an *in vitro* model of experimentally induced intestinal inflammation. The signalling pathways involved, including NF-κB and the mitogen-activated protein kinase family such as c-Jun NH$_2$-terminal kinases (JNK), extracellular signal-regulated kinases and p38, were also evaluated. The results show that the cocoa-rich diet decreases the nuclear levels of NF-κB and the expression of pro-inflammatory enzymes such as cyclo-oxygenase-2 and inducible NO synthase induced by AOM in the colon. Additionally, the experiments in Caco-2 cells confirm that cocoa polyphenols effectively down-regulate the levels of inflammatory markers induced by TNF-α by inhibiting NF-κB translocation and JNK phosphorylation. We conclude that cocoa polyphenols suppress inflammation-related colon carcinogenesis and could be promising in the dietary prevention of intestinal inflammation and related cancer development.

Key words: Colorectal cancer: NF-κB: Cyclo-oxygenase-2: Inducible nitric oxide synthase

Colorectal cancer (CRC) is a major healthcare problem and the third most common cause of all cancer deaths[1]. Colon carcinogenesis is a complex multistep process from small benign precursor lesions to metastatic carcinomas[2]. During this progression, chronic inflammation is causally linked to carcinogenesis and acts as a driving force in the premalignant and malignant transformation of cells[3].

The early stages of colon carcinogenesis involve an increase in the pro-inflammatory enzymes cyclo-oxygenase-2 (COX-2) and inducible NO synthase (iNOS)[4]. COX-2 and iNOS are implicated in chronic inflammation, which creates a microenvironment contributing to the development of preneoplastic lesions in colon carcinogenesis[5]. More importantly, inhibition of these two enzymes has shown protective effects against colon tumour development in different animal models,

suggesting that they are crucial targets for mucosal inflammation and colon tumorigenesis[6]. Likewise, the redox-sensitive eukaryotic transcription factor NF-κB that regulates the expression of iNOS and COX-2 has been related to inflammation-induced colon carcinogenesis and its inhibition also prevents cancer progression[7]. Therefore, the use of chemo-preventive compounds that suppress inflammation seems to be a useful strategy to control the development and progression of CRC.

Accordingly, considerable attention has recently been focused on the identification of dietary bioactive compounds with anti-inflammatory activities as an alternative natural source for the prevention of inflammation-associated diseases[8]. Evidence from epidemiological studies suggests that a high consumption of fruits and vegetables and the intake

of certain non-nutrients that are present in foods reduce the risk of CRC[9]. These beneficial effects have been partly attributed to polyphenolic phytochemicals, which have a wide range of pharmacological properties, including antioxidant, anti-inflammatory and anti-carcinogenic activities[10]. The molecular mechanisms underlying their chemo-preventive effects have been associated with the modulation of signalling cascades, gene expression involved in the regulation of cell proliferation and apoptosis and the suppression of chronic inflammation[11].

Cocoa is a rich source of bioactive compounds such as polyphenols and methylxanthines, mainly theobromine and, in a much smaller quantity, caffeine. Cocoa contains high amounts of the flavonoids $(-)$-epicatechin, $(+)$-catechin and procyanidins B_2 and B_1, although other polyphenols such as quercetin, isoquercitrin (quercetin 3-O-glucoside), quercetin 3-O-arabinose, hyperoside (quercetin 3-O-galactoside), naringenin, luteolin and apigenin have also been found in minor quantities[12]. At present, cocoa and its phenolic compounds have attracted a great deal of interest because of their potential ability to act as highly effective chemo-preventive agents[13,14]. As antioxidants, cocoa flavanols have been shown to protect cell constituents, limiting the risk factor for cancer and other chronic diseases[15]. Accordingly, we have recently shown that a cocoa-rich diet could prevent the early stages of colon carcinogenesis by reducing oxidative stress and cell proliferation and by inducing apoptosis[16]. Additionally, cocoa flavanols can display other chemo-preventive properties which may be independent of conventional antioxidant activities. In particular, recent in vitro studies have demonstrated that cocoa phenolic compounds exhibited a variety of potential anti-inflammatory effects in intestinal cells[17], which may contribute to their cancer chemo-preventive activity. However, those mechanisms have not yet been investigated in in vivo studies of colon carcinogenesis.

Considering this, in the present study, we have used the well-defined azoxymethane (AOM)-induced colon cancer model in rats to investigate the anti-inflammatory effect of a cocoa-rich diet. In addition, to gain further insight into the mechanisms involved, we tested the anti-inflammatory effect of a cocoa polyphenolic extract (CPE) in an in vitro model of inflammation using human intestinal Caco-2 cells and TNF-α as a pro-inflammatory stress. Caco-2 cells constitute a widely known and well-established in vitro model of the human intestinal barrier commonly used to test the ability of dietary substances to modulate intestinal inflammation[18–20]. The present results indicated that cocoa polyphenols could suppress intestinal inflammation in vivo and in vitro through the inhibition of NF-κB signalling and the down-regulation of pro-inflammatory enzyme expression.

Materials and methods

Materials and chemicals

AOM, o-phthalaldehyde, gentamicin, penicillin G and streptomycin were purchased from Sigma Chemical. Anti-extracellular signal-regulated kinases (ERK), anti-phospho-ERK (p-ERK), anti-JNK, anti-phospho-JNK (p-JNK), anti-phospho-p38 and anti-β-actin were obtained from Cell Signaling Technology. Anti-p38, anti-p65 (NF-κB), anti-iNOS and anti-COX-2 were purchased from Santa Cruz Biotechnology. The RNA isolation kit was obtained from Applied Biosystems/Ambion, primers for RT-PCR were from Isogen, dNTP and RT were from Promega and Taq polymerase was obtained from Roche. Recombinant murine TNF-α was from PreproTech. Materials and chemicals for electrophoresis and the Bradford reagent were from BioRad Laboratories S.A. Cell-culture dishes were from Falcon and cell-culture medium and fetal bovine serum (FBS) from Biowhittaker Europe (Innogenetics).

