

RESEARCH ARTICLE

Catechols in caffeic acid phenethyl ester are essential for inhibition of TNF-mediated IP-10 expression through NF- κ B-dependent but HO-1- and p38-independent mechanisms in mouse intestinal epithelial cells

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Scope: Caffeic acid phenethyl ester (CAPE) is an active constituent of honeybee propolis inhibiting nuclear factor (NF)- κ B. The aims of our study were to provide new data on the functional relevance and mechanisms underlying the role of CAPE in regulating inflammatory processes at the epithelial interface in the gut and to determine the structure/activity relationship of CAPE.

Methods and results: CAPE significantly inhibited TNF-induced IP-10 expression in intestinal epithelial cells. Using various analogues, we demonstrated that substitution of catechol hydroxyl groups and addition of one extra hydroxyl group on ring B reversed the functional activity of CAPE to inhibit IP-10 production. The anti-inflammatory potential of CAPE was confirmed in ileal tissue explants and embryonic fibroblasts derived from TNF^{AARE/+} mice. Interestingly, CAPE inhibited both TNF- and LPS-induced IP-10 production in a dose-dependent manner, independently of p38 MAPK, HO-1 and Nrf2 signaling pathways. We found that CAPE did not inhibit TNF-induced I κ B phosphorylation/degradation or nuclear translocation of RelA/p65, but targeted downstream signaling events at the level of transcription factor recruitment to the gene promoter.

Conclusion: This study reveals the structure–activity effects and anti-inflammatory potential of CAPE in the intestinal epithelium.

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Keywords:

Caffeic acid phenethyl ester / Inflammatory bowel diseases / Intestinal epithelium / Nuclear factor κ B / Polyphenols

1 Introduction

Caffeic acid phenethyl ester (CAPE) is proposed to have a wide range of biological activities via modulation of cellular

processes such as immune responses, cell division and apoptosis [1, 2]. Recent publications illustrate the promising

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Abbreviations: CAPE, caffeic acid phenethyl ester; CAPEA, caffeic acid phenethyl amine; ChIP, chromatin immunoprecipitation; HO-1, heme oxygenase-1; IEC, intestinal epithelial cells; I κ B, inhibitor of κ B; IKK, I κ B kinase; IP-10, interferon- γ inducible 10 kDa protein; MAPK, mitogen-activated protein kinase; MEF, mouse embryonic fibroblasts; MIP-2, macrophage inflammatory protein 2; NAC, N-acetylcysteine; NF- κ B, nuclear factor κ B; Nrf2, nuclear factor-erythroid 2 p45-related factor 2; PDC, phenethyl dimethyl caffeate; SEAP, secreted alkaline phosphatase; TNF, tumor necrosis factor; WT, wild type

role of CAPE as a potential prophylactic and therapeutic agent with emphasis on anti-inflammatory effects [3–5]. CAPE was first isolated as an active phenolic constituent of honeybee propolis possessing cytotoxic and antitumor activities [6]. In subsequent experiments, CAPE potently inhibited activation of the nuclear factor (NF)- κ B signaling pathway [7]. However, the molecular mechanisms underlying NF- κ B inhibition by CAPE are largely unknown. Previously, we assessed the functional diversity of various polyphenols with respect to the modulation of NF- κ B, interferon regulatory factor and Akt activation that shape inflammatory responses involved in cytokine induction [8, 9]. Although our understanding of how most of these non-nutritive polyphenols modulate cellular functions *in vitro* has improved, the structural features responsible for mechanistic effects remain unclear. The limited structural complexity of CAPE makes it a good model compound for structure function analysis. However, most studies on CAPE do not show evidence for the specific pharmacophore (a set of structural features with biological activity that is recognized at receptor sites of target proteins) responsible for anti-oxidative and/or anti-inflammatory properties [10–12].

Interferon- γ inducible 10 kDa protein (IP-10) has been described as a chemoattractant, which binds to the chemokine (C-X-C motif) receptor 3 (CXCR3) receptors on monocytes and activated Th1 lymphocytes upon challenge with tumor necrosis factor (TNF) or LPS [13]. Recently, we have shown that IP-10 protein expression by intestinal epithelial cells (IEC) is up-regulated in the TNF ^{Δ ARE/+} mouse model of experimental ileitis [14]. One of the most important signaling mediators related to inflammatory processes is NF- κ B, which plays a key role in coordinating the transcriptional induction of several cytokines such as TNF- α and IL-1 and chemokines such as IL-8 and IP-10. The critical process of NF- κ B activation has been comprehensively

described [15, 16]. Typically, NF- κ B binds to DNA as homodimers or heterodimers of five possible subunits, including RelA (p65), c-Rel, RelB, p100/p52 and p105/p50, where p52 and p50 are truncated forms of p100 and p105, respectively. These dimeric complexes bind at NF- κ B-binding sites in target genes with distinct preferences, distinguishable affinity and specificity, whereby different combinations of these heterodimers act as transcriptional activators or repressors. Regulation of NF- κ B is mediated by the inhibitor of κ B (I κ B). In activated cells, I κ B α is phosphorylated by the activated I κ B kinase (IKK) complex followed by ubiquitylation and degradation by the 26S proteasome. Liberated NF- κ B translocates to the nucleus where it binds to NF- κ B-specific DNA sequences resulting in transcription of many pro-inflammatory cytokines [17, 18]. NF- κ B activation or suppression in epithelial cells seems to be critical for tissue homeostasis in the gut mucosa, as observed in various experimental conditions [19–21].

The first aim of the present study was to identify structural features of CAPE that are essential for anti-inflammatory properties, as measured by production of IP-10 in the IEC line Mode-K. In addition, we aimed at providing new insights into the mechanistic significance of CAPE in the regulation of NF- κ B activity. Finally, we tested the physiological relevance of our *in vitro* findings using samples from TNF ^{Δ ARE/+} mice.

