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# CHEMOPROTECTIVE EFFECT OF CAFFEIC ACID PHENETHYL ESTER ON PROMOTION IN A MEDIUM-TERM RAT HEPATOCARCINOGENESIS ASSAY

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**Caffeic acid phenethyl ester (CAPE), a natural honeybee product exhibits a spectrum of biological activities including anti-microbial, anti-inflammatory, antioxidant and anti-tumoral actions. CAPE is also chemopreventive against intestinal, colon and skin cancer. Our aim was to extend the study of its chemoprotective features to the promotion of hepatocarcinogenesis. Male Wistar rats were subjected to a protocol under a modified promotion regimen of the resistant hepatocyte model. The altered hepatic foci (AHF) were quantitatively analyzed by histochemistry and image processing. When given during promotion, CAPE (20 mg/kg) decreased the expression of number and area γ-glutamyl transpeptidase (GGT) positive AHF by 91% and 97%, respectively. When *GGT* expression was analyzed by RT-PCR, CAPE drastically decreased and prevented expression of almost all *GGT* transcripts at this stage of the carcinogenic process. Glutathione s-transferase placental form (GST-P), another protein marker for preneoplastic lesions was measured by Western blot and a decrease of 82% was observed. Additionally, we evaluated the effect of CAPE on the expression of nuclear factor NF-κB and found an 85% decrease in nuclear localization of the p65 subunit of NF-κB; however, their repressor, IκBα was not modified. Our results showed that CAPE given during promotion in hepatocarcinogenesis protects against induction of GGT-positive AHF, GST-P protein, *GGT* mRNA expression and translocation of p65. This phenomenon was independent of IκBα degradation.**


**Key words:** *caffeic acid phenethyl ester; chemoprevention; hepatocarcinogenesis*

Chemoprotectors have often been detected when studying the effect of substances purified from extracts of natural products to which folk medicine has attributed therapeutic properties. This is the case of caffeic acid phenethyl ester (CAPE), one of the esters of propolis. CAPE exhibits a broad spectrum of biological activities including anti-microbial, anti-inflammatory, anti-viral, antioxidant and anti-tumoral actions.[1–4] In at least 3 different carcinogenesis models, CAPE has been shown to inhibit induction of preneoplastic lesions or tumors. CAPE inhibits the azoxymethane-induced colon aberrant crypt foci formation in F344 rats by >50%.[5] It also represses tumor induction in mouse skin, and the concomitant formation of DNA 5-hydroxymethyl-2′-deoxyuridine, when the carcinogenic process is initiated by dimethylbez-(a)anthracene and promoted with 12-*O*-tetradecanoylphorbol-13-acetate (TPA).[6] Additionally, CAPE inhibits the effect of tumor promoter TPA-induced processes associated with carcinogenesis in mouse skin and in bovine lens.[7] CAPE inhibits intestinal tumor formation by 63% in a mice model of familial adenomatous polyposis, with a germline mutation in the *Apc* gene, that is manifested by appearance of spontaneous intestinal tumors. This protective effect was associated with the reversal of alterations in enterocyte growth by apoptosis, normalization of their proliferation and restitution of their migration.[8] The antioxidant activity of CAPE, has also been demonstrated in TPA-treated human HL-60 and HeLa cells, where it reduced the levels of intracellular $H_2O_2$, and oxidized bases in DNA.[4,9,10] CAPE also induces growth suppression in human cells, including glioblastoma multiforme (GBM-18) and melanoma (HO-1) cells.[11] Moreover, it induces cell death in 5 adenovirus-transformed rat fibroblast cells whereas it only arrests normal parental cell division.[12] The molecular mechanism of CAPE as chemoprotector remains unknown. Nevertheless, CAPE antiinflammatory properties have been attributed to the suppression of eicosanoid synthesis, through inhibition of arachidonic acid release from cell membrane and the expression of cyclooxygenase-2 (COX-2) gene, and to the inhibition of COX-1 and COX-2 activity.[13] In addition, it has been reported that CAPE completely and specifically blocks the activation of nuclear factor NF-κB induced by a wide variety of inflammatory agents and blocks the production of reactive oxygen species (ROS) in human neutrophils.[14,15]