Cocoa

Natural Forastero cocoa powder (a kind gift from Nutrexpa) was used for the present study.

For the in vivo experiments, diets were prepared from an AIN-93G formulation (Panlab S.L.). The 12% cocoa diet was produced by adding 120 g/kg cocoa to AIN-93G at the expense of starch and cellulose. As a result, the cocoa diet was slightly richer in proteins (2·6%) and lipids (1·2%) but, in order to make both diets isoenergetic (15 048 kJ/kg), the content in carbohydrate was slightly reduced (5%). The composition of the diets is given in Table 1.

For the in vitro experiments, a CPE was prepared from the same Natural Forastero cocoa powder by sequentially washing 1 g of cocoa with 40 ml of 16 mM-HCl in 50% aqueous methanol (50:50, v/v, 1 h at room temperature, constant shaking) and 40 ml of acetone–water (70:30, v/v, 1 h at room temperature, constant shaking) in 50 ml centrifuge tubes. After centrifugation (15 min, 3000 g), supernatants from each extraction step were combined and made up to 100 ml. The desiccated extract was dissolved in distilled

Table 1. Composition of the experimental control and cocoa-rich diets

Components (g/kg dry weight)	Control	Cocoa
Casein	140	140
Dextrose	155	155
Sucrose	100	100
Fat	40	40
t-BHQ	0·008	0·008
Mineral mix	35	35
Vitamin mix	10	10
L-Cys	1·8	1·8
Choline bitartrate	2·5	2·5
Cellulose	100	59·2
Starch	415·7	336·5
Cocoa powder	–	120
Total cocoa polyphenols*	–	2·4
Cocoa flavonoids†		
Procyanidin B_1	–	0·042
Catechin	–	0·139
Procyanidin B_2	–	0·159
Epicatechin	–	0·459
Theobromine†	–	0·891

t-BHQ, tert-butylhydroquinone.
* Cocoa polyphenols were determined by the Folin–Ciocalteu method.
† Main cocoa flavonoids and theobromine were determined by liquid chromatography–MS[17].

water and kept frozen until assay. The total polyphenol content of the CPE was determined by the spectrophotometric method of Folin–Ciocalteu using gallic acid as the standard. A Beckman DU640 spectrophotometer (Beckman Instruments, Inc.) was used. The phenolic fraction of the CPE was previously analysed by liquid chromatography–MS[21]. Accordingly, the polyphenolic profile of the CPE showed that monomeric epicatechin and catechin were the major flavanols in the extract, together with appreciable amounts of procyanidins B_1 and B_2. Additionally, theobromine was present in high amounts while only traces of caffeine were detected in the extract (Table 1).

Animals and experimental design

A total of forty male Wistar Han rats (5 weeks old) were purchased from Harlan Laboratories Models, S.L. The animals were placed individually in stainless-steel wire-bottomed metabolism cages housed in a room under controlled conditions (19–23°C, 50–60% humidity and 12 h light–dark cycles). After 1 week of acclimatisation, rats were randomly assorted into four different experimental groups (Fig. 1(A)) and were provided with food and water *ad libitum*. Of these four groups, two received a standard diet (control) and two were fed with the cocoa-rich diet (cocoa). At 2 weeks later, one control and one cocoa group were intraperitoneally injected with AOM (20 mg/kg body weight) and the other control and cocoa groups with saline, once per week for two consecutive weeks. Rats were killed in the 8th week and the entire colon was resected and cleaned with PBS. Sections (1 cm) from the most distal portion of the colon were fixed in 10% neutral-buffered formalin for histological and immunohistochemical analyses. The remaining midsection of the colon was used for mRNA analyses. The animals were treated according to Institutional Care Instructions (Bioethical Commission from Consejo Superior de Investigaciones Científicas, CSIC, Spain).

Cell culture and treatments

Caco-2 cells were a kind gift from Dr Sonia de Pascual-Teresa (ICTAN-CSIC, Madrid, Spain). Cells (ECACC 86010202) were originally obtained from the European Collection of Cell Cultures (ECACC) and were used between passages 40 and 60. Caco-2 cells were maintained in a humidified incubator containing 5% CO_2 and 95% air at 37°C and were grown on 100 mm-diameter culture plates in Dulbecco's modified Eagle's medium F12 from Biowhittaker, supplemented with 10% Biowhittaker fetal bovine serum and 50 mg/l of each of the following antibiotics: gentamicin; penicillin; streptomycin. The plates were changed to a fetal bovine serum-free medium the day before the assay.

For the TNF-α treatment, different concentrations of TNF-α (10, 20 and 40 ng/ml), diluted in serum-free culture medium, were added to the cell plates. The concentration range was determined according to the literature[22] and the absence of cytotoxicity was verified by the crystal violet method. For the CPE treatment, 10 μg/ml of CPE diluted in serum-free culture medium and filtered through a 0·2 μm membrane were added to the cell plates for 20 h. This concentration of the CPE in cell cultures is comparatively equivalent to realistic concentrations in the human gut and evoked a significant protection against oxidative stress in Caco-2 cells[23]. In the present study, the culture medium containing the CPE was removed after 20 h pretreatment and a fresh culture medium containing pro-inflammatory TNF-α added for 24 h.