2 Materials and methods

2.1 Chemicals

The structures of the compounds used in the present study are shown in Fig. 1. CAPE, phenethyl 3-methyl caffeate (P3MC) and phenethyl dimethyl caffeate (PDC) were

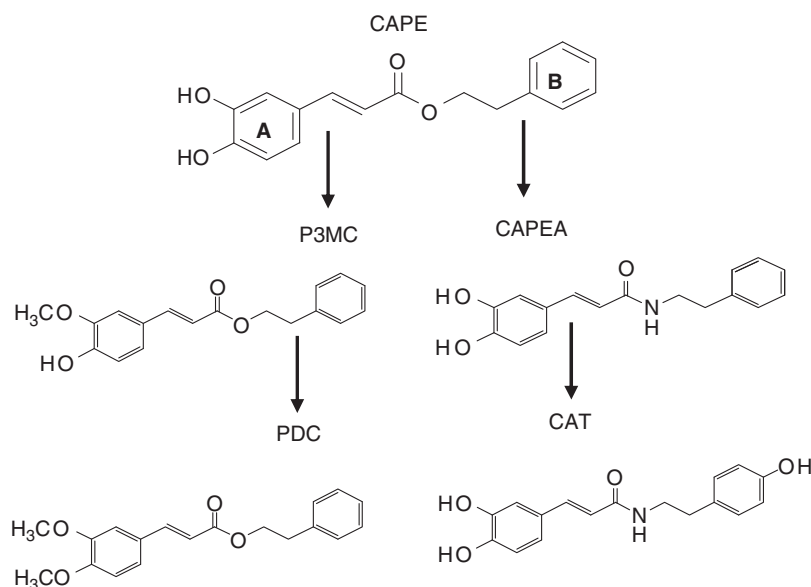


Figure 1. Chemical structures of CAPE and analogues. Phenethyl 3-methyl caffeate (P3MC) was substituted with one methoxyl, while phenethyl dimethyl caffeate (PDC) had two substitutions on the hydroxyl groups of the catechol ring (A). Caffeic acid phenethyl amine (CAPEA) was prepared by substituting the ester with an amide, while Caffeic acid tyramine (CAT) was prepared by adding one extra hydroxyl on the benzene ring (B) of CAPEA.

purchased from LKT Laboratories (St. Paul, MN, USA). Caffeic acid phenethyl amine (CAPEA) and caffeic acid tyramine (CAT) were synthesized at the Food Chemistry and Molecular Sensory Science department (TU München) using standard protocols. TNF was purchased from Invitrogen. LPS, SB203580 and BAY11-7082 were purchased from Sigma-Aldrich. For all treatments, TNF and LPS were used at a final concentration of 10 and 100 ng/mL, respectively.

2.2 Cell culture

The small IEC line Mode-K (passage 13–25) [22] was cultivated in high glucose DMEM medium (Invitrogen) containing 10% v/v FBS, 1% Antibiotic/Antimycotic (PAA) and 2 mM of L-Glutamine (Invitrogen). Cells were grown at 37°C in tissue culture plates (Cell Star, Greiner bio-one) in a humidified atmosphere containing 5% CO₂. They were split every third day and grown to 80% confluency before stimulation.

2.3 Mouse embryonic fibroblasts (MEF)

For preparation of embryonic fibroblasts, C57BL/6 wild type (WT) and TNF^{ΔARE/+} mice were killed by cervical dislocation at day 13.5 of pregnancy. Embryos were taken out, placed in separate wells and killed by decapitation. Heart and liver were removed. Embryonic tissues were rinsed with pre-warmed PBS, transferred into 3 mL of 0.25% Trypsin/EDTA (supplemented with 2% chicken serum), and minced into small pieces. After incubation time of 15 min at 37°C, 10 mL of MEF medium was added [DMEM medium supplemented with 10% FBS, 1% Antibiotic/Antimycotic (PAA), 2 mmol/L L-Glutamine, 100 μM non-essential amino acids (PAA) and 1 mM sodium pyruvate (Sigma)]. The suspensions were transferred into 15 mL falcon tubes leaving cell debris to settle. Supernatants were transferred and centrifuged (430 × g, 5 min, RT). Pellets were re-suspended in 7 mL MEF medium. Cells from each embryo were seeded separately in T25 flasks and incubated at 37°C, 5% CO₂. The medium was changed after 24 h and cells were incubated for additional 24 h. Following genotyping, MEF were pooled together (passage 1) in MEF freezing medium (70% MEF medium supplemented with 20% FBS and 10% DMSO) and stored in cryo-vials at –180°C until use. Stimulation experiments were carried out using MEF between passages 1–7. Nrf2^{+/+} and Nrf2^{-/-} MEF (a generous gift from Dr. Alben Dinkova-Kostova and Prof. Masayuki Yamamoto) were maintained in culture as described previously [23].

2.4 Tissue explants

Ileal tissues obtained from 12-wk-old WT and TNF^{ΔARE/+} mice were flushed with PBS and cut open into 4-mm-long tissue pieces, which were placed onto Netwell inserts in

Mode-K medium with the serosal side touching the transwell membrane (Corning Life Sciences). After incubation with or without CAPE (20 μM) for 24 h, supernatants were collected while the tissue was mashed in lysis buffer containing 1% Triton X-100, 150 mM NaCl, 10 mM Tris pH 7.4, 1 mM EDTA, 1 mM EGTA as well as complete Mini (protease) and Phos-Stop (phosphatase) inhibitors (Roche). Tissue homogenates were centrifuged (16 000 × g, 4°C, 10 min) and protein-containing supernatants were used for immunoblotting.

2.5 Cell viability and cytotoxicity

Cells were grown to 80% confluency in 96-well plates followed by pre-incubation with test substances for 24 h. Proliferation and cytotoxicity assays were performed using the cell counting kit-8 (Dojindo) according to manufacturer's instructions. The absorbance was measured at 450 nm using a Multiskan spectrophotometer (Thermo scientific).

2.6 ELISA analysis

Mode-K cells and TNF^{ΔARE/+} MEF were pre-incubated with test compounds (10 μM) for the indicated times followed by stimulation with or without TNF or LPS for additional 24 h. Secreted IP-10, TNF and MIP-2 proteins in the supernatants were measured using mouse-specific DuoSet ELISA kits, according to manufacturer's instructions (R&D Systems). All experiments were performed in triplicate and repeated at least three times.

2.7 Reporter (SEAP) gene assay for NF-κB transcriptional activity

The secreted alkaline phosphatase (SEAP) gene is under the control of an NF-κB inducible endothelial leukocyte adhesion molecule 1 (ELAM1) composite promoter, which drives the expression of a reporter gene that is induced in the presence of NF-κB or repressed in its absence. The SEAP gene reporter assay was carried out as described previously [9]. Briefly, *pNlFty-SEAP* transfected Mode-K cells were pre-incubated with CAPE and its analogues for 1 h before TNF stimulation for 24 h. Subsequent assays were carried out according to manufacturer's recommendations (InvivoGen). Samples were measured at 405 nm in a Multiskan spectrophotometer (Thermo scientific).