Animal models offer several advantages and have proved to be valuable for the study of multistep tumorigenesis at different levels.[16] This is the case of resistant hepatocyte model based on the induction of altered hepatic foci (AHF).[17] We used a medium-term rat hepatocarcinogenesis model, with an alternative regimen of selection/promotion on the resistant hepatocyte model similar to the Semple-Roberts model.[18] The hepatocarcinogenic process can be dissected with the aid of this carcinogenesis assay, which allowed us to evaluate possible chemoprotective substances that modulate the carcinogenic process at specific stages, and study their biological mechanisms of action.

Because there is information about the protective effect of CAPE in several *in vivo* carcinogenic assays when given during promotion, the aim of this work was to explore the preventive effect and mechanism of CAPE on the induction of AHF in the rat liver, in a medium-term assay during the promotion stage.

## MATERIAL AND METHODS

### *Animals*

Male Wistar rats (180–200 g) obtained from the Production Unit of Experimental Laboratory Animals (UPEAL-Cinvestav, México D.F., México.), had access to food and water at all times; food cups were replenished 3 times weekly. During treatment, rats were transferred to the holding room, under controlled conditions of temperature and light. All animals received humane care and the study protocols were in compliance with the institutional guidelines for use of laboratory animals.

*Abbreviations:* 2-AAF, 2-acetylaminofluorene; AHF, altered hepatic foci; CAPE, caffeic acid phenethyl ester; CCT, complete carcinogenic treatment; COX-2, cyclooxygenase-2; DEN, diethylnitrosamine; GGT, γ-glutamyl transpeptidase; GST-P, glutathione s-transferase placental form; PH, partial hepatectomy; ROS, reactive oxygen species; TPA, 12-*O*-tetradecanoylphorbol-13-acetate.

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*Experimental protocol*

Chemical reagents were purchased from Sigma Chemical, Co. (St. Louis, MO) and Research Organics, Inc. (Cleveland, OH). CAPE was obtained by esterification of caffeic acid with phenethyl alcohol.[19]

The modified model of Semple-Roberts used here presents the following features: (*i*) AHF generated rapidly and initiator dose-dependent[20] and (*ii*) all animals sacrificed 40 weeks or more after initiation present liver tumors; both AHF and tumors express the 2 positive markers γ-glutamyl transpeptidase (GGT) and glutathione s-transferase placental form (GST-P). Animals were killed 25 days after DEN administration, the time of maximum occurrence of AHF. Briefly, animals were initiated with 200 mg/kg diethylnitrosamine (DEN); 1 week later, 2-acetylaminofluorene (2-AAF) dissolved in dimethyl sulfoxide suspended in a 1% aqueous solution of carboxymethylcellulose to a final concentration of 10 mg/ml was orally administered with a stainless steel feeding tube, at doses of 20 mg/kg/day on 3 consecutive days before partial hepatectomy (PH). PH was carried out at Day 10. CAPE was dissolved in corn oil, three doses of 20 mg/kg weight were administered 12 hr before each of the 2 first 2-AAF doses, and another 72 hr after PH (Fig. 1). Livers were excised, quickly frozen in liquid nitrogen and stored at −80°C until analysis.

*Histochemical GGT staining and analysis*

Histological liver sections of 15 μm were obtained in a cryostat (Slee cryostat MTC, Germany) fixed in absolute ethanol during 5 min at 4°C and stained according to Rutenburg.[21] γ-Glutamyl-4-methoxy-2-naphtylamine (GMNA) at a ratio of 1:20 with 25 mM Tris pH 7.4, 0.5 mg/ml glycyl-glycine and 4-benzoylamino-2,5-diethoxybenzene-diazonium chloride hemi[zinc chloride] salt (fast blue bb salt) were added to sections and the reaction kept during 30 min; next, the slices were washed with phosphate buffer solution, and added with 0.1 M cupric sulfate solution for 2 min. Finally, images of the GGT positive foci were captured with a digital camera (Color View 12, Soft Imaging System GmbH) and preneoplastic lesions were quantified with image analysis software (AnalySIS Soft Imaging System GmbH). Three slices were randomly selected from each rat liver, 60 histological sections were prepared from each liver and 12 of these histological preparations were randomly selected and analyzed.

