

Evidence that the Anticarcinogenic Effect of Caffeic Acid Phenethyl Ester in the Resistant Hepatocyte Model Involves Modifications of Cytochrome P450

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Caffeic acid phenethyl ester (CAPE), a natural component of propolis, shows anticarcinogenic properties in the modified resistant hepatocyte model when administered before initiation or promotion of hepatocarcinogenesis process; however, information about the mechanism underlying this chemoprotection is limited. The aim of this work was to characterize the effect of CAPE on cytochrome P450 (CYP), which is involved in diethylnitrosamine (DEN) metabolism during the initiation stage of chemical hepatocarcinogenesis. Male Fischer-344 rats were treated as in the modified resistant hepatocyte model. Liver samples were obtained at four different times: at 12 h after pretreatment with CAPE and at 12 and 24 h and 25 days after DEN administration. Liver damage was determined by histology with hematoxylin and eosin, measurement of total CYP levels and enzyme activity, and γ -glutamyl transpeptidase-positive (GGT⁺) staining of hepatocyte foci. CAPE administration prevented DEN-induced necrosis at 24 h. It also decreased *O*-dealkylation of 7-ethoxy-resorufin (EROD), *O*-dealkylation of 7-methoxyresorufin (MROD), and 7-pentoxy-resorufin activities at 12 h after its administration and EROD and MROD activities at 12 h after administration of DEN. CAPE treatment decreased GGT⁺ foci by 59% on day 25. Our results suggest that CAPE modifies the enzymatic activity of CYP isoforms involved in the activation of DEN, such as CYP1A1/2 and CYP2B1/2. These findings describe an alternative mechanism for understanding the ability of CAPE to protect against chemical hepatocarcinogenesis.

Key Words: chemoprevention; cytochrome P450; DEN metabolism; hepatocarcinogenesis.

Liver cancer is the fifth most frequent cancer and the third most frequent cause of cancer death worldwide (Parikh and

Hyman, 2007). Although millions of people live with cancer or have received medical treatment for it, prevention remains the best option. Chemoprevention is based on the hypothesis that disruption of the biological events involved in any stage of carcinogenesis can decrease cancer incidence. There are three principal stages in carcinogenesis: initiation, when mutations occur and cells are initiated; promotion, when clonal expansion of initiated cells takes place and forms preneoplastic lesions; and progression, when preneoplastic lesion becomes tumors through an increase in genetic and metabolic changes. These stages constitute a lengthy process during which chemoprevention can be applied (Klaunig and Kamendulis, 2004).

Caffeic acid phenethyl ester (CAPE), a natural component of propolis collected from honeybee (*Apis mellifera*) hives, has been studied since 1987 (Bankova *et al.*, 1987). It shows a broad spectrum of biological properties, including activity as an anti-inflammatory (Michaluart *et al.*, 1999), antioxidant (Oktem *et al.*, 2005; Song *et al.*, 2002; Sud'ina *et al.*, 1993), and anticarcinogen (Carrasco-Legleu *et al.*, 2004, 2006; McEleny *et al.*, 2004; Na *et al.*, 2000).

The anticarcinogenic properties of CAPE have been studied in the modified resistant hepatocyte model. Its effects have been tested when it was administered before the initiation and promotion stages. Administering CAPE in several doses during promotion caused a 90% decrease in the induction of γ -glutamyl transpeptidase-positive (GGT⁺) foci on day 25; decreases in markers of preneoplastic lesions, GGT activity, and the amount of glutathione-*S*-transferase class *Pi* (GST-*p*) protein were also observed. CAPE administration 12 h before exposure to diethylnitrosamine (DEN) caused an 85% decrease in the translocation of the p65 subunit of nuclear factor kappa B into the nucleus; it reduced GGT⁺ foci by 84% and GST-*p* levels by 90% on day 25. In addition, in primary hepatocyte culture, it lowered liver thiobarbituric reactive species and it prevented DNA damage. The protective effects of CAPE on initiation have been attributed to its antigenotoxic,

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antioxidative, and free radical scavenging activities, though it may also be due to an ability to interfere with DEN bioactivation (Carrasco-Legleu *et al.*, 2004, 2006).