2.8 Chromatin immunoprecipitation (ChIP)

ChIP was performed using the ChIP kit (Cell Signaling; #9003) as described by the manufacturer. Briefly, Mode-K cells were pretreated for 4 h with CAPE before addition of TNF. After 30 min, cells were fixed in 1% formaldehyde

(10 min, RT). The chromatin digest was normalized according to the purified DNA concentration and immunoprecipitated against anti-phospho-RelA^{ser536}, anti-acH3, anti-acetyl CBP/p300 (all from Cell signaling) and NF- κ B p50 (D-17) (Santa Cruz) antibodies. H3 and normal rabbit IgG antibodies (Cell Signaling) were used as positive and negative controls, respectively. Immunoprecipitated DNA, together with purified DNA as input control (2% of the total chromatin extract) was used as template for PCR amplification using the following promoter-specific primers: IP-10, F-5'-aaggagcacaagagggg, R-5'-attggctgacttggag; SimpleChip mouse RPL30 (Cell Signaling). PCR products were resolved by electrophoresis on 1% agarose gels.

2.9 RT-PCR

Total RNA was extracted from cells using the Isol-RNA-Lysis according to manufacturer's instructions (5-Prime). Nucleotide concentrations were determined using the Nanodrop spectrophotometer (PqLab). Complementary DNA (cDNA) was obtained from 1 μ g RNA by reverse transcription using random primers (Invitrogen) and M-MLV reverse transcriptase (Invitrogen). Real-time qPCR was performed using the UPL master mix and primer sets designed by the Universal Probe Library on the LightCycler 480 (Roche). Crossing point (Ct) values were obtained using the Second Derivative Maximum method. Coefficients of regulation between treated and control samples were calculated from triplicate samples according to the $\Delta\Delta$ Ct method [24]. Primer pairs (mouse UPL, Roche) were: GAPDH, F-5'-tcactcatggcaaatcaa, R-5'-ttgatgttagtgggtctcg (probe no. 9); IP-10, F-5'-gctgccgtcattttctgc, R-5'-tctactggcccgtcatc (probe no. 3); TNF, F-5'-tgccatgtctcagcctcttc, R-5'-gaggccatttgggaacttct (probe no. 49); MIP-2, F-5'-ctggttcaga-aatcatcca-3', R-5'-cttcggttgaggacagc-3' (probe no. 63); NF- κ B RelA, F-5'-cccagaccgagtcctc-3', R-5'-ttctcgctctggacc tgc-3' (probe no. 47); NF- κ B p105, F-5'-cactgctcaggtcactgtc-3', R-5'-acttgaggccctcatcgtc-3' (probe no. 69); NF- κ B p100, F-5'-tggaacagccaacagc-3', R-5'-tacctcaaacggtccac (probe no. 76); NF- κ B RelB, F-5'-gtgacctcttccctgtcact-3', R-5'-aacctttgtagctgcttatgt-3' (probe no. 80).

2.10 Western blotting

Cells were lysed in SDS-PAGE sample buffer and 10 μ g proteins resolved on a 10 or 15% SDS-polyacrylamide gel. Antibodies against phospho-RelA (Ser536), phospho-p38 MAPK, p38 MAPK, phospho-I κ B α , I κ B α , phospho-ERK, ERK, phospho-SAPK/JNK, SAPK/JNK, phospho-AMPK, AMPK (Cell Signaling), p65/RelA (Santa Cruz), IP-10 (R&D systems), heme oxygenase-1 (HO-1) (Stressgen) and β -actin (MP Biomedicals) were used and the protein bands were detected with the Amersham ECL detection kit (GE Healthcare).

2.11 Immunofluorescence and confocal microscopy

Cells were grown on cover-slips in 6-well plates to 80% confluency, treated with CAPE for 1 h followed by TNF for 20 min and fixation with 4% formaldehyde in PBS (15 min, RT). Cover-slips were rinsed three times in PBS (5 min each) before blocking (1 h) in 5% normal goat serum supplemented with 0.5% Triton X-100. After overnight incubation at 4°C in anti-RelA primary antibody [1:100 dilution; sc-372, (Santa Cruz)], cover-slips were rinsed three times as above and Alexa-Fluor 488 goat-anti-rabbit secondary antibody (Invitrogen) was added. After 2 h at room temperature in the dark, rinsed slides were covered in VectaShield HardSet mounting medium with DAPI (Vector Laboratories). Cellular localization of RelA was determined using a Leica SP2 confocal laser scanning microscope (Leica Microsystems).

2.12 Feeding experiment in TNF^{ARE/+} mice

The animal use protocol was approved by the Bavarian Animal Care and Use Committee (No. 55.2-1-54-2531-88-09). Twelve-week-old heterozygous TNF^{ARE/+} (kindly provided by Dr. George Kollias) and WT C57BL/6 mice ($n = 5$ per treatment/genotype group) were fed gelatin pellets (15% w/v gelatin and 20% sucrose) with or without CAPE (10 mg/kg body weight) three times a wk for six wks in addition to their usual diet [8]. Mice were killed by cervical dislocation at the age of 18 weeks and samples were prepared for histological scoring and IEC isolation as previously described [8].

2.13 Statistical analysis

Values were expressed as mean $\pm$ SD of triplicate measurements representative of at least two independent experiments. Statistical analyses were performed using the SigmaPlot 11 (Systat Software). Mean values were compared by ANOVA (data were tested for normal distribution and equality of variances). The Holm–Sidak test was used for pairwise comparisons. For all tests, the bilateral α risk was $\alpha = 0.05$.