*Semiquantitative RT-PCR assay*

Total RNA of liver tissue was isolated by the method developed by Chomczynski and Sacchi.[22] After RNase-free DNase I treatment (Boehringer-Mannheim, Germany) 1μg of RNA was reverse transcribed into cDNA and amplified with a one step RT-PCR commercial kit (Gibco BRL/Life Technologies, Inc., Gaithersburg, MD) at a final volume of 12.5 μl. α-Actin was used as an internal reference gene. The 2 primer sequences of rat GGT and α-actin used were: CTCTGCATCTGGCTACCCAC, GGATGCTGGGTTGGAAGAGG and CCAAGGCCAACCGCGAGAAGATGAC, GGTACATGGTGGTGCCGCCAGAC (sense and antisense), respectively. The mixture was heated to 45°C for 30 min in a GeneAmp PCR System 2400 (Perkin-Elmer, Corp., CT). The program was denaturation (94°C, 30 sec), annealing (59°C, 45 sec), and extension (70°C, 45 sec). To determine the adequate number of cycles for a semi-quantitative analysis of our samples, a kinetic analysis was carried out, GGT and α-actin were amplified in 40 and 30 sequential cycles, and the PCR products were 418 bp and 586 bp respectively. The reverse transcription RT-PCR-amplified samples were visualized on 2% agarose gels, stained with ethidium bromide and captured with a digital camera (Kodak electrophoretic documentation and Analysis System 120).

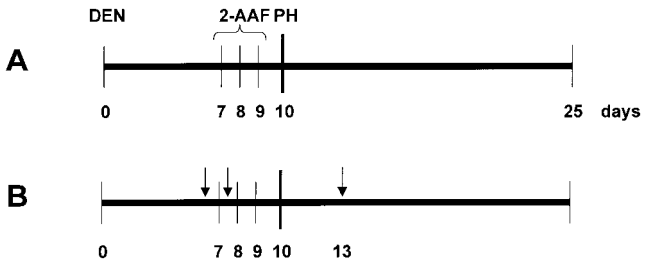


**FIGURE 1** – CAPE administration on hepatocarcinogenesis protocol. (*a*) CCT, the treatment of rats was initiated with 200 mg/kg of DEN i.p.; at Day 7, 8 and 9 2-AAF was administered by gavage at doses of 20 mg/kg/day; a PH was carried out on Day 10 and the rats were sacrificed at Day 25. (*b*) CAPE (↓) was given 12 hr before the first and second doses of 2-AAF and the third 3 days after PH.

*Western blot analysis*

To detect p65 nuclear protein, the nuclei sub-cellular fraction was obtained as described previously.[23,24] For detection of p65 and GST-P in the cytosolic fraction, liver homogenates were prepared with lysis buffer (10 mM Tris-Cl pH 7.4, 0.15 M NaCl and 1 mM phenylmethylsulphonyl fluoride) and were spun at 9,000 rpm in a microcentrifuge at 4°C for 15 min. The supernatants were used as cytosolic extracts. The nuclear and cytoplasmic protein concentration was determined according the Lowry method[25] and bicinchoninic acid,[26] respectively. SDS/PAGE was carried out under reducing conditions on 12% polyacrylamide gels. The resolved proteins were transferred onto nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA) and then probed with a rabbit polyclonal anti-GST-P (DAKO, Carpinteria, CA), p65 (Upstate, NY), IκBα (Santa Cruz Biotechnology, Santa Cruz, CA) and horseradish peroxidase-conjugated secondary antibody (Zymed, San Francisco, CA) and detected by Enhanced Chemiluminescence detection reagent (Santa Cruz Biotechnology) and developed with Konica Film (Tokio, Jpn.). These membranes were then re-probed with goat polyclonal anti-lamin B (Santa Cruz Biotechnology) and anti-actin monoclonal antibody (Cinvestav, Mexico City), as loading controls of nuclear and cytoplasmic extracts.