It is not clear which of several isoforms of cytochrome P450 (CYP) participate in DEN metabolism. Isoforms proposed to be the principal bioactivators are CYP2E1 (Amelizard *et al.*, 1988; Sook-Jeong *et al.*, 1990; Verna *et al.*, 1996; Yamazaki *et al.*, 1992a,b) or CYP2B1/2 (Bellec *et al.*, 1996; Yamazaki *et al.*, 1992b). It is also possible that all or any of these three isoforms can bioactivate DEN (Espinosa-Aguirre *et al.*, 1997). In fact, evidence suggests that CYP1A1/2 plays an important role in this process (Bellec *et al.*, 1996; Yamazaki *et al.*, 1992b). In summary, CYP2E1, CYP1A1/2, and CYP2B1/2 may be DEN bioactivators.

Experimental evidence concerning the effect of CAPE on liver CYP regulation is limited. An alcoholic extract of propolis, which contains CAPE, has been shown to reduce the enzyme activity associated with the isoforms CYP1A1/2, thereby reducing the bioactivation of carcinogens like benzo[a]pyrene. Therefore, both these CYP isoforms have been proposed as DEN bioactivators (Jen *et al.*, 2000).

The aim of the present study was to analyze the protective effect of CAPE on liver CYP isoforms involved in DEN metabolism when administered before cancer induction in a resistant hepatocyte model of chemically induced hepatocarcinogenesis. The effects of CAPE were assessed during the first 24 h post-administration of DEN and included assays of tissue damage, total CYP levels, enzyme activity, and induction of GGT⁺ foci on day 25 in Fischer-344 male rats.

MATERIALS AND METHODS

Animals

Male Fischer-344 rats (180–200 g) were obtained from the Unit for Production of Experimental Laboratory Animals (UPEAL-Cinvestav, México City, México). Animals had free access to food (PMI Nutrition International, Richmond, Indiana, Feeds, Inc., Laboratories Diet) and water at all times; each rat consumed approximately 12–15 g of food and 10–15 ml of water per day. After treatment, the animals were transferred to the holding room and kept under controlled conditions of 12 h light/12 h dark, 50% relative humidity, and 21°C. Animal care followed institutional guidelines for the use of laboratory animals.

Animal Treatments

Animals were subjected to three different treatments to test chemoprevention, to confirm the chemoprotective effect when DEN or 2-acetylaminofluorene (2-AAF) is absent, and to perform microsomal assays.

Chemoprotective effect of CAPE on GGT⁺ foci. Animals were treated according to the modified resistant hepatocyte model of Semple-Robert (Carrasco-Legleu *et al.*, 2004, 2006). In the first group, rats were administered 200 mg/kg DEN ip on day 0. On days 7, 8, and 9, 20 mg/kg of 2-AAF suspended in carboxymethyl cellulose and dimethylsulfoxide (DMSO) were administered by gavage before partial hepatectomy (PH) on day 10 ($n = 8$). A second group was treated with a single 20 mg/kg ip dose of CAPE (kindly provided by Dr Javier Hernández-Martínez, CIAD, Hermosillo, México, with 99% of purity, obtained according to Grunberger *et al.*, 1988) using DMSO as vehicle and a third group was treated using DMSO 12 h before administration

of DEN ($n = 8$ for CAPE pretreatment and $n = 8$ for DMSO control group). All groups were sacrificed by exsanguination under ether anesthesia on day 25 after DEN administration. Livers were excised, washed in saline solution, frozen in 2-methyl butane, and stored at -80°C . Other liver sections were formalin-fixed and paraffin-embedded for histology (weight of the rats, 220–230 g; liver weight, 5–7 g).