3 Results

3.1 CAPE inhibits IP-10 expression in a dose- and time-dependent manner

CAPE inhibited IP-10 induction in Mode-K cells in a dose-dependent manner, i.e. both intracellular (Fig. 2A) and secreted IP-10 protein levels (Supporting Information Fig. S1A) were reduced in the presence of CAPE. Since we obtained a marked and reproducible inhibition using 10 μ M and the test compounds were less cytotoxic at this concentration (80% viability after 24 h) (Supporting Information Fig. S1B), 10 μ M

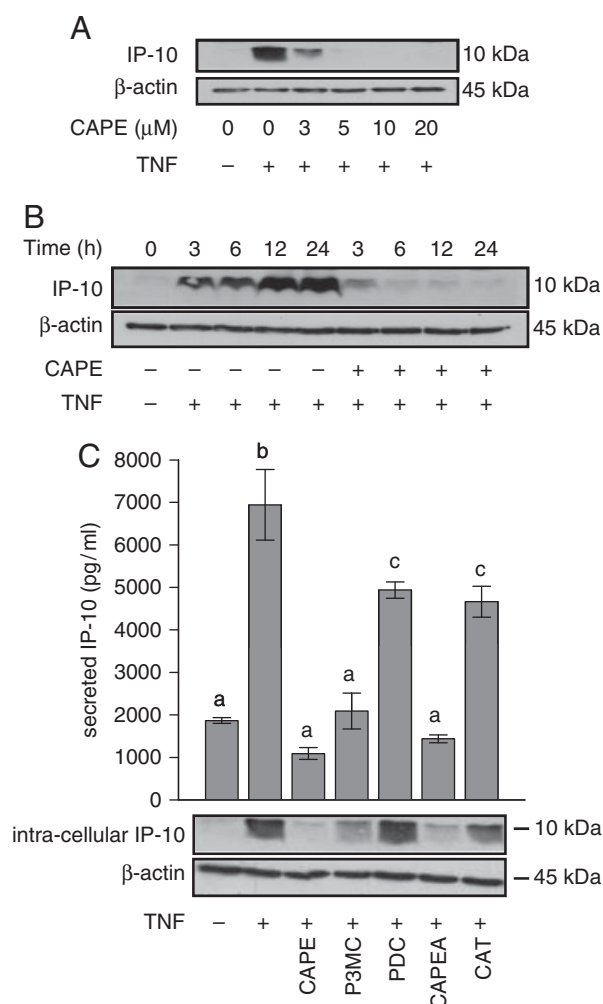


Figure 2. CAPE inhibits IP-10 in a dose-, time- and structure-dependent manner. (A) Cells were pre-incubated with the indicated concentrations of CAPE for 1 h before TNF (10 ng/mL) for additional 24 h. (B) Cells were pre-incubated with CAPE for 1 h followed by TNF for the indicated time. (C) Cells were pre-incubated with 10 μ M of test compounds followed by TNF for additional 24 h. The results are mean $\pm$ SD from triplicate measurements representative of three independent experiments; means without a common letter differ, $p < 0.05$.

was used as the concentration of reference in all subsequent experiments, unless otherwise stated. To determine how long CAPE sustained inhibition of TNF-induced IP-10 production, we performed time-course experiments. CAPE abolished IP-10 expression from 3 to 24 h of TNF stimulation, indicating that CAPE-mediated inhibitory functions occurred at early time points and persisted over time (Fig. 2B).

3.2 Hydroxyl groups determine the anti-inflammatory effects of CAPE

To investigate the structure/activity relationship of CAPE, we used a variety of CAPE analogues (Fig. 1) and examined

Table 1. The half maximal inhibitory concentration 50 (IC_{50}) of CAPE and its analogs for cell cytotoxicity and IP-10 inhibition in Mode-K cells

Compound	Cytotoxicity (μ M)	IP-10 inhibition (μ M)
CAPE	61.0	0.85
P3MC	76.8	1.78
PDC	> 100.00	> 100.00
CAPEA	85.2	0.98
CAT	90.2	27.91

IC_{50} values were generated from three independent experiments (CAPE concentrations in the range of 0–200 μ M) using the Four Parameter Logistic Equation in SigmaPlot 11 (Systat Software).

their activity via measurement of cell viability and TNF-induced IP-10 expression in Mode-K cells. Both ELISA and Western blot analyses revealed that CAPE, P3MC and CAPEA significantly reduced IP-10 expression ($p < 0.05$), while PDC and CAT were significantly less active (Fig. 2C). These observations prompted us to determine the half maximal inhibitory concentrations 50 (IC_{50}) of all five compounds. The IC_{50} values were in the micromolar (cytotoxicity) and nanomolar (IP-10 inhibition) range and showed that CAPE was most potent (Table 1). The gradual decrease in cytotoxic IC_{50} CAPE > P3MC > PDC suggested that the catechol hydroxylic groups in ring A define functional effects, i.e. the presence of one hydroxyl group retains activity while substitution of both hydroxyl groups in ring A leads to a loss of activity. Also, substitution of the ester functional group with an amide group (CAPEA) revealed only limited functional consequences, i.e. led only to a slight decrease in IC_{50} . However, further modification of CAPEA to CAT by addition of a hydroxyl group to ring B markedly reduced the IC_{50} (Table 1). Importantly, we found that the effect of CAPE treatment on Mode-K cells was specific. CAPE, as well as P3MC and CAPEA, did not affect the expression of another TNF-induced chemokine of the CXC family, namely macrophage inflammatory protein 2 (MIP-2), to the same extent as IP-10, i.e. return to basal levels as in the absence of TNF (Supporting Information Fig. S2).

3.3 CAPE inhibits NF- κ B by attenuating transcription factor recruitment to the gene promoter

To elucidate anti-inflammatory mechanisms underlying IP-10 inhibition, we investigated the influence on NF- κ B activity. Therefore, we examined the effect of CAPE and analogues on promoter activity using the SEAP reporter assay. Compared with control samples, SEAP expression was up-regulated after TNF administration. However, it was significantly reduced after treatment with CAPE and CAPEA, but not P3MC, PDC and CAT (Fig. 3A). Importantly, CAPE completely abolished NF- κ B transactivation revealing that the catechol hydroxylic groups are essential

for its bioactivity. The influence of CAPE on gene transcription was confirmed by measurement of IP-10 mRNA levels. CAPE completely blocked IP-10 mRNA (Fig. 3A) and protein (Fig. 2C) synthesis indicating that it most likely targets the de novo synthesis of IP-10 mRNA and protein via the NF- κ B pathway [17]. The most important mechanism that prevents spontaneous NF- κ B activation is the cytoplasmic retention via I κ B α . I κ B α phosphorylation, ubiquitination and subsequent proteasomal degradation is essential for full NF- κ B activation. To further investigate molecular mechanisms of CAPE-mediated inhibition of the NF- κ B pathway, we checked for TNF-induced I κ B α degradation and RelA phosphorylation. CAPE neither inhibited I κ B α degradation nor RelA phosphorylation (Fig. 3B). Even at increasing concentrations, CAPE did not reverse the I κ B α degradation process after 30 min of TNF stimulation (Fig. 3C). We also compared CAPE and BAY-11-7082 with respect to their ability to inhibit NF- κ B activity. BAY-11-7082 selectively inhibits NF- κ B activation by blocking TNF-induced degradation of I κ B α without affecting constitutive