*Statistical analysis*

Number and area of GGT-positive AHF and GST-P protein expression of the group treated with CAPE were compared to the complete carcinogenic treatment (CCT) group (carcinogenic treatment plus corn oil). Results were analyzed by Student's *t*-test. Differences were considered statistically significant at $p < 0.05$.

## RESULTS

*Effect of CAPE on the medium-term assay*

CAPE was tested for its chemopreventive effect in hepatocarcinogenesis assay, induced by DEN as initiator and 2-AAF as promoter following the scheme depicted in Figure 1. In this *in vivo* assay, GGT-positive AHF were generated and determined histochemically. Figure 2 shows representative sections of each treatment. After CAPE treatment during promotion, we observed only a few GGT-positive AHF (Fig. 2*c*) resembling those depicted in a liver section of a non-treated (NT) rat (Fig. 2*a*). Rats that received CCT presented copious AHF; this average-quantity was taken as 100% (Fig. 2*b*). In 3 different groups of 3 rats each in which DEN, 2-AAF or hepatectomy were omitted, AHF were not detected using 0.01 $mm^2$ as a low-end measure of GGT foci (data not shown).

The histochemically developed GGT-positive liver foci were analyzed by image analysis. We found that CAPE given during promotion drastically decreased the expression in number and area of GGT-positive AHF (Fig. 3). CAPE had a protective effect of 91% and 97% respectively related to number and area of nodules, and these differences were statistically different from animals that received the CCT ($p = 0.001$ and $p = 0.01$).

*Effect of CAPE on* GGT *mRNA expression*

Figure 4 shows that administration of CAPE during promotion blocks the transcription of *GGT* observed in the CCT group. CAPE effect is so pronounced that the level of *GGT* expression is below that encountered in the liver of NT rats.

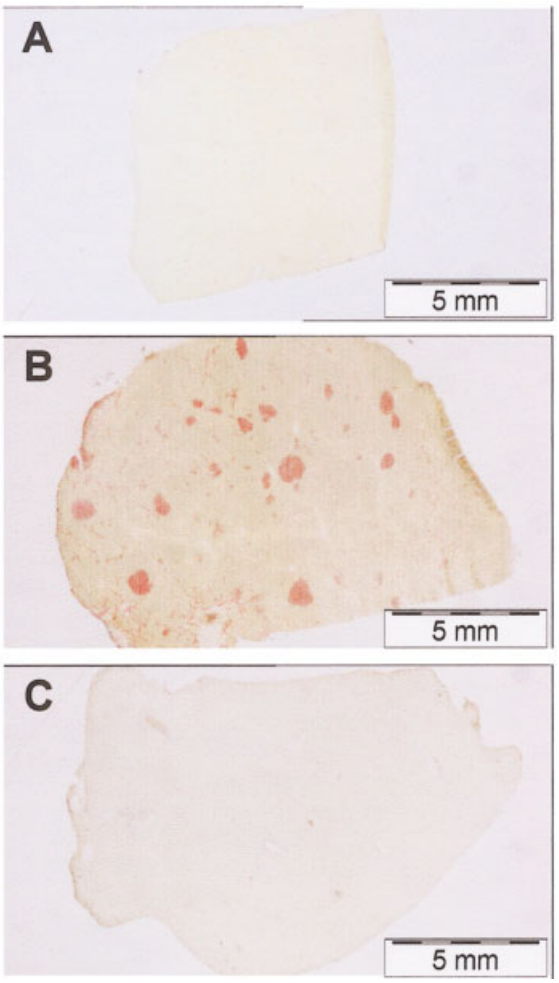


**FIGURE 2** – Histochemically stained sections showing the effect of CAPE on the induction of GGT-positive AHF. Liver sections representative of each treatment. (*a*) NT, (*b*) CCT, (*c*) CCT plus CAPE during promotion.