Effect of DEN and 2-AAF on GGT⁺ foci. One group received only DEN treatment (200 mg/kg) ($n = 6$) and the other received only 2-AAF treatment (20 mg/kg) ($n = 6$). Both received a PH on day 10 and were sacrificed by exsanguination under ether anesthesia on day 25. Livers were excised, washed in saline solution, frozen in 2-methyl butane, and stored at -80°C . Other liver sections were formalin-fixed and paraffin-embedded for histology (weight of the rats, 220–230 g; liver weight, 5–7 g).

Microsomal assays. Two groups received only DEN treatment (200 mg/kg) and were sacrificed 12 and 24 h after its administration ($n = 4$). Another group received CAPE alone (20 mg/kg) in DMSO vehicle ($n = 4$) and was sacrificed 12 h after its administration. Finally, two more groups received DEN together with CAPE and were sacrificed 12 and 24 h after DEN administration ($n = 4$). Animals were sacrificed under ether anesthesia by perfusion with physiological solution via the portal vein and processed immediately to obtain microsomes (Mayer *et al.*, 1990). The treatment did not affect the weight of the rats or the weight of their livers.

Histochemistry of GGT and Hematoxylin and Eosin

Liver sections of 20 μm thickness were stained for GGT activity (Rutenburg *et al.*, 1969). Images of the GGT⁺ foci were captured with a digital camera (Color View 12, Soft Imaging System GmbH, Germany) and quantified with AnalySIS software (AnalySIS, Soft Imaging System GmbH). Only GGT⁺ areas larger than 0.01 mm^2 were registered to avoid detecting background. In addition, paraffin-fixed sections of 5 μm thickness were processed for routine histological examination by staining with hematoxylin and eosin (H&E) ($n = 5$).

Total CYP Levels and Enzyme Activities

Total liver CYP content was measured according to Omura and Sato (1964). To detect enzyme activity of CYP1A1, microsomal *O*-dealkylation of 7-ethoxyresorufin (EROD) was assayed. In contrast, *O*-dealkylation of 7-methoxyresorufin (MROD) was used for CYP1A2 and *O*-dealkylation of 7-pentoxymethoxyresorufin (PROD) for CYP2B1/2. All these reactions were followed fluorimetrically at 37°C with excitation at 530 nm and emission at 585 nm (Burke *et al.*, 1985; Lubet *et al.*, 1985; Nerurkar *et al.*, 1993). The enzyme activity of CYP2E1 was measured by *p*-nitrophenol hydroxylase (PNPH) assay, which detects the formation of 4-nitrocatechol by colorimetric assay at 510 nm (Reinke and Moyer, 1985) ($n = 4$). Protein was measured with the Bio-Rad DC Protein Assay Kit (Richmond, CA) (Lowry *et al.*, 1951; Peterson, 1979).

Statistical Analysis

The data were analyzed using one-way ANOVA and further analyzed using Bonferroni's pairwise multiple comparison *post hoc* test (Klockars *et al.*, 1995). Analyses were made using SigmaStat 3.1.1 software (Systat Software, Inc., Point Richmond, CA). In all tests, the level of significance was $p < 0.05$.

RESULTS

CAPE Treatment Reduces GGT⁺ Foci

GGT⁺ foci were considered to be preneoplastic lesions, and their number reached a maximum approximately 25 days after initiation of the carcinogenic treatment (Pérez-Carreón *et al.*, 2006). CAPE has been shown to reduce GGT⁺ foci in Wistar rats with a precise end point, which allows measurement of the

