Caffeic Acid Phenethyl Ester, an Inhibitor of Nuclear Factor-κB, Attenuates Bacterial Peptidoglycan Polysaccharide-Induced Colitis in Rats

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ABSTRACT

Caffeic acid phenethyl ester (CAPE) is an anti-inflammatory component of propolis (honeybee resin). CAPE is reportedly a specific inhibitor of nuclear factor-κB (NF-κB). The aims of our study were 1) to evaluate the effect of CAPE on cytokine production, NF-κB, and apoptosis in two cell lines; 2) to assess the effect of CAPE on NF-κB in rats with peptidoglycan-polysaccharide (PG-PS)-induced colitis; and 3) to evaluate the efficacy of CAPE against this colitis. In vitro experiments used rat macrophage (NR8383) and colonic epithelial cell (SW620) lines. NF-κB was evaluated by electrophoretic mobility shift assay. Cytokines and apoptosis were measured by enzyme-linked immunosorbent assay. Colitis was induced by intramural injections of PG-PS into the distal colon. CAPE (30 mg/kg) or vehicle was administered once daily to rats by intraperitoneal injection, for 1 week. Various macroscopic and biochemical indices were measured on day 21. CAPE (30 μg/ml) significantly inhibited NF-κB and TNF-α production in the macrophage cell line. In macrophages, CAPE significantly increased DNA fragmentation. CAPE exhibited generally similar effects in the colonic epithelial cell line. CAPE treatment reduced the mean level of colonic NF-κB in rats. CAPE also induced a significant reduction in gross colonic injury. Moreover, colonic cytokine levels (TNF-α and IL-1β) were significantly reduced in CAPE-treated rats. In summary, CAPE inhibits NF-κB, causes a reduction of pro-inflammatory cytokine production, and induces apoptosis in macrophages. These mechanisms likely contributed to the attenuation of PG-PS-induced colitis by CAPE.

Caffeic acid phenethyl ester (CAPE) is a phenolic antioxidant, which is an active anti-inflammatory component of propolis (honeybee resin) (Mirzoeva and Calder, 1996; Michaulart et al., 1999). Various investigators have demonstrated that CAPE has anti-inflammatory properties both in vitro and in vivo (Huang et al., 1996; Mirzoeva and Calder, 1996; Michaulart et al., 1999; Orban et al., 2000). CAPE was also proposed to be a specific inhibitor of the transcription factor nuclear factor-κB (NF-κB), which may account for its anti-inflammatory actions (Natarajan et al., 1996). Specifically, in a human histiocyte cell line, CAPE only inhibited NF-κB, as opposed to other transcription factors such as AP1, TFIID, and Oct-1. In this regard, it was proposed that CAPE directly inhibited the interaction of NF-κB with DNA (Natarajan et al., 1996). CAPE also clearly induces apoptosis in various cell types (Chiao et al., 1995; Orban et al., 2000; Chen et al., 2001).

An initial goal of our study was to assess the anti-inflammatory and apoptosis-inducing effects of CAPE in macrophage and colonic epithelial cell lines. We utilized these cells because they clearly have relevance to the pathogenesis of inflammatory bowel disease (IBD) (Rogler et al., 1998). In this regard, we were specifically interested in evaluating the effects of CAPE on the activation of NF-κB in these cell types. Although CAPE has demonstrated efficacy in various animal models of inflammation, it has apparently not been tested previously in an animal model of IBD. Therefore, another important goal of this study was to evaluate the efficacy of CAPE in a relevant animal model of human Crohn’s Disease [bacterial peptidoglycan polysaccharide (PG-PS)-induced colitis in Lewis rats] (Yamada et al., 1993; Sartor et al., 1996).