*Effect of CAPE on GST-P protein expression*

As shown by Western blot, CAPE greatly diminished the expression of GST-P protein (Fig. 5). This protein, a preneoplastic lesion marker in several hepatocarcinogenesis models[27] was represented in arbitrary units with respect to the average obtained from 3 NT rats. CAPE significantly decreased GST-P protein expression on promotion by 82 % with respect to the CCT (Fig. 5*a*). CCT increased 124% with respect to the NT group and CAPE treatment reduced GST-P expression even below the level of NT rats (Fig. 5*b*).

In sum, these results show qualitative and quantitative CAPE protective activity in the promotion stage of the liver carcinogenic process.

*Effect on NF-κB translocation by CAPE*

We examined the effect of CAPE on the activation of transcription factor NF-κB by Western blot analysis. Because factor activation requires nuclear translocation of the p65 subunit of NF-κB, we measured the cytoplasmic and nuclear pool of the p65 protein. Results in Figure 6 indicate that CAPE inhibited the activation of NF-κB, given that p65 decreased in nuclear fractions by 85% (Fig. 6*a*) and increased by 78% at cytoplasmic level (Fig. 6*b*) with respect to the CCT group. It is known that the translocation of NF-κB is preceded by phosphorylation and proteolytic degradation of IκBα, therefore, IκBα protein at cytoplasmic level was analyzed by Western blot. Figure 6*c* shows that treatment of CAPE in promotion did not modify the IκBα protein.

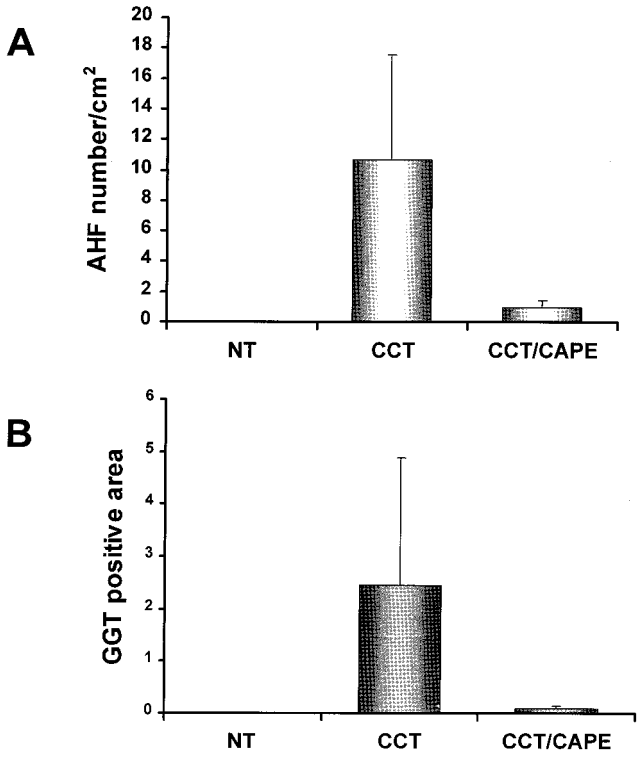


**FIGURE 3** – Quantification of the CAPE effect upon number/cm$^2$ and % area of GGT-positive AHF. (*a*) AHF quantity number/cm$^2$. (*b*) The percent of GGT-positive area/tissue area. Twelve histological sections of the liver per rat from each treatment were randomly chosen and analyzed. NT ($n$ = 3), CCT ($n$ = 3), CCT plus CAPE ($n$ = 5). Statistically different from CCT group, $p < 0.05$.

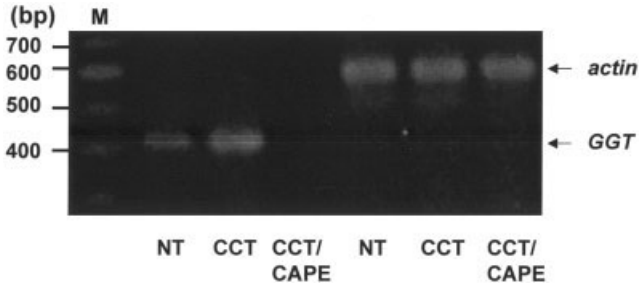


**FIGURE 4** – Effect of CAPE on *GGT* mRNA expression in liver by RT-PCR. Column M shows weight markers. The series on the extreme right represents α-actin transcripts. This experiment was made with a pool of total RNA isolated from 3 animals.