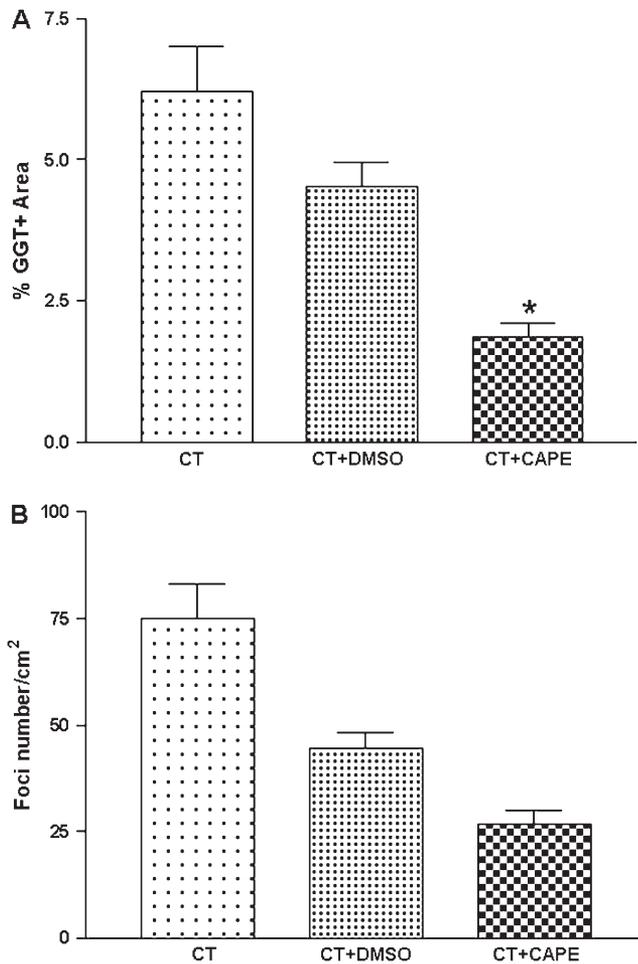


FIG. 1. Effect of CAPE on the induction of GGT⁺ liver foci. To determine the effect of CAPE in the induction of GGT⁺ foci, three groups of male Fischer-344 rats were studied: complete carcinogenic treatment (CT), complete carcinogenic treatment plus DMSO (CT + DMSO), and complete carcinogenic treatment plus CAPE (CT + CAPE) pretreatment. Each group was sacrificed on day 25 to analyze induction of GGT⁺ liver foci ($n = 8$). (A) %GGT⁺ area; (B) foci number per square centimeter. *Significantly different from CT + DMSO by the Bonferroni test ($p < 0.05$). Values are mean \pm SE.