Materials and Methods

A rat macrophage cell line (NR8383) and a human colonic epithelial cell line (SW620) were obtained from the American Type Culture Collection (Manassas, VA). NR8383 cells were grown in F-12K culture medium, whereas SW620 cells were grown in RPMI-1640 culture medium. PG-PS (product 10S from Streptococcus group A bacteria) was purchased from Lee Laboratories (Grayson, GA). CAPE was obtained from Sigma Chemical Co. (St. Louis, MO). Female Lewis rats (weighting 125–150 g) were purchased from Charles River Laboratories (Southbridge, MA). Animals were housed one to a cage. In the animal housing area, rats were maintained on a 12-h light/dark cycle. All animals were provided with a standard rodent diet.
and water ad libitum. Rats were acclimated to these environmental conditions for at least 5 days prior to their use in colitis studies.

**Evaluation of NF-κB Binding from Cell Lines.** NF-κB was detected in nuclear protein extracts by an electrophoretic mobility shift assay (EMSA). The EMSA was performed using a specific 32P-oligonucleotide sequence for NF-κB, which was obtained from Promega (Madison, WI). This EMSA technique was described previously (Fitzpatrick et al., 2000).

With the NR8383 cell line, cells were incubated with CAPE (3–30 μg/ml) or vehicle (0.2% DMSO) for 1 h. The cells were then stimulated for 4 h with 100 μg/ml PG-PS. A nuclear extract was prepared for use in the EMSA. Equal amounts (4 μg) of nuclear protein [based on a modified Bradford method from Bio-Rad Laboratories ( Hercules, CA)] were used for all treatment conditions tested in the EMSA. With the SW620 cell line, cells were also incubated with CAPE or vehicle (0.2% DMSO) for 1 h. The cells were then stimulated for 4 h with 20 ng/ml TNF-α. Equal amounts of nuclear protein (4 μg) were again used for all treatment conditions tested in the EMSA.

Densitometry data were obtained by scanning the autoradiographs with an EagleSight computer system and software package [Stratagene, La Jolla, CA]. This system allowed quantification and comparison of the nuclear levels of NF-κB by means of a relative densitometry analysis.

**Evaluation of Cytokine Production from Cell Lines.** NR8383 cells (0.5 million/ml) were exposed to various concentrations of CAPE (3–30 μg/ml) or vehicle (0.2% DMSO) for 24 h. The Cell Death Detection Elisa Plus assay (Roche Molecular Biochemicals, Mannheim, Germany) was used to measure DNA fragmentation. Data are representative studies) was 14 ± standard error. IC50 values (in vitro studies) were calculated via a GraphPad Prism software package (i.e., by regression analysis). Statistical analyses between or among treatment groups were done in conjunction with a Sigma-Stat statistical software program. Drug (CAPE) treatment data was usually compared with data from a parallel vehicle-treated condition, using a two-tailed unpaired t test analysis. If a normal data distribution was not present, the Mann-Whitney test was used. A difference of p < 0.05 was considered significant for all statistical analyses.

**Results**

**CAPE Inhibits NF-κB in Two Cell Lines.** Supershift experiments (data not shown), which were conducted in both cell lines, demonstrated that the NF-κB DNA binding complex consisted of p50 and p65 subunits. Similar results have been reported previously by several other investigators (e.g., Wahl et al., 1998). As shown in Fig. 1A, CAPE dose-dependently inhibited the PG-PS-stimulated nuclear binding of NF-κB in a rat macrophage (NR8383) cell line. The mean percent inhibitions obtained with CAPE treatment were: 31.2 ± 6.8 (3 μg/ml), 41.5 ± 14.0 (10 μg/ml), and 62.3 ± 11.0 (30 μg/ml). All these mean reductions in the nuclear binding of NF-κB were statistically significant (p < 0.05 versus vehicle treatment). The calculated IC50 value (mean of two representative studies) was 14 μg/ml. CAPE also inhibited NF-κB in the colonic epithelial (SW-620) cell line (Fig. 1B). The mean percentage inhibitions obtained with CAPE treatment were: 40.5 ± 1.3 (3 μg/ml), 43.7 ± 1.2 (10 μg/ml), and 72.3 ± 7.8 (30 μg/ml). All these mean reductions in the nuclear binding of NF-κB were also statistically significant (p < 0.05 versus vehicle treatment). The calculated IC50 value was 8 μg/ml. A larger concentration of CAPE (100 μg/ml) was clearly toxic (i.e., by trypan blue exclusion) in both cell types.