Histological and immunohistochemical analyses

Samples from the distal colon fixed in 10% neutral-buffered formalin were sectioned and stained with haematoxylin and eosin for histological evaluation. NF-κB-p65 immunohistochemistry was performed in histological sections of colonic mucosa. Briefly, serial sections of the colon were deparaffinised in xylene, rehydrated in graded ethanol, washed in PBS and heated in a microwave at 98°C, with 0·01 M-sodium citrate buffer (pH 6·0). Peroxide blocking was performed with 0·3% H_2O_2 in methanol at room temperature for 5 min. The sections were incubated with rabbit polyclonal anti-NF-κB-p65 (dilution 1:100) at 4°C overnight. A secondary antibody, goat anti-rabbit, was then applied for 30 min in a 1:200 dilution. Slices were incubated with horseradish peroxidase-conjugated streptavidin and then visualised with 3,3′-diaminobenzidine and counterstained with Harris's
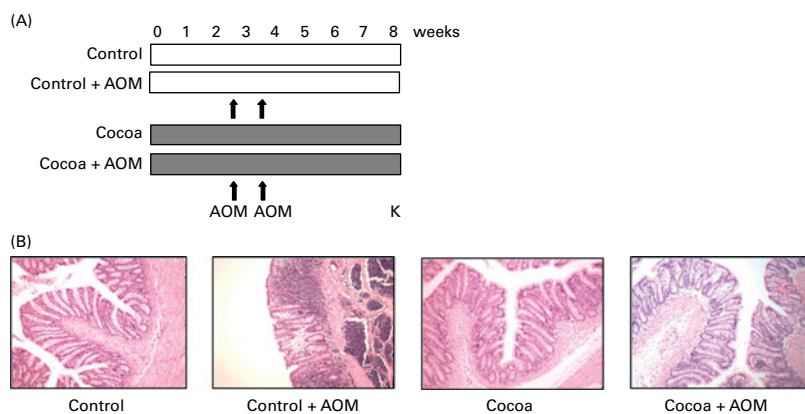
Fig. 1. (A) Experimental design for azoxymethane (AOM, 20 mg/kg body weight)-induced colon carcinogenesis in rats. (B) Representative haematoxylin and eosin-stained sections of the colon of rats injected with saline or AOM and fed with the control (□) or cocoa-enriched diet (■) (100×). K, killing.

haematoxylin. NF-κB (p65) expression was detected as nuclear and cytoplasmic brown staining. The number of nuclear NF-κB (p65)-positive epithelial cells was examined under light microscopy at 400× magnification (a Leica DM LB2 microscope and a digital Leica DFC 320 camera; Leica Microsystems) and quantified by a percentage score (mean values with their standard errors) with grading between 0 and 4: 0, no nuclear staining; 1, 1–10% positive cells; 2, 10–30% positive cells; 3, 30–60% positive cells; 4, 60–100% positive cells. As a positive internal control, we used NF-κB/p65 expression in lymphocytes and endothelial cells of the tissue sample, which showed positive nuclear staining in all runs. Negative controls were used during the optimisation of the method.

Determination of cyclo-oxygenase-2 and inducible nitric oxide synthase expression in colon tissues

Total RNA was extracted from the rat distal colon using the RNA isolation kit (Totally RNA kit; Applied Biosystems/Ambion) as described in the manufacturer's manual. Total RNA (1 µg) was subjected to RT and the complementary DNA products were amplified by PCR using the following couples of primers: 5′-CCCTTCCGAAGTTTCTGGCAGCAGC-3′ and 5′-GGCTGTCAGAGAGCCTCGTGGCTTTGG-3′ for amplification of iNOS; 5′-GGAGAGACTATCAAGATAGTGATC-3′ and 5′-ATGGTCAGTAGACTTTTACAGCTC-3′ for COX-2; 5′-ACCACAGTCCATGCCATCAC-3′ and 5′-TCCACCACCCTGT-TGCTGTA-3′ for glyceraldehyde 3-phosphate dehydrogenase as a housekeeping gene. The samples were incubated in a Thermo Cycler (PCR Express; Thermo Hybaid) using the following parameters: 94°C for 1 min, 64°C for 1 min and 72°C for 1 min (thirty-five cycles) followed by a 10 min extension at 72°C for COX-2 amplification; 94°C for 1 min, 55°C for 1 min and 72°C for 1 min (thirty-five cycles) followed by a 10 min extension at 72°C for iNOS and 92°C for 1 min, 55°C for 1 min and 72°C for 1 min (thirty-five cycles) followed by a 10 min extension at 72°C for glyceraldehyde 3-phosphate dehydrogenase amplification. The PCR products were electrophoresed on a 1·5% agarose gel containing ethidium bromide. The gel was photographed under UV transillumination and the bands were quantified by laser scanning densitometry (Molecular Dynamics). Band intensity was normalised to values for glyceraldehyde 3-phosphate dehydrogenase that was used as an internal control.

Determination of IL-8 secretion by Caco-2 cells

Concentration of IL-8 in cell-culture supernatants was determined using a human IL-8 ELISA kit (BD Biosciences) according to the manufacturer's instructions. The ELISA ninety-six-well microplates were analysed using a microplate reader at 570 nm (Bio-Tek).