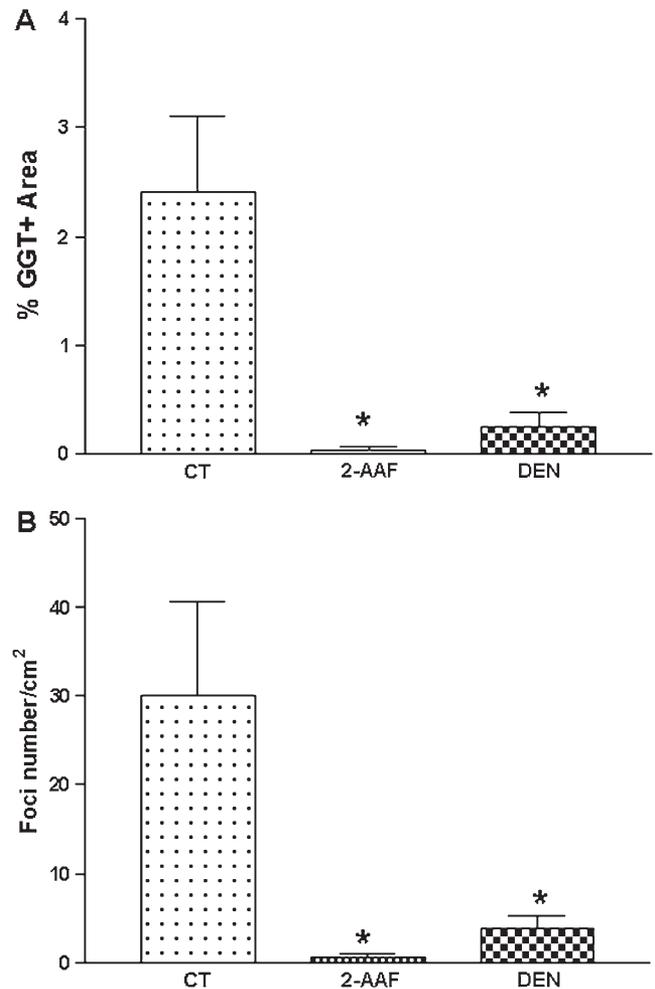


FIG. 2. Effect of omitting DEN and 2-AAF from the modified resistant hepatocyte model. To determine the effect of omitting DEN and 2-AAF, male Fischer-344 rats were treated in three groups: complete treatment (CT), with only 2-AAF, and with only DEN. Each group was sacrificed on day 25 to analyze induction of GGT⁺ liver foci ($n = 6$). (A) %GGT⁺ area; (B) foci number per square centimeter. *Significantly different from CT + DMSO by the Bonferroni test ($p < 0.05$). Values are mean \pm SE.

chemoprotective effect in a few days (Carrasco-Legleu *et al.*, 2004, 2006). These results have since been confirmed in male Fischer-344 rats, where CAPE reduces the induction of GGT⁺ foci. The only significant difference in the experimental animals, compared to the vehicle-only controls, was GGT⁺ area (59% in the experimental animals; Fig. 1A), but the number of foci per square centimeter was reduced too (40%; Fig. 1B). These results show that a single dose of the chemoprotector CAPE, prior to DEN administration, reduced the preneoplastic lesions in our model, probably by interfering with the effects of DEN at the initiation stage.

DEN Is Required to Produce GGT⁺ Foci

To determine whether CAPE interferes with the effects of DEN in the modified resistant hepatocyte model, the effect of

omitting DEN during induction of chemical hepatocarcinogenesis was tested as in previous experiments (Tsuda *et al.*, 1980). We observed that administering 2-AAF alone caused 98% and 98.4% inhibition of the total number of foci induced per square centimeter and the GGT⁺ area, respectively, once treatment was complete. These values were higher than the corresponding results (87.4% and 89.7%) obtained when only DEN was administered (Fig. 2). The results of omitting DEN were therefore similar to the results seen when CAPE was administered before initiation. Therefore, we cannot exclude that CAPE may be blocking DEN activation.

CAPE Treatment Prevents Necrosis Induced by DEN

Liver cell necrosis induced by DEN plays an important role in the early stages of experimental hepatocarcinogenesis

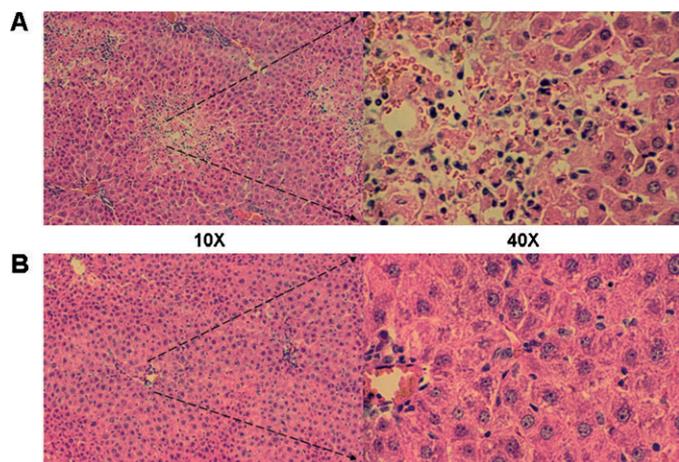


FIG. 3. CAPE protects the liver against tissue damage induced by DEN. To examine the effect of CAPE on liver tissue damage induced by DEN 24 h after its administration, male Fischer-344 rats were treated in two groups: DEN only and DEN after CAPE pretreatment (DEN + CAPE) ($n = 5$). Each group was sacrificed 24 h after administration of DEN, and tissue was stained with H&E. (A) DEN only; (B) DEN + CAPE.

leading to the formation of preneoplastic lesions such as GGT⁺ foci (Ying *et al.*, 1981). Generalized necrosis of the liver was observed by H&E staining at 24 h after the DEN administration (Fig. 3A). With DEN + CAPE, however, the focal necrosis areas were minimal and the livers resembled those of untreated rats (Fig. 3B). These results indicate that CAPE administration reduces the toxicity of DEN—very likely by affecting its metabolism—and consequently alters DEN's activity at the initiation stage in the resistant hepatocyte model.