**CAPE Inhibits Cytokine Production in Two Cell Lines.** CAPE also inhibited PG-PS-stimulated TNF-α production from the rat macrophage cell line. However, a strict dose-related effect was not observed. Moreover, all utilized concentrations of CAPE inhibited TNF-α production by >50%. At CAPE concentrations of ≥10 μg/ml, TNF-α was inhibited by 79% (Fig. 2A). CAPE (30 μg/ml) also inhibited TNF-α-stimulated IL-8 production (by 60%) in the SW-620 colonic epithelial cell line (Fig. 2B). However, lower concentrations of CAPE (3 or 10 μg/ml) were ineffective in this cell line.

**CAPE Induces Apoptosis in Two Cell Lines.** As illustrated in Fig. 3, CAPE induced apoptosis (increased DNA...
CAPE Attenuates Colonic NF-κB and PG-PS-Induced Colitis in Rats. Treatment of Lewis rats with CAPE for a 1-week period resulted in a significant reduction in the gross colonic injury of Lewis rats (Fig. 4). Specifically, on day 21 after the induction of PG-PS-induced colitis, the gross colonic injury scores were 0.5 ± 0.3 (vehicle/saline), 4.7 ± 0.2 (vehicle/PG-PS), and 3.5 ± 0.4 (CAPE/PG-PS). CAPE treatment resulted in a reduction of gross colonic injury to approximately 50% of the level found in PG-PS-treated rats on day 14 (Fig. 4).

CAPE treatment also reduced other parameters of PG-PS-induced colitis in rats. In particular, colonic IL-1β and TNF-α levels were reduced by 51% and 81%, respectively, in CAPE-treated animals (Table 1). In conjunction with this study, the mean body weight gains (in grams) during the 1-week dosing period were 14 ± 7 (vehicle/saline), 3 ± 1 (vehicle/PG-PS), and -5 ± 3 (CAPE/PG-PS).

The mean colonic NF-κB levels (i.e., in densitometry units) of Lewis rats, as measured by EMSA, were 0.011 ± 0.01 (vehicle/saline), 0.061 ± 0.01 (vehicle/PG-PS), and 0.055 ± 0.01 (CAPE/PG-PS). Therefore, there was a significant up-regulation (approximately 6-fold) in the mean colonic NF-κB level of PG-PS-treated rats compared with those administered only saline (Fig. 5). Overall, there was only a slight reduction in the mean level of colonic NF-κB in CAPE-treated animals. However, as illustrated in this figure, there was a significant association ($r = 0.574, p < 0.01$) between colonic NF-κB levels and gross colonic injury in individual Lewis rats.

CAPE Slightly Reduces the Arthritis Associated with PG-PS-Induced Colitis in Rats. As indicated in Table 1, CAPE treatment resulted in a slight reduction in the arthritis associated with PG-PS administration to Lewis rats. Specifically, the mean ankle joint diameters (in mm) on day 21 were 5.44 ± 0.04 (vehicle/saline), 7.28 ± 0.39 (vehicle/PG-PS), and 6.85 ± 0.29 (CAPE/PG-PS).

Discussion

Our study demonstrates that CAPE is an effective in vitro inhibitor of NF-κB and relevant cytokines in two different cell lines. Similar to our previous results with gliotoxin (another NF-κB inhibitor), we found that CAPE was also an effective inhibitor of NF-κB and cytokines in both our rat macrophage and colonic epithelial cell lines (Fitzpatrick et al., 2000). Interestingly, CAPE appeared to be a more effective inducer of apoptosis in macrophages, as opposed to colonic epithelial cells. In this regard, other investigators have also reported that CAPE can preferentially induce apoptosis in macrophages and colonic epithelial cells. However, as illustrated in this figure, there was a significant association ($r = 0.574, p < 0.01$) between colonic NF-κB levels and gross colonic injury in individual Lewis rats.