Preparation of cell lysates for Western blotting

To detect ERK, p-ERK, JNK, p-JNK, p38, p-p38, iNOS and COX-2 levels, Caco-2 cells were lysed at 4°C in a buffer containing 25 mM-HEPES (pH 7·5), 0·3 M-NaCl, 1·5 mM-MgCl₂, 0·2 mM-EDTA, 0·5 mM-1,4-dithiothreitol, 0·1% Triton X-100, 200 mM-β-glycerolphosphate, 0·1 mM-Na₃VO₄, 2 µg leupeptin/ml and 1 mM-phenylmethylsulphonyl fluoride. The supernatants were collected, assayed for protein concentration using the Bradford reagent, aliquoted and stored at −80°C until used for Western blot analyses.

Preparation of nuclear and cytosolic cellular extracts

To evaluate the cytosolic and nuclear NF-κB-p65 content, cells were resuspended at 4°C in 10 mM-HEPES, pH 7·9, 1·5 mM-MgCl₂, 10 mM-KCl, 0·5 mM-1,4-dithiothreitol and 0·2 mM-phenylmethylsulphonyl fluoride (buffer A), allowed to swell on ice for 10 min, and then vortexed for 10 s. The samples were centrifuged at 10 000 g for 2 min and the supernatant containing the cytosolic fraction was stored at −80°C. The pellet was resuspended in cold buffer B (20 mM-HEPES, pH 7·9, 25% glycerol, 420 mM-NaCl, 1·5 mM-MgCl₂, 0·2 mM-EDTA, 0·5 mM-1,4-dithiothreitol, 0·2 mM-phenylmethylsulphonyl fluoride, 2·5 µg leupeptin/ml and 2·5 µg aprotinin/ml) and incubated on ice for 20 min for high salt extraction. Cellular debris was removed by centrifugation at 13 000 g for 10 min at 4°C, and the supernatant fraction containing the nuclear protein extract was stored at −80°C. Protein content was measured using the Bradford reagent.

Protein determination by Western blotting

Equal amounts of protein (40 µg) were separated by SDS−PAGE and transferred to polyvinylidene difluoride filters (Protein Sequencing Membrane). Membranes were probed with the corresponding primary antibody followed by incubation with peroxide-conjugated anti-rabbit Ig (GE Healthcare). Blots were developed with the enhanced chemiluminescence (ECL) system (GE Healthcare). Anti-growth factor receptor-bound protein 2 and anti-poly (ADP-ribose) polymerase (PARP) antibodies were used as markers for the cytosolic and nuclear extracts, respectively. Normalisation of the Western blot was ensured by β-actin and band quantification was carried out with a scanner (HP Scanjet G2710; HP) and Scion Image software (Scion Corporation).

Statistical analysis

Following assurance of the normal distribution of data, Student's t test was used to assess the statistical significance of aberrant crypt foci (ACF) formation between the control + AOM and cocoa + AOM groups. In the rest of the comparisons, one-way ANOVA was used followed by a Bonferroni test when variances were homogeneous or by the Tamhane test when variances were not homogeneous. Differences were considered as statistically significant if $P < 0.05$ (statistical package SPSS version 19.0; SPSS, Inc.).

Results

General observations and histological changes

During the study period, rats fed with the cocoa diets had food intakes similar to their corresponding control animals (data not shown). However, the body weight of the animals fed with the cocoa diet was slightly but significantly reduced when compared with the control groups (about 10%) at weeks 5–7. As described elsewhere, this effect has been attributed to a reduced fat adipose tissue in rats consuming a cocoa diet[24,25]. On the other hand, there was no difference among the colon weights or lengths of any group of animals at the time of killing (data not shown). Furthermore, gross observation of liver, lung and kidney indicated no pathological alterations in any organ among the groups.

After haematoxylin and eosin staining, sections from the control + AOM group showed signs of diffuse inflammation with the destruction of the epithelial architecture and the loss of crypts (Fig. 1(B)). The epithelial crypts were distorted and irregularly distributed in the lamina propria, which contains high numbers of inflammatory cells, including lymphocytes, plasma cells and polymorphonuclear leucocytic cells. The preneoplastic lesions in the epithelial layer of colonic mucosa were identified after careful histological examination involving dysplastic aberrant crypts, hypercellularity with enlarged hyperchromatic nuclei, loss of polarity and decreased mucine excretion. Treatment with cocoa reduced the morphological alteration associated with AOM administration, protecting the mucosal architecture, reducing crypt and epithelial damage and showing scarce infiltration of inflammatory cells. No histological modification was present in the colon of rats fed with control and cocoa diets.

Cocoa-enriched diet reduced the colonic expression of cyclo-oxygenase-2, inducible nitric oxide synthase and NF-κB (p65) induced by azoxymethane

Next, we investigated the effect of cocoa administration in the expression of inflammatory enzymes COX-2 and iNOS that are often elevated in the initial phase of carcinogenesis induced by AOM. As shown in Fig. 2(A), injection of AOM markedly elevated the expression of COX-2 and iNOS mRNA in the colon, while cocoa feeding effectively suppressed this elevation. It is worth noting that cocoa feeding alone was able to significantly reduce COX-2 expression in non-AOM rats to levels below those of control non-AOM rats.

Given that the expression of both inflammatory proteins is regulated by NF-κB, the levels of the p65 subunit of NF-κB in the nucleus of colonocytes (active form) were then measured. To this end, immunohistochemical staining was performed in the local colonic tissue. As shown in Fig. 2(B), there was a little expression of immunoreactive NF-κB-p65 in the control or cocoa distal colon tissues that was mainly localised in stromal cells. On the contrary, strong NF-κB-p65 expression was detected in both epithelial and stromal cells in the colon of the control + AOM or cocoa + AOM group. Nevertheless, the scores for NF-κB-p65 in the cocoa groups were significantly smaller than those of the control + AOM group, indicating that cocoa supplementation significantly reduces NF-κB activity in colonic mucosa.