I κ B α phosphorylation. The results confirmed that CAPE does not inhibit I κ B α degradation (Supporting Information Fig. S3A). It has been shown that I κ B α expression is induced by NF- κ B providing a negative feedback that terminates NF- κ B activation [25]. However, NF- κ B activation is known to be biphasic following TNF stimulation (the initial phase lasts one hour followed by a persistent second phase that depends on the input stimuli concentration), and some genes require persistent TNF stimulation to be fully activated [25]. To investigate the influence of CAPE on prolonged TNF stimulation, we monitored I κ B α phosphorylation and degradation leading to NF- κ B activation over eight hours. The results showed that CAPE neither altered the extent of TNF-induced RelA phosphorylation nor I κ B α phosphorylation/degradation (Supporting Information Fig. S3B). In addition, we investigated the influence of CAPE on RelB, p100, p105 and p65 mRNA expression. The results indicated that, in combination with TNF, CAPE induced the expression of relB and p105, a p50 precursor (Supporting Information Fig. S3C). We next investigated

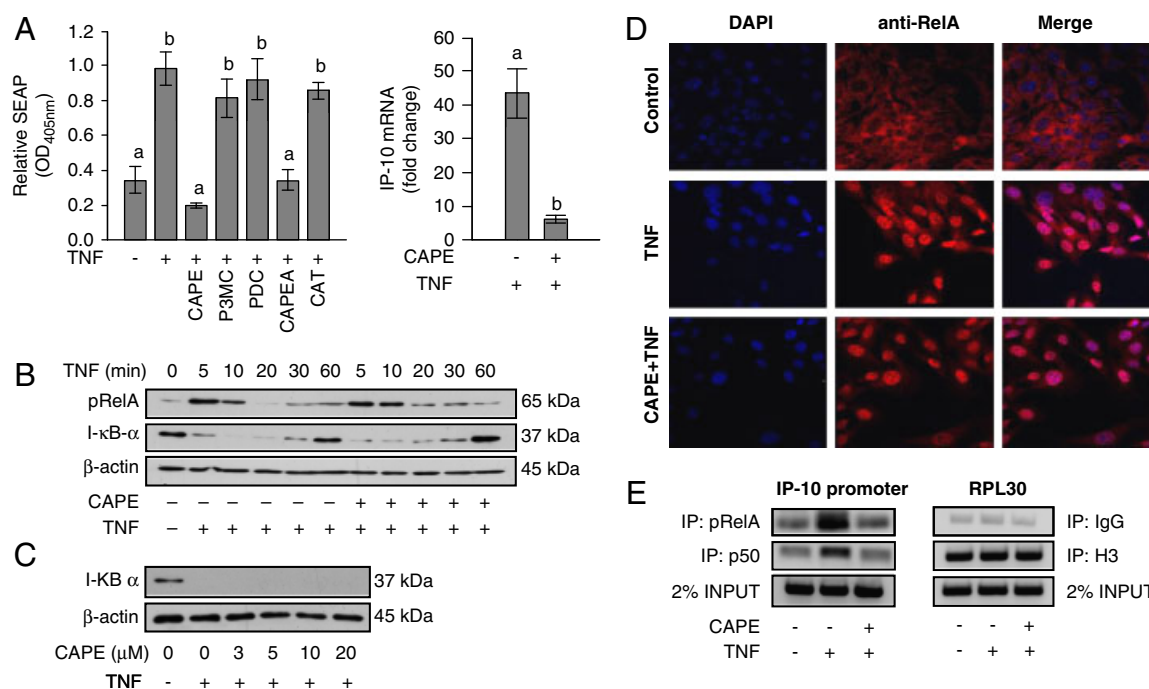


Figure 3. CAPE attenuates NF- κ B activity at the promoters of target genes. (A) CAPE targets the NF- κ B site of the ELAM1 promoter. Mode-K cells were stably transfected with a pNiFty plasmid expressing the SEAP gene under the control of an NF- κ B-inducible ELAM1 composite promoter. Transfectants were pre-incubated for 1 h with 10 μ M of indicated compounds before TNF stimulation for additional 24 h. In addition, CAPE inhibited IP-10 mRNA expression (Mode-K cells were pre-incubated with 10 μ M CAPE for 1 h before TNF stimulation for 4 h). The results are mean $\pm$ SD of triplicate measurements representative of three independent experiments; means without a common letter differ, $p < 0.05$. (B to D) CAPE neither inhibited TNF-induced I κ B α degradation nor RelA phosphorylation and subsequent nuclear translocation. (B) Time course for Mode-K cells treated with or without CAPE for 1 h followed by TNF induction. (C) Dose dependence for Mode-K cells treated with or without CAPE for 1 h followed by TNF for 30 min. (D) Confocal immunofluorescence images (400 $\times$) of cells labeled with anti-RelA (red) showing cytoplasmic staining (control) and nuclear translocation (TNF-stimulated) and DAPI (blue) signals were generated from Mode K cells treated with or without CAPE for 1 h followed by TNF for 20 min. The results are representative of two independent experiments. (E) CAPE reduces recruitment of p50/RelA heterodimer to the IP-10 promoter. Cells were pre-incubated with CAPE for 4 h before TNF stimulation for 30 min followed by RelA, p50, H3 and IgG antibody immunoprecipitation of the enriched chromatin; H3 and IgG were included as positive and negative controls against the ribosomal protein L30 (RPL30) gene.