CAPE inhibits the nuclear binding of NF-κB in the rat macrophage cell line. Representative EMSA results showing NF-κB binding activity under various conditions. Lanes 1 and 2, CAPE (3 μg/ml) + PG-PS; lanes 3 and 4, CAPE (10 μg/ml) + PG-PS; lanes 5 and 6, CAPE (30 μg/ml) + PG-PS; lanes 7 and 8, vehicle + PG-PS; and lane 9, vehicle + no PG-PS. Based on a relative densitometry analysis, IC₅₀ of CAPE for inhibiting the nuclear binding of NF-κB was calculated to be 14 μg/ml. B, CAPE inhibits the nuclear binding of NF-κB in the colonic epithelial cell line. Representative EMSA results showing NF-κB binding activity under various conditions. Lanes 1 and 2, CAPE (3 μg/ml) + TNF-α (20 ng/ml); lanes 3 and 4, CAPE (10 μg/ml) + TNF-α; lanes 5 and 6, CAPE (30 μg/ml) + TNF-α; lanes 7 and 8, vehicle + TNF-α; and lane 9, vehicle + no TNF-α (background level in this picture). Based on a relative densitometry analysis, IC₅₀ of CAPE for inhibiting the nuclear binding of NF-κB was calculated to be 5 μg/ml.
redox state of a particular cell type (Chiao et al., 1995; Chen et al., 2001; Orban et al., 2000).

NF-κB is up-regulated in macrophages and epithelial cells of patients with IBD (Ellis et al., 1998; Rogler et al., 1998; Bantel et al., 2000). Moreover, macrophages have been reported to play an important role in the pathogenesis of PG-PS-induced intestinal injury in Lewis rats (Yamada et al., 1993; Sartor et al., 1996). In our study, the mean colonic NF-κB level was only slightly attenuated in CAPE-treated Lewis rats. Nevertheless, there was a significant correlation between the colonic NF-κB level and gross colonic injury in rats. Specifically, clear evidence of a reduced colonic NF-κB level and associated gross colonic injury was present in three CAPE-treated animals (Fig. 5). In these rats, the colonic NF-κB levels were similar to those found in vehicle/saline-treated animals. These rats also had gross colonic injury scores of <4. It is possible that the inherent pharmacokinetic properties of CAPE may have resulted in the lack of more prominent reductions in all treated animals. Specifically, it is possible that relevant pharmacological concentrations of CAPE may not have been consistently achieved in the rat colon. A similar suggestion was put forth previously by other investigators, regarding the in vivo anti-inflammatory profile of CAPE (Orban et al., 2000). The dose of CAPE used in this study was sufficient to induce in vivo biological effects in other rat inflammatory models (Michaulart et al., 1999; Orban et al., 2000). Larger doses, which might lead to higher in vivo drug levels, were not pursued because of concerns with possible toxicity. In this regard, some weight loss was observed in our CAPE-treated animals.

In this study, CAPE significantly reduced the gross colonic injury associated with the chronic reactivation phase (days 14–21) of PG-PS-induced colitis. However, CAPE exhibited only partial inhibition of this damage to approximately 50% of the level initially found in PG-PS-treated rats on day 14. CAPE also significantly, but partially, reduced the increased colonic cytokine levels (IL-1β and TNF-α) associated with chronic PG-PS-induced colitis. Such attenuations in these pro-inflammatory cytokines may have contributed to the attenuation of gross colonic injury in rats. This statement is supported by results from a previous study, which suggested that IL-1 plays an important pathogenic role in PG-PS-induced intestinal damage (McCall et al., 1994). In our studies, CAPE (at 30 μg/ml) effectively reduced chemokine (i.e., IL-8) production in vitro, but had only a slight effect on colonic neutrophil infiltration (myeloperoxidase activity) in vivo. In this regard, it is known that PG-PS-induced colitis has a multi-factorial pathogenesis (Sartor et al., 1996). Therefore,