Cocoa polyphenolic extract treatment protects TNF-α-induced inflammatory marker expression in Caco-2 cells

In order to confirm that the positive effects of the cocoa diet could be related to its polyphenol content and to gain further
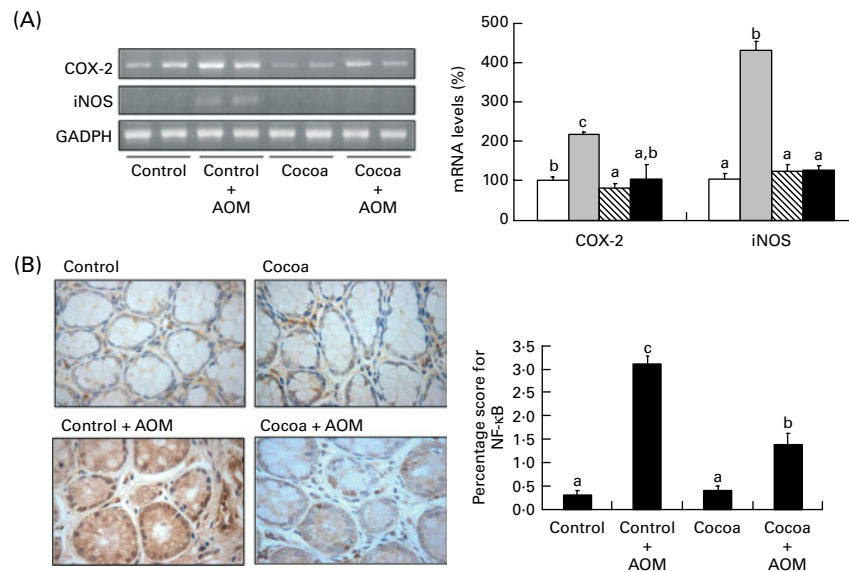

Fig. 2. Effect of the cocoa-enriched diet on the colonic expression of cyclo-oxygenase-2 (COX-2), inducible nitric oxide synthase (iNOS) and NF-κB induced by azoxymethane (AOM). (A) Representative RT-PCR analysis and percentage values of mRNA levels of COX-2 and iNOS in the distal colon mucosa of rats injected with saline or AOM and fed with the control or cocoa-enriched diet. (B) Representative photographs for immunohistochemical staining of NF-κB subunit p65 (dark brown)-positive cells (400× magnification) and the percentage score in colon tissues from rats injected with saline or AOM and fed the control or cocoa-enriched diet. Values are means from six to eight rats in each group, with standard deviations represented by vertical bars. [a,b,c] Mean values with unlike letters were significantly different (P<0.05). GADPH, glyceraldehyde 3-phosphate. ☐, Control; ▨, control + AOM; ◪, cocoa; ■, cocoa + AOM.

insight into the mechanism involved, we next investigated the anti-inflammatory activity of a CPE obtained from cocoa used in the *in vivo* experiment. To this end, the effects of the CPE on intestinal inflammation were investigated in TNF-α-stimulated Caco-2 cells, an *in vitro* model of experimentally induced intestinal inflammation. Accordingly, Caco-2 cells were exposed for 24 h to different concentrations of TNF-α and the levels of the pro-inflammatory marker IL-8 were evaluated. Fig. 3(A) shows that IL-8 secreted in the culture medium by Caco-2 cells in response to TNF-α augmented in a dose-dependent manner, being significantly increased at 40 ng/ml of TNF-α. Since this was the minimal dose able to induce the inflammation of Caco-2 cells, it was the one selected for the subsequent experiments. Likewise, the levels of the inflammatory enzymes COX-2 and iNOs, evaluated by Western blot, were significantly enlarged in Caco-2 cells after 24 h of stimulation with 40 ng/ml of TNF-α (Fig. 3(B) and (C)). To determine whether the CPE could reduce these inflammatory responses, Caco-2 cells were then pretreated for 20 h with 10 μg/ml of CPE supplying 132 nM-epicatechin, 40 nM-catechin and 43 nM-procyanidin B2. Since cocoa polyphenols can be largely found in the gastrointestinal tract even at micromolar concentrations, 10 μg/ml of CPE represent a realistic concentration in the human gut. After that, CPE-treated cells were further stimulated with TNF-α (40 ng/ml) for 24 h. As shown in Fig. 4, pretreatment of Caco-2 cells with CPE significantly reduced the IL-8 release (Fig. 4(A)) and down-regulated the levels of COX-2 and iNOS in Caco-2 cells stimulated with TNF-α (Fig. 4(B) and (C)).