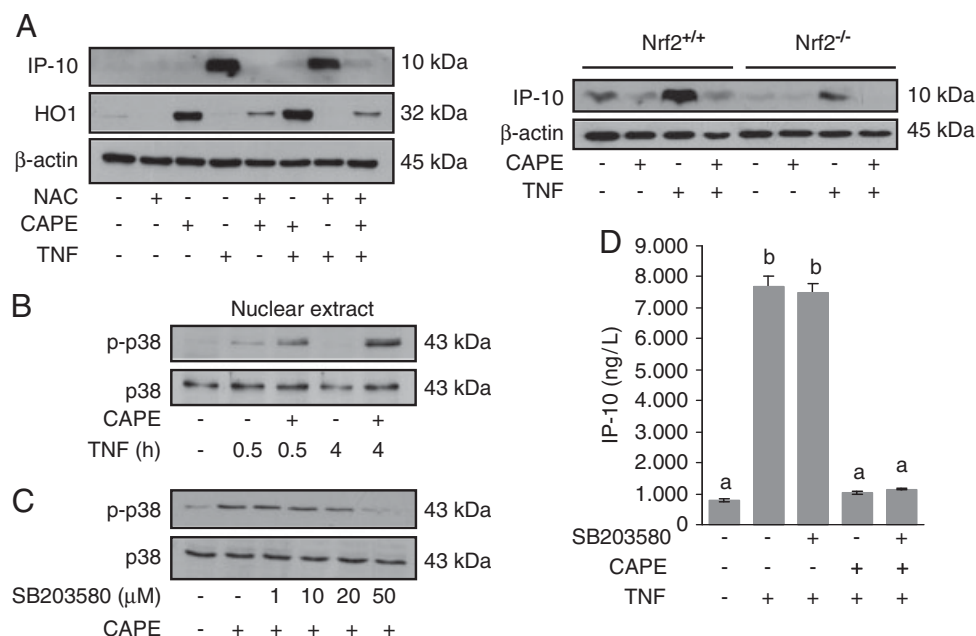


Figure 4. Accumulation of HO-1 and phospho-p38 MAPK had no influence on CAPE-mediated IP-10 inhibition. (A) Co-treatment of Mode-K cells with NAC (HO-1 inhibitor) (20 mM) for 16 h did not reverse CAPE mediated IP-10 inhibition. CAPE also abolished IP-10 expression in Nrf2^{-/-} MEF (a generous gift from Dr. Albena Dinkova-Kostova and Prof. Masayuki Yamamoto). Nrf2^{+/+} and Nrf2^{-/-} MEF cells were pre-incubated with 10 μM CAPE for 1 h before TNF stimulation for 24 h. (B) CAPE increases phosphorylation of p38 MAPK in a time-dependent manner. Mode-K cells were pre-incubated with or without CAPE for 1 h followed by TNF for the indicated time. (C) Mode-K cells were pre-incubated with indicated amounts of SB203580 for 30 min prior to CAPE treatment for 4 h. (D) Inhibition of p38 MAPK phosphorylation does not reverse CAPE-mediated IP-10 inhibition. Cells were incubated with or without SB203580 (50 μM) then CAPE for 1 h followed by TNF for 24 h.

whether CAPE targets nuclear translocation of RelA. In comparison to non-treated cells, CAPE treatment had no effect on RelA translocation as demonstrated by immunofluorescence analysis (Fig. 3D). Indeed, RelA was localized in the cytoplasm in non-treated cells and quickly shifted into the nucleus following TNF stimulation, even when cells were pre-incubated with CAPE. These observations pointed at a process in which NF-κB activity is controlled at the level of transcription factor binding to the gene promoter and chromatin remodeling. To investigate this assumption, we analyzed IP-10 promoter regions for NF-κB binding using ChIP analysis, with emphasis on the functional specificity of the p50/RelA heterodimer. CAPE reduced TNF-induced IP-10 promoter occupancy of both p50 and phospho-RelA NF-κB subunits (Fig. 3E), suggesting that both subunits are essential for IP-10 expression.

3.4 CAPE-mediated IP-10 inhibition is independent of HO-1, Nrf2 and p38 MAPK signaling pathways

Several polyphenols exhibit a strong anti-oxidative capacity. Since HO-1 is a key player in response to oxidative stress, we assessed the role of HO-1 activation and underlying nuclear factor-erythroid 2 p45-related factor 2 (Nrf2) signaling pathway in CAPE-mediated inhibition of IP-10 production. As shown in Fig. 4A, CAPE (10 μM) strongly induced HO-1

protein expression, yet inhibition of CAPE-induced HO-1 protein levels using N-acetylcysteine (NAC) did not affect inhibition of IP-10 production. By using Nrf2-knockout MEF, we also showed that CAPE-induced IP-10 inhibition seems not to be dependent upon Nrf2 signaling (Fig. 4A). Activation of mitogen-activated protein kinases (MAPKs), like p38, has also been associated with anti-inflammatory effects of polyphenols. Interestingly, the levels of phospho-p38 MAPK increased markedly in the nucleus within 4 h of CAPE treatment (Fig. 4B). To determine whether the p38 MAPK pathway activation is required for the observed inhibitory effects, Mode-K cells were co-treated with CAPE and increasing amounts of SB203580, a p38 MAPK inhibitor (Fig. 4C). Interestingly, SB203580 did not reverse the inhibitory effect of CAPE on IP-10 expression (Fig. 4D), suggesting that the accumulation of phospho-p38 MAPK in the nucleus does not play a direct role in CAPE-mediated IP-10 inhibition. Additionally, CAPE was not able to activate ERK but phosphorylated SAPK/JNK and AMPK in a time-dependent manner (Supporting Information Fig. S4).

3.5 CAPE suppressed IP-10 and TNF expression *ex vivo* but failed to reduce ileitis in TNF^{ΔARE/+} mice

To bring to the test the relevance of our *in vitro* experiments in TNF-stimulated Mode-K cells, we generated embryonic

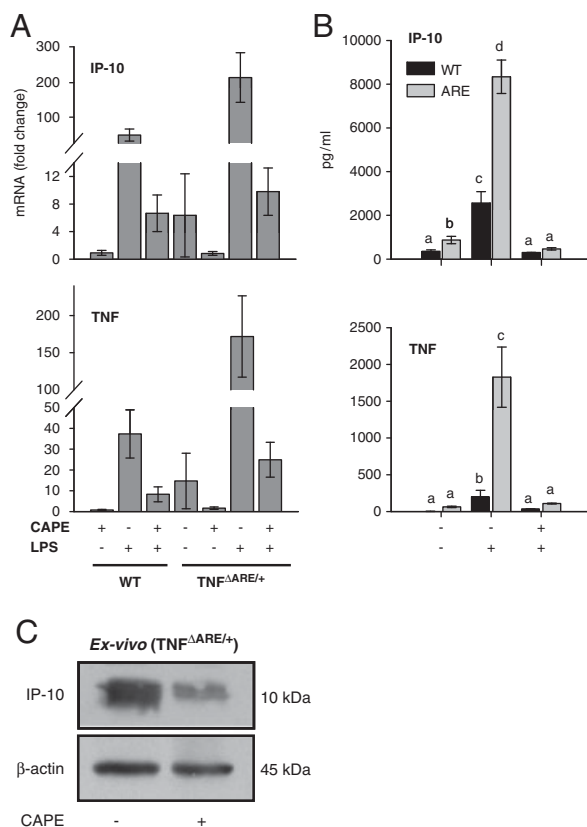


Figure 5. CAPE suppresses IP-10 and TNF mRNA and protein expression ex vivo. WT and $TNF^{\Delta ARE/+}$ MEF were pre-incubated with or without CAPE for 1 h followed by LPS stimulation for 4 h (A) (mRNA expression) or 24 h (B) (protein concentrations in culture supernatant). The results are mean $\pm$ SD of triplicate measurements representative of three separate experiments; means without a common letter differ, $p < 0.05$. (C) IP-10 inhibition in ileum tissue explants. Washed ileal tissues from WT and $TNF^{\Delta ARE/+}$ mice placed onto Netwell inserts were incubated with or without CAPE (20 μ M) for 24 h. Tissue were mashed in lysis buffer, centrifuged and used for Western blotting.