**Fig. 3.** A, CAPE induces apoptosis (DNA-fragmentation) in a rat macrophage (NR8383) cell line. Cells were exposed to culture medium (no treatment), vehicle (0.2% DMSO), or CAPE (3–30 μg/ml) for 24 h. DNA fragmentation was evaluated by ELISA. n = 8 to 9 per treatment group. *, p < 0.05 versus vehicle treatment. B, CAPE induces apoptosis (DNA fragmentation) in a colonic epithelial (SW620) cell line. Cells were exposed to culture medium (no treatment), vehicle (0.2% DMSO), or CAPE (3–30 μg/ml) for 24 h. DNA fragmentation was evaluated by ELISA. n = 7 to 8 per treatment group. *, p < 0.05 versus vehicle treatment.
conceivably other/additional chemotactic factors may be involved in regulating neutrophil influx into the colon. As mentioned above, it is also possible that a sufficient level of CAPE (e.g., 30 μg/ml) was not consistently maintained in the rat colon. Therefore, based on our in vitro findings (Fig. 2B), chemokine production may not have been dramatically altered in all CAPE-treated rats. It is somewhat difficult to gauge the “clinical” significance of these reductions with CAPE treatment. Particularly, because the PG-PS model is not well-suited for examining symptoms of colitis (e.g., diarrhea, rectal bleeding). Nevertheless, the overall results obtained with CAPE on gross colonic injury and measured biochemical parameters were better than those that we obtained previously in this rat colitis model with olsalazine (L. R. Fitzpatrick and T. Le, unpublished data).

In summary, we found that CAPE inhibits NF-κB and cytokine production in two relevant cell types for IBD. In terms of in vivo relevance, CAPE reduced these parameters in conjunction with stimulation of rat macrophages by PG-

Fig. 5. There is a significant association between the colonic levels of NF-κB and gross colonic injury (PG-PS colitis model). NF-κB was determined by EMSA and relative densitometry analysis. (n = 29 rats, r = 0.574, p < 0.01 by linear regression analysis).

Table 1: Effect of CAPE (30 mg/kg) on PG-PS-induced colitis in rats (day 21)—data summary

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Vehicle + Saline (n = 6)</th>
<th>Vehicle + PG-PS (n = 12)</th>
<th>CAPE + PG-PS (n = 11)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colonic injury (score, 0–5 scale)</td>
<td>0.5 ± 0.3</td>
<td>4.7 ± 0.2**</td>
<td>3.5 ± 0.4**</td>
</tr>
<tr>
<td>Colon weight (g/5 cm)</td>
<td>0.30 ± 0.01</td>
<td>1.10 ± 0.24*</td>
<td>0.99 ± 0.16</td>
</tr>
<tr>
<td>MPO activity (units/cm of colon)</td>
<td>14 ± 1</td>
<td>149 ± 32*</td>
<td>114 ± 19</td>
</tr>
<tr>
<td>IL-1β (pg/mg of tissue)</td>
<td>0.4 ± 0.1</td>
<td>8.6 ± 1.7*</td>
<td>4.4 ± 1.0**</td>
</tr>
<tr>
<td>TNF-α (pg/mg of tissue)</td>
<td>0.21 ± 0.06</td>
<td>0.57 ± 0.11*</td>
<td>0.28 ± 0.06**</td>
</tr>
<tr>
<td>Rear ankle joint (diameter in mm)</td>
<td>5.44 ± 0.04</td>
<td>7.28 ± 0.39*</td>
<td>6.85 ± 0.29</td>
</tr>
</tbody>
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*p < 0.05 vs. vehicle + saline.

**p < 0.05 vs. vehicle + PG-PS.
PS. Moreover, CAPE induced apoptosis in macrophages. There was also in vivo evidence that CAPE reduced colonic levels of NF-κB, as well as relevant pro-inflammatory cytokines. The aforementioned pharmacological profile of CAPE likely contributed to the observed attenuation of PG-PS-induced colitis by this compound.

References


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