Cocoa polyphenolic extract regulates NF-κB and mitogen-activated protein kinase in Caco-2 cells

Finally, to elucidate the mechanism responsible for the anti-inflammatory action of cocoa polyphenols, we examined the upstream pathways for COX-2 and iNOS stimulation, which are rapidly activated after TNF-α treatment. Accordingly, the nuclear and cytosolic levels of NF-κB were evaluated by Western blot. As shown in Fig. 5(A) and (B), stimulation of
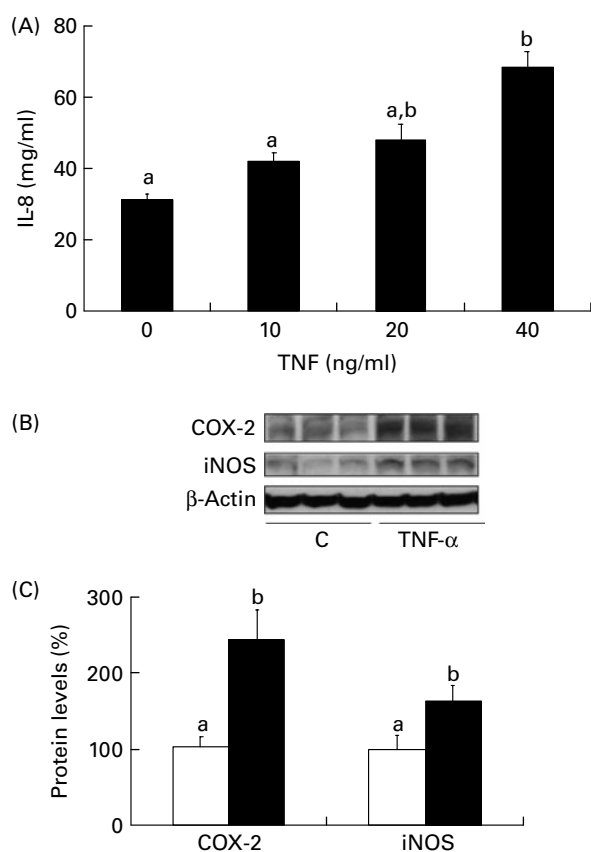
Fig. 3. Effect of TNF-α on IL-8 secretion and cyclo-oxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) expression in Caco-2 cells. Values are means, with standard deviations represented by vertical bars. (A) Cells were treated with 10, 20 and 40 ng/ml TNF-α for 24 h and IL-8 secretion was evaluated by ELISA. (B) Cells were treated with 40 ng/ml TNF-α for 24 h and COX-2 and iNOS levels were evaluated by Western blot. Representative bands of three different experiments. (C) Percentage values of COX-2 and iNOS levels relative to the control condition. [a,b] Mean values with unlike letters were significantly different ($P < 0.05$). □, Control; ■, TNF-α.
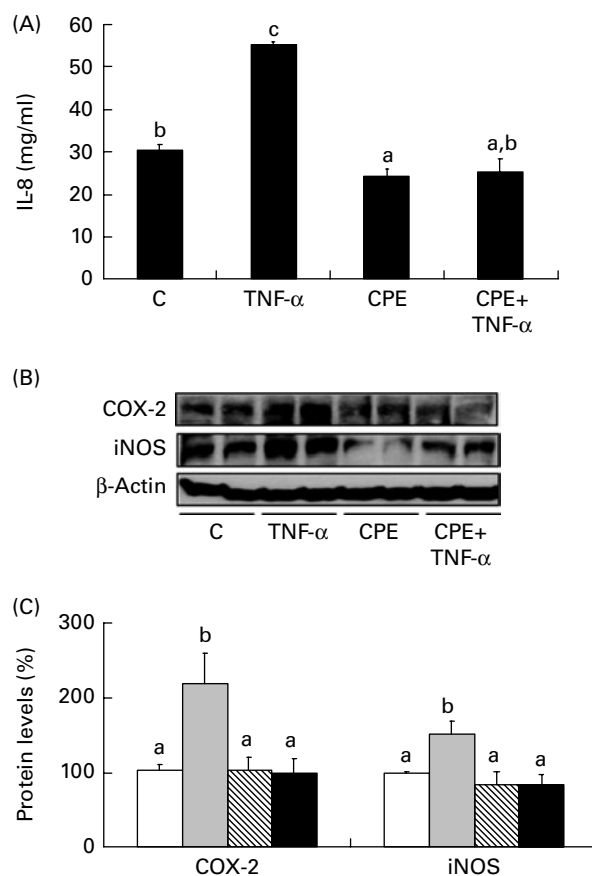
Fig. 4. Effect of the cocoa polyphenolic extract (CPE) treatment in TNF-α-induced IL-8 secretion and cyclo-oxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) expression in Caco-2 cells. C (□): control, untreated cells; TNF-α (▨): cells treated with 40 ng/ml TNF-α for 24 h; CPE (▨): cells treated with 10 μg/ml CPE for 20 h; CPE + TNF-α (■): cells pretreated with 10 μg/ml CPE for 20 h and then treated with 40 ng/ml TNF-α for 24 h. (A) IL-8 secretion was evaluated by ELISA. (B) COX-2 and iNOS levels were evaluated by Western blot. Representative bands of three different experiments. (C) Percentage values of COX-2 and iNOS levels relative to the control condition. Values are means, with standard deviations represented by vertical bars. [a,b,c] Mean values with unlike letters were significantly different ($P < 0.05$).

Caco-2 cells with TNF-α during 60 min increased accumulation of the p65 subunit of NF-κB in the nucleus, while 20 h pretreatment of cells with the CPE significantly prevented TNF-α-induced NF-κB translocation.

The induction of NF-κB by TNF-α is partially mediated by members of the mitogen-activated protein kinase family (MAPK), such as c-Jun NH$_2$-terminal kinases (JNK), ERK and p38. Then, we determined the activation of these MAPK by TNF-α by measuring the levels of phosphorylated JNK, ERK and p38. As shown in Fig. 5(C) and (D), all MAPK were strongly activated upon 60 min of TNF-α stimulation. The CPE pretreatment did not block ERK and p38 activation but strongly inhibited the activation of JNK. In addition, 20 h of CPE treatment induced the activation of ERK and p38 in Caco-2 cells.