## DISCUSSION

One feature of the resistant hepatocyte model is the induction of persistent nodules that allows studies of chemopreventive agents in the sequence from the nodule to cancer.[28,29] In the model used here, rat livers presented foci and nodules fairly early and they were quite easily detected by their characteristic staining-pattern for GGT and GST-P markers.[30,31] Furthermore, 12 of 12 rats treated with DEN, 2-AAF and PH developed liver tumors 40 weeks after initiation (data not included). Therefore, as an early endpoint of chemoprotective capacity of CAPE we chose Day 25 after initiation, this being a period of maximum expression of preneoplastic lesions.

At this early endpoint, our results demonstrated a CAPE prevention of preneoplastic induction manifested by diminution in size and number of GGT$^+$ foci and nodules. Accordingly with the CAPE

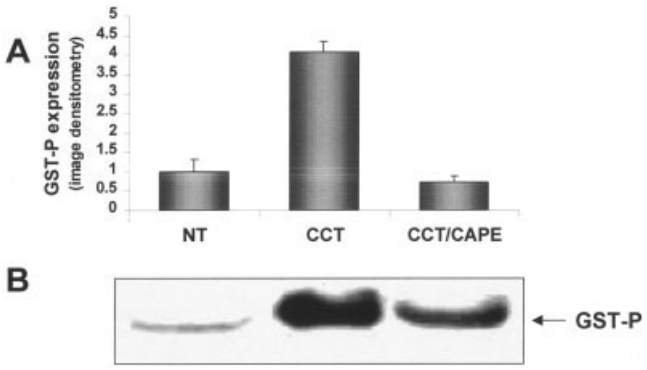


**FIGURE 5** – CAPE effect on GST-P expression by Western blot analysis. (*a*) Densitometric analysis of GST-P expression in control and CAPE treated groups. (*b*) A representative Western blot of GST-P of different rats. Statistically different from CCT group; ($p < 0.05$).

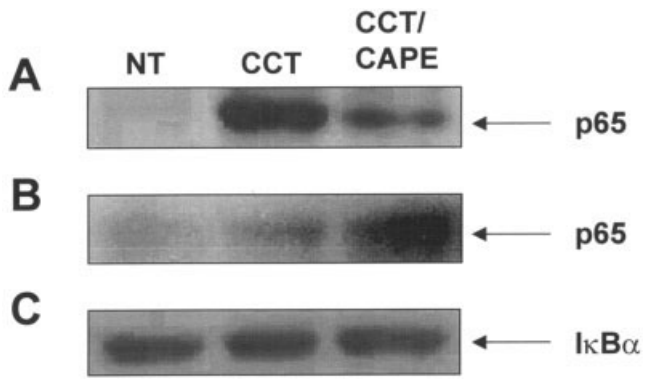


**FIGURE 6** – Effect of CAPE on NFκB activation. (*a*) CAPE decreased nuclear p65. (*b*) p65 increased in cytoplasm and (*c*) IκBα cytosolic was not modified. Data are representative of 3 animals.

chemoprotective ability, the growth inhibition of preneoplastic lesions spares few foci and nodules of cells that specifically express GGT and GST-P, so an apparent reduction in GGT mRNA levels and the concomitant decrease in the amount of GST-P protein were detected. These observations add an example of the CAPE protective effect to other reports in intestine, colon and skin *in vivo* models.[5,6,8]

GST-P and GGT have been considered among the best markers for foci, nodules and tumors induced with the resistant hepatocyte model. Nevertheless, they are not universal and have shown to be poor in foci and nodules induced by peroxisome proliferators. This type of compounds, putatively carcinogens, under certain experimental conditions inhibit the appearance of $GGT^+$ and $GST\text{-}P^+$ foci and nodules induced by genotoxic carcinogens, though this has been considered more as a chemopreventive activity than a direct inhibition of marker expression.[32,33] With these exceptions, both markers are reliable and are expressed all along the process in most hepatocarcinogenic medium-term models and are also found to be increased in hepatocellular tumors.[17,29,34]