fibroblasts using the $TNF^{\Delta ARE/+}$ mouse model of experimental ileitis. It is here worthwhile to note that $TNF^{\Delta ARE/+}$ mice produce excessive amounts of TNF, a hallmark for chronic inflammation in the gastrointestinal tract. Both transcriptional (Fig. 5A) and post-transcriptional (Fig. 5B) analysis confirmed that CAPE significantly inhibited IP-10 as well as TNF gene and protein expression. It also showed that CAPE not only targeted TNF signaling, but also the LPS-induced TLR4 signaling pathway. To determine the expression of IP-10 in the ileum, we performed ex vivo experiments using explant tissues from 12-week-old mice. The results clearly showed that CAPE suppressed intracellular expression of IP-10 (Fig. 5C). We then tested the efficacy of CAPE in the $TNF^{\Delta ARE/+}$ mouse model of ileitis. After six weeks of feeding, CAPE did not antagonize the development of Crohn's disease-like ileitis (Supporting Information Fig. S5A). When compared with sham-fed control mice, $TNF^{\Delta ARE/+}$ mice were characterized by similar

levels of leukocyte infiltration in the mucosa and submucosa (Supporting Information Fig. S5B).

4 Discussion

In the present study, we demonstrate anti-inflammatory effects of CAPE on IP-10 expression by providing novel data on structure/activity relationship and molecular mechanisms underlying NF- κ B pathway inhibition. We focused on the chemokine IP-10 as endpoint target because IP-10 has been described as a master effector molecule of inflammatory responses [13, 26, 27].

We observed a time- and concentration-dependent inhibition of TNF-induced IP-10 expression by CAPE in intestinal epithelial Mode-K cells. By comparing the activity of several analogous compounds obtained by synthetic modification, we found that catecholic hydroxyls are more reactive in comparison to methoxyl groups. Substitution of one of the hydroxyl in ring A (P3MC) resulted in reduced activity and methoxylation of both hydroxyls (PDC) rendered the compound inactive. The ability of P3MC to inhibit IP-10 expression but not SEAP activity when compared with CAPE can be attributed to methoxylation of the reactive hydroxyl group in ring A lowering the potency to reduce NF- κ B-induced gene expression. Inhibition of TNF-induced IP-10 production by P3MC and CAPE depends on downstream events within the NF- κ B signaling pathway as influenced by different NF- κ B dimer combinations, whereas the SEAP assay is primarily under the control of κ B-binding repeats. Of course, we also cannot exclude the fact that signaling pathways other than NF- κ B play a substantial role in the inhibition of IP-10 production by P3MC. These observations in Mode-K cells are in agreement with a recent study showing that methylation of the hydroxyl residues in polyphenols resulted in a loss in potency against human leukemia cells [28]. In addition, Wang et al. [29] and Lee et al. [30] have also shown very recently that methoxy derivatives of CAPE are characterized by impaired or diminished cytoprotective and NF- κ B inhibitory functions, respectively. In our experiments, the importance of hydroxyl groups in mediating anti-inflammatory mechanisms was also supported by the fact that CAPE retained most of its biological activity after the ester was replaced with an amide (CAPEA), suggesting that the more stable amides are as active as the corresponding structurally flexible (less rigid and more volatile) esters. However, modification of CAPEA to CAT by further addition of a hydroxyl group to ring B resulting in the formation of a phenol ring led to a total loss of activity. One possible explanation is that the proximity of hydroxyl residues of the catechol structure facilitates formation of sterically important reactive intermediates [31, 32]. The subtle structural differences between PDC and CAT may account for loss of activity as a result of: (i) increased polarity and, as a consequence, reduced cell membrane permeability; (ii) disturbed hydrophobic interactions in

target protein binding sites. These results confirm the recent findings by Wang et al. and Lee et al. showing that the phenethyl moiety of CAPE determines activity, i.e. caffeic acid or *n*-alkyl derivatives were characterized by loss of cytoprotection and anti-inflammatory activity [29, 30]. Taken together, our molecular similarity analyses revealed that the reactivity of the catecholic hydroxyl groups control the potency of CAPE, that is, its ability to interact with target proteins.

We next aimed at providing novel insights into the molecular mechanisms underlying CAPE activity, focusing primarily on the functional effects of the p50/RelA heterodimer, which has been shown to transactivate many inflammatory genes [33–35]. The major regulatory step that prevents spontaneous NF- κ B activity is the retention of the NF- κ B/I κ B α complex in the cytoplasm. Dissociation of this complex results in I κ B α degradation and NF- κ B liberation to the nucleus. While others reported inhibitory effects of CAPE on the IKK complex and I κ B α phosphorylation in human CD4⁺ T cells and the gastric epithelium [36, 37], we could show that phosphorylation of NF- κ B and I κ B α , degradation of I κ B α and RelA nuclear translocation were unchanged in Mode-K cells after CAPE treatment, suggesting that effects are cell/tissue type-specific. However, our results are in agreement with two previous reports which demonstrated that CAPE has no effect on I κ B α degradation in osteoclasts [38] and U937 cells [7]. Concentrations also likely influence effects. For instance, Lee et al. also obtained results that contradict ours, i.e. partial prevention of I κ B α degradation by CAPE, but using higher concentrations (> 25 μ M) in HCT116 cells [30]. The non-canonical NF- κ B signaling pathway is a result of p100 to p52 processing by the proteasome. The liberated p52 forms active p52/RelB complexes that induce transcriptional process responses differing from the I κ B α -dependent canonical pathway. Processing of p100 and I κ B α degradation regulate different NF- κ B dimers and as a consequence non-canonical and canonical pathways regulate distinct NF- κ B target genes that are stimuli specific. In our experiments, CAPE did not alter p100 mRNA expression, yet induced transcripts of the p50 precursor p105. Consistent with a previous report that polyphenols affect recruitment of transcription factors to gene promoter regions [8], we found that CAPE inhibits NF- κ B (p50/RelA heterodimer) binding to the IP-10 promoter. This is also in line with a genetic analysis study by Hoffmann et al., who found that both p50 and RelA subunits are essential for IP-10 expression [17]. Whereas CAPE inhibited p50 and RelA binding onto the IP-10 promoter, its failure to inhibit the coordinated degradation and re-synthesis of I κ B α may be attributed to selective gene activation in the I κ B α promoter [39]. Importantly, Hoffmann et al. also showed that, whereas most regulatory genes are RelA subunit-dependent, the p50 subunit is dispensable for some of these genes including the neutrophils chemokine MIP-2 that controls mucosal lymphocyte migration in IEC [40]. MIP-2 can be induced by many of the