Discussion

The present study was performed to investigate the potential anti-inflammatory effect of cocoa polyphenols and to elucidate the molecular mechanism involved. In a previous study, we demonstrated that a cocoa-rich diet could prevent the early stage of CRC in rats[16]. The chemo-preventive effects elicited by cocoa were due at least in part to its ability to prevent oxidative stress and cell proliferation and to induce apoptosis. Herein we show for the first time that cocoa flavonoids also prevent colon inflammation in AOM-treated rats and in TNF-α-stimulated Caco-2 cells, which contributes to its anti-carcinogenic activity.

The AOM colon cancer model has been extensively used to examine the chemo-preventive effect of numerous compounds on CRC[26]. Administration of the colon-specific carcinogen AOM to rodents induces oxidative stress and inflammation that results in chromosomal instability and DNA damage in proliferating cells contributing to malignant transformation[27]. In the present study, we showed that feeding animals with a cocoa-rich diet strongly prevents inflammatory tissue damage and cellular infiltration in colonic mucosa. This result is in concordance with previous reports indicating that some polyphenolic compounds including genistein[28], limonin[29] and silibinin[30] possess anti-inflammatory effects *in vivo* on the colonic tissue. More importantly, two single doses of a CPE reduced colon damage and neutrophil infiltration in an acute model of dextran sulphate sodium-induced ulcerative colitis in mice[31]. Indeed, we have recently shown that a cocoa rich-diet prevents the development of colonic preneoplastic lesions (ACF) induced by AOM[16]; therefore, we suggest that the reduction of intestinal inflammation by cocoa could be one of the causal mechanisms for the prevention of the early steps of colon carcinogenesis.

The anti-inflammatory activity of a cocoa diet was thus investigated as one underlying mechanism of its efficacy in inhibiting AOM-induced ACF formation in the rat colon. We found that a cocoa-rich diet markedly inhibited the expression of enzymes associated with inflammation, such as iNOS and COX-2, which are increased both in human CRC and in AOM-induced rat colon carcinogenesis[9]. Increased expression of iNOS and, to a lesser extent, of COX-2 occurred early in AOM-induced colon carcinogenesis in rats[32]. iNOS expression is frequently observed in dysplastic, but not in hyperplastic, ACF, indicating that iNOS plays an important role in the early stages of tumour formation[32]. On the other hand, tumorigenic mechanisms of COX-2 include the inhibition of apoptosis via increased Bcl-2 and the activation
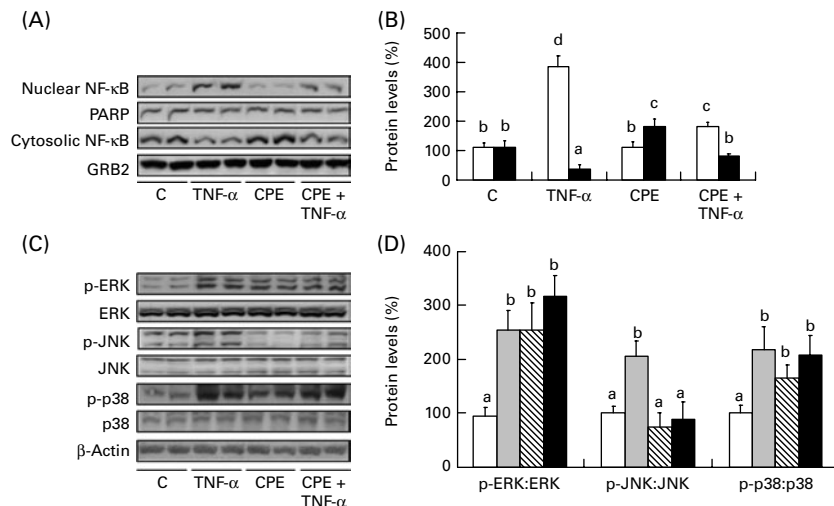

Fig. 5. Effect of cocoa polyphenolic extract (CPE) on NF-κB translocation and phosphorylated levels of extracellular signal-regulated kinases (ERK), c-Jun NH$_2$-terminal kinases (JNK) and p38 MAPK (p38) induced by TNF-α in Caco-2 cells. C (□): control, untreated cells; TNF-α (▨): cells treated with 40 ng/ml TNF-α for 1 h; CPE (▨): cells treated with 10 μg/ml CPE for 20 h; CPE + TNF-α (■): cells pretreated with 10 μg/ml CPE for 20 h and then treated with 40 ng/ml TNF-α for 1 h. NF-κB levels were determined by Western blot in the nuclear or cytosolic cellular compartment. Values are means, with standard deviations represented by vertical bars. (A) Representative bands of three different experiments. (B) Percentage values of nuclear (□) and cytosolic NF-κB (■) levels relative to the control condition. Phosphorylated and total levels of ERK, JNK and p38 were determined by Western blot analysis using phospho- and total specific antibodies. (C) Bands are representative of two to three different experiments. (D) Percentage values of the p-ERK:ERK, p-JNK:JNK and p-p38:p38 ratios relative to the control condition. [a,b,c,d] Mean values with unlike letters were significantly different ($P < 0.05$).

of proliferation via MAPK or phosphoinositide 3-kinase–AKT signalling pathways[33]. More importantly, the effect of a cocoa-rich diet preventing iNOS and COX-2 expression induced by AOM is consistent with our previous report showing that a cocoa-rich diet prevents the increase in proliferation evoked by AOM and induces apoptosis[16]. Therefore, as previously described by other naturally occurring phytochemicals, such as β-carotene, epigallocatechin gallate or silibinin[11], the reduction in the pro-inflammatory mediators iNOS and COX-2 in association with the inhibition of ACF formation suggests that a cocoa-rich diet is effective for the primary prevention of inflammation-related carcinogenesis in the rat colon.