Modulation of CYP Liver Concentration and Enzyme Activities

Our first approach to analyzing the metabolism of DEN was to measure total liver CYP content; the results show that the total content was not altered by any treatment (Table 1). Next, several enzyme activities associated with the isoforms

TABLE 1
Changes in Total CYP Levels in the Liver

Treatments	Total CYP (nmol of CYP/mg protein)
NT	0.50 ± 0.09
DMSO	0.43 ± 0.01
CAPE	0.32 ± 0.07
DEN-12 h	0.35 ± 0.03
DEN-CAPE-12 h	0.28 ± 0.04

Note. Male rats were treated ip with a single dose of DE (200 mg/kg), with or without ip pretreatment with a single dose of CAPE (20 mg/kg) with DMSO as the vehicle. Several groups were evaluated: not treated (NT), vehicle control with DMSO (DMSO), CAPE alone (CAPE), DEN alone (DEN-12 h), and DEN and CAPE 12 h after DEN administration (DEN-CAPE-12 h). Each value is the mean ± SD from $n = 4$.

CYP1A1, CYP1A2, CYP2B1/2, and CYP2E1 were determined to assess the effect of CAPE on specific CYP isoforms involved in DEN bioactivation. Vehicle alone (DMSO) increased EROD and MROD activities by 39% and 62%, respectively, and it decreased PNP activity by 27% compared to untreated rats. CAPE treatment, however, showed the opposite effect with respect to DMSO. By 12 h after CAPE treatment, the EROD, MROD, and PROD activities had decreased by 54%, 60%, and 67%, respectively; in contrast, CYP2E1 activity increased by 89%. Pretreatment with CAPE 12 h before DEN administration reduced EROD, MROD, and PROD activities by 64%, 65%, and 22%, respectively (Table 2). Similar results were obtained when these activities were assayed 24 h after DEN administration (data not shown). These results show that CAPE administration modifies the enzyme activity related to CYP isoforms proposed to function as DEN bioactivators, which suggests that these events are involved in CAPE chemoprotection in resistant hepatocyte model.

DISCUSSION

During the last 20 years, the chemoprotective properties of CAPE have been studied intensively in several kinds of cancer models and several mechanisms of action have been proposed to explain its various properties (Carrasco-Legleu *et al.*, 2004, 2006; McEleny *et al.*, 2004; Na *et al.*, 2000).

In the present study, we examined the chemopreventive effect of CAPE on the initiation stage in the modified resistant hepatocyte model when the compound was administered prior to DEN treatment. The main findings of this study are that CAPE reduced the induction of GGT⁺ foci by 59%, preventing the tissue damage that is characteristic of DEN treatment, and it modified the enzyme activity associated with CYP1A1, CYP1A2, CYP2B1/2, and CYP2E1 in a time-dependent manner.