RelA-containing dimeric complexes including p50/RelA, p52/RelA and RelA/RelA. Recently, it was shown that NF- κ B and AP-1 activation is required for MIP-2 up-regulation and silencing of NF- κ B alone may not be sufficient to reduce inflammation in acute pancreatitis [41]. Data from the literature have also demonstrated that CAPE does not affect AP-1 promoter activity [7]. Taken together, we propose that CAPE targets NF- κ B DNA-binding sites of the p50/RelA heterodimer without compromising other essential NF- κ B functions. This suggests that although CAPE targets activity within the core of NF- κ B binding in the promoter regions, it may in fact have selective effects, for instance, inhibition of IP-10 pro-inflammatory signals without completely blocking MIP-2, an acute phase response gene.

Since CAPE exhibits a strong radical scavenging activity [42] and HO-1 and Nrf2 have been shown to interact with inflammatory pathways [43, 44], we determined whether the combined effect of reduced NF- κ B activity and increased anti-oxidative stress responses was a prerequisite for the anti-inflammatory effect of CAPE. We could demonstrate a strong induction of HO-1 after CAPE treatment. Using NAC to inhibit CAPE-induced HO-1 induction, we found that IP-10 inhibition occurred independently of the presence or absence of HO-1 proteins. Because induction of HO-1 is mediated by the transcription factor Nrf2 [45], we also aimed at analyzing the impact of Nrf2 signaling on CAPE-induced IP-10 expression. Using Nrf2 knockout MEF, we found that Nrf2 is not required for inhibition of IP-10 protein expression by CAPE. Via knockdown of Nrf2 using shRNA in HCT116 cells, Lee et al. had previously proposed that CAPE-mediated Nrf2 activation is associated with inhibition of the NF- κ B pathways [30]. However, differences observed after specific knockdown were marginal at concentrations below 25 μ M and related only to NF- κ B reporter gene activity, whereas our data refer to the endpoint readout of interest IP-10. Also, our data goes along the line of the work on interaction between anti-inflammatory and -oxidative properties by Liu et al. [46], who found that a variety of phase 2 inducers, i.e. chemicals inducing for instance NQO1, were capable of inhibiting NO production by mouse peritoneal macrophages, independently of macrophage origin (WT or Nrf2^{-/-} mice). Another important result of the present study is that CAPE potentiates phosphorylation and accumulation of p38 MAPK in the nucleus. Phosphorylation of p38 MAPK under conditions of oxidative/electrophilic stress has been shown to be synergistic to NF- κ B activity via phosphorylation of RelA by MSK1, a p38 MAPK substrate [47]. However, based on our findings using the p38 MAPK inhibitor SB203580, we propose that NF- κ B-dependent IP-10 inhibition is not related to the p38 MAPK pathway activation. As for the effect of CAPE on p-AMPK and JNK activation, further work is needed to draw conclusion on their impact on IP-10 inhibition.

Epithelial cells are in direct contact with the gut luminal content including bacteria and their metabolites and other chemical stimuli that trigger chemokine production in a

dysregulated gut homeostasis. Experimental evidence has demonstrated up-regulation of the chemokine IP-10 in ileitis [14], brain ischemia [48] and adipocyte maturation [49]. In the present study, we have shown that CAPE abolishes expression of both TNF- and LPS-induced IP-10 expression in embryonic fibroblasts from TNF^{ΔARE/+} mice. Furthermore, IP-10 production was also inhibited in ileum tissue explants from TNF^{ΔARE/+} mice after CAPE treatment. Nevertheless, we found that oral treatment with CAPE had no influence on ileitis in TNF^{ΔARE/+} mice under the experimental conditions used. In inflammation, many chemokines are expressed at different times following cell stimulation, thus failure of CAPE to inhibit MIP-2 gives a hint into the complexity of signals that are involved in leukocyte trafficking under inflammatory conditions. Hence, we should not expect blockade of a single chemokine secreted by the epithelium to be sufficient to suppress disease progression. Furthermore, a prerequisite for in vivo activity is that active compounds must reach target cells (distal epithelial cells in the present study). Whereas CAPE permeated IEC membranes at micromolar concentrations in the in vitro assays, this was most likely not the case in vivo, possibly due to rapid absorption in the upper GI tract and subsequent excretion or degradation by intestinal micro-organisms [50]. Contrary to our findings, it has been previously reported that CAPE attenuates peptidoglycan-polysaccharide-induced colitis in rats [51]. However, the authors used an acute model of inflammation and daily intraperitoneal injection of CAPE (30 mg/kg body weight) for one week, which contrasts to our dietary treatment (10 mg/kg, three times a week over six weeks) in a chronic inflammatory model of ileitis.

In summary, we showed that CAPE has a strong inhibitory effect on TNF-induced IP-10 expression in IECs. We provided evidence that the catechol moiety controls CAPE potency. In addition, we showed that CAPE exhibits its anti-inflammatory effects by disrupting the p50/RelA NF-κB heterodimer binding to the IP-10 promoter. Although feeding experiments in TNF^{ΔARE/+} mice failed to prevent TNF-driven Crohn's disease ileitis, the anti-inflammatory potential of CAPE was confirmed in ileal tissue explants and embryonic fibroblasts derived from TNF^{ΔARE/+} mice.

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provided essential materials; J. O. M., C. B., I. S. and T. C. analyzed and interpreted data; J. O. M., N. W., T. C. and D. H. wrote paper; J. O. M. and D. H. had primary responsibility for final content.

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