The concurrence of the selective mito inhibitory effect of 2-AAF on normal hepatocytes together with the 2/3 hepatectomy proliferative stimulus, constitutes the driving force for the evolution of preneoplastic lesions. It is known that absence of 2-AAF administration or abstention of hepatectomy prevent formation of preneoplastic lesions,[18] therefore, the effect of CAPE upon one of these processes, either at the metabolic level of 2-AAF interfering with CYP 1A2[35] activity or blocking proliferation induced by hepatectomy, will prevent their development. Nevertheless a very attractive possibility is that, by a common mechanism, CAPE blocks the proliferative effect of hepatectomy and interferes with the mito inhibitory activity of 2-AAF, an effect that prevents the promotion process in the hepatocarcinogenesis model.

Propositions have been forwarded for the chemoprotective mechanisms CAPE activity in mouse skin carcinogenesis. CAPE inhibits cancer induction and its antioxidant activity is suggested as the mechanism of protection at the level of promotion. Furthermore, the anticancer and anti-inflammatory properties of CAPE can be understood by an inhibition in expression of COX-2.[13] This enzyme produces prostaglandins that are inflammation mediators, and chronic inflammation increases the risk of cancer. Nevertheless, our determination at the early endpoint of 25 days of the COX-2 protein and the expression of its transcripts were not modified by CAPE (data not shown).

As reported in U937 cells, CAPE inhibits the activation of the nuclear transcription factor NF-κB induced by phorbol ester.[14] Some evidence indicates that NF-κB activation leads to tumor cell proliferation, invasion, angiogenesis, and metastasis. Suppression of these events in the carcinogenic process, are additional targets in cancer prevention.[36] Our results demonstrate that carcinogenic treatment activates NF-κB, and CAPE, when given during promotion, blocks this activation. There is evidence that NF-κB activation is an essential step in proliferation-induced liver regeneration after hepatectomy,[37] additionally, 2-AAF through the generation of ROS, activates NF-κB.[38] Although the mechanism of NF-κB activation in the resistant hepatocyte model is not known, a very attractive possibility is that this event is induced by the over production of ROS and the chemopreventive CAPE effect may be due to its ability to alter the redox state of the cell, as the intracellular thiols are known to regulate NF-κB activation.[14] The latter hypothesis is consistent with the antioxidant properties assigned to CAPE of its electrophilic α,β-unsaturated carbonyl groups that can selectively react with nucleophils such as thiols.[39] Therefore, the antioxidative ability[4,11,40] reported for CAPE can be postulated as the general mechanism of action of its hepatoprotective activity on the induction of preneoplastic lesions. In a similar way, antioxidants such as butylated hydroxyanisole, butylated hydroxytoluene and catechol show an inhibitory effect in $GST\text{-}P^+$ foci development induced in the resistant hepatocyte model.[41]

Our study is the first report that demonstrates a nuclear translocation of NF-κB produced by the carcinogenic treatment of an alternative resistant hepatocyte model and the prevention of this phenomenon by CAPE. NF-κB activation requires the degradation of IκBα that holds NF-κB in the cytoplasm in a dormant complex state. Even though the latter is one of the pathways suggested for NF-κB regulation, in our model nuclear translocation was not associated to a IκBα degradation. These results show an NF-κB activation by an alternative pathway independent of IκBα degradation, a situation that has been described before.[42]

With these results it is valid to suggest that CAPE exerts chemoprotective activity against induction of preneoplastic lesions and that it very likely inhibits tumor formation in the experimental model used.[28,43,44] In conclusion, CAPE prevents the appearance of GGT-positive foci in a medium-term assay of hepatocarcinogenesis through the prevention of NF-κB activation, which justifies further studies on the mechanism of action of CAPE.

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