One of the most important anti-inflammatory mechanisms of some phenolic compounds is the activation of NF-κB[11,19]. NF-κB is considered to be an important transcription factor in the tumorigenic process because its constitutive activation exerts strong proliferative and anti-apoptotic properties in cancer cells[3]. In resting cells, NF-κB is sequestered in the cytoplasm by interaction with inhibitors, such as IκB and related proteins. In response to stimulation, IκB is phosphorylated, ubiquitinated and degraded, allowing NF-κB dimers (p65 and p50) to translocate to the nucleus for promoting the transcription of target genes including the pro-inflammatory enzymes iNOS and COX-2[34]. Interestingly, the results of the present study show that the increased levels of NF-κB (p65) in the nucleus of epithelial colonic cells of AOM-treated rats were significantly decreased by the cocoa diet, suggesting that the inhibitory effects of the cocoa diet on COX-2 and iNOS expression may be mediated by the inhibition of NF-κB activation. These findings are consistent with other studies showing that natural chemo-preventive agents suppress both iNOS and COX-2 levels by regulating NF-κB signalling[35,36]. Overall, the suppression of NF-κB signalling by a cocoa-rich diet contributes to the reduction in AOM-induced formation and the development of colonic ACF in rats.

In response to oxidative and pro-inflammatory stimuli, NF-κB is activated, at least in part, by a series of upstream kinases, including those belonging to the MAPK family proteins, such as ERK, JNK and p38 MAPK. Recent *in vitro* studies have demonstrated that cocoa phenolic compounds can regulate several signal transduction pathways[22,37] and transcription factors[38,39], and thus modulate the expression of specific genes to exert their function[40]. In the present study, we confirmed that a CPE obtained from the same cocoa used in the *in vivo* study significantly reduced the increase in inflammatory markers such as IL-8 secretion, COX-2 and iNOS expression induced by the pro-inflammatory agent TNF-α in Caco-2 cells. In addition, we showed that CPE selectively reduced both the phosphorylation of JNK and the nuclear translocation of NK-κB induced by TNF-α, indicating that this pathway could be an important mechanism contributing to the reduction of intestinal inflammation associated with cocoa feeding. Similar results have been found in previous reports indicating that several natural compounds could contribute to the inhibition of inflammation by blocking MAPK signalling and NF-κB[11]. More importantly, it has recently been demonstrated that JNK inhibition could prevent

inflammatory bowel disease[41]. Altogether, the mechanisms by which cocoa polyphenols reduced the expression of inflammatory markers seem to be partly due to their ability to inhibit JNK phosphorylation and NF-κB activation.

Considerable evidence has demonstrated that oxidative stress is involved in the link between chronic inflammation and cancer[42]. In fact, the activation of NF-κB by nearly all stimuli can be blocked by antioxidants, such as cysteine, *N*-acetylcysteine, thiols, green tea polyphenols and vitamin E[43,44]. Other than flavonoids, CPE also contains a high quantity of theobromine. In this regard, we have previously tested the potential participation of dimethylxanthine in the protection against oxidative stress in a cell culture model[21] and the results showed that the contribution of theobromine to the antioxidative ability of CPE is negligible. Accordingly, we suggest that the preventive effect of CPE in the inflammatory process is mostly provided by the flavonoid fraction. However, the potential synergistic effects between the bioactive cocoa components, flavanols and methylxanthines, need much further investigation and cannot be ruled out.

Interestingly, for many individuals, cocoa products constitute a larger proportion of the diet than foodstuffs containing bioactive compounds with similar properties such as green tea, wine or soyabeans[45–47]. Indeed, when compared with other flavonoid-containing foodstuffs, cocoa products exhibit a high concentration of procyanidins that are poorly absorbed in the intestine and consequently their beneficial effects would be restricted to the gastrointestinal tract where they may have an important antioxidant and anti-inflammatory local function[48]. Furthermore, using the body surface area normalisation method[49], the amount of cocoa employed in the present animal study is equivalent to a daily dose of 78 g cocoa, containing 1560 mg polyphenols, for a 60 kg human. Although this phenolic intake could be considered realistic, the amount of cocoa required is certainly above a recommended individual ingestion. Accordingly, a human intervention trial has been conducted using 20 g/d of polyphenol-rich dark chocolate containing 1000 mg polyphenols[50]. This dose of 20 g/d of dark chocolate represents 5–10 % of dietary energy, an amount that most people could readily incorporate into their diet.

In summary, the present study shows that cocoa polyphenols posses a potent anti-inflammatory effect *in vivo* and *in vitro* on the colonic tissue. At the molecular level, the inhibition of JNK/MAPK and NF-κB activation and the down-regulation of iNOS and COX-2 expression seem to play an important role in the prevention of intestinal inflammation. Therefore, together with their inherent antioxidant effects, cocoa polyphenols are able to reduce colon inflammation as a complementary mechanism of chemo-prevention in the early stages of chemical carcinogen-induced colonic ACF formation and development. Taken together, the present data provide evidence that cocoa polyphenols would offer a natural approach to improve individual health status including the prevention of intestinal inflammation with minimal toxicity.

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