The metabolites produced during bioactivation of DEN induce mutations in the DNA and increase oxidative stress that triggers several signaling pathways, ultimately leading to the production of initiated cells. DEN requires metabolic activation by CYP to give α -hydroxynitrosamines, and these intermediate compounds decompose spontaneously to give acetaldehyde and mono-*N*-ethyl-nitrosamines, followed by ethyl-diazo hydroxides and nitrogen-separated pairs. The ethyl-diazo hydroxides and nitrogen-separated pairs can lead to the formation of diazoalkanes or carbocations and ultimately to the alkylation of nucleophiles, and these very reactive species are known to induce cancer in mammals. Nearly all the DEN is biotransformed within the first 12 h after its administration (Verna *et al.*, 1996). In our modified resistant hepatocyte model, every GGT⁺ focus is assumed to originate from a single initiated cell that underwent neoplastic conversion. Thus, because CAPE treatment reduces the occurrence of GGT⁺ foci, it is likely to interfere with the initiating activity of DEN. Since both carcinogens, DEN and 2-AAF, are required to induce liver

TABLE 2
Effect of Treatments on Enzyme Activity in Hepatic Microsomes

Treatment	Alkoxyresorufin <i>O</i> -dealkylation activity (pmol resorufin/min/mg protein)			PNPH activity (nmol 4-nitrocatechol/ min/mg protein)
	EROD	MROD	PROD	
NT	6.1 ± 0.9	6.4 ± 0.7	3.6 ± 0.7	1.5 ± 0.4
DMSO	8.5 ± 0.6 ^a	10.4 ± 2.7 ^a	3.9 ± 0.5	1.1 ± 0.1 ^a
CAPE	3.9 ± 0.7 ^{a,b}	4.2 ± 1.8 ^{a,b}	1.3 ± 0.1	2.0 ± 0.2 ^b
DEN-12 h	5.0 ± 2.4 ^{a,b}	3.4 ± 0.5 ^{a,b}	8.6 ± 2.3 ^{a,b}	1.1 ± 0.3 ^a
DEN-CAPE-12 h	1.8 ± 0.4 ^{a,b}	1.2 ± 0.2 ^{a,b,c}	6.7 ± 0.5 ^{a,b}	0.9 ± 0.1 ^a

Note. Male rats were treated ip with a single dose of DEN (200 mg/kg), with or without ip pretreatment with a single dose of CAPE (20 mg/kg) with DMSO as the vehicle. Several groups were evaluated: not treated (NT), vehicle control with DMSO (DMSO), CAPE alone (CAPE), DEN alone (DEN-12 h), and DEN and CAPE 12 h after DEN administration (DEN-CAPE-12 h). Significantly different.

^aFrom the NT group

^bFrom the DMSO group.

^cFrom DEN-12 h by the Bonferroni test ($p < 0.05$). Each value is the mean ± SD from $n = 4$.

cancer, the absence of one of them results in a drastic reduction in the occurrence of GGT⁺ foci.

Detection of tissue damage produced by the carcinogen 24 h after its administration confirmed that CAPE interferes with cell initiation. CAPE protected the liver from necrosis, suggesting that it blocks DEN activity at the initiation stage. CAPE has previously been proposed to act as an active antioxidant scavenger of reactive species produced during bioactivation of DEN (Carrasco-Legleu *et al.*, 2004, 2006). Several mechanisms of action have been proposed, and CAPE could in fact be acting like garlic powder, which blocks the bioactivation of carcinogens (Park *et al.*, 2002).

CYP1A, CYP2B, and CYP2E subfamilies constitute the main groups of enzymes involved in the activation of environmental mutagens/carcinogens. These isoforms represent 11% of total CYP protein in rat liver, and all of them are involved in the metabolism of *N*-nitroso-dialkylamines (Bellec *et al.*, 1996; Escobar-García *et al.*, 2001). CYP1A1/2, CYP2B1/2, and CYP2E1 have been shown to participate in DEN bioactivation in rats (Amelizard *et al.*, 1988; Bellec *et al.*, 1996; Espinosa-Aguirre *et al.*, 1997; Sook-Jeong *et al.*, 1990; Verna *et al.*, 1996; Yamazaki *et al.*, 1992a,b). Our results show that CAPE decreased the enzyme activity associated with CYP1A1/2 and CYP2B1/2 12 h after administration of CAPE. The level of activity for CYP1A1 and CYP1A2 remained low until 12 h after DEN administration. Together, these results suggest that CAPE can modulate the activity of CYP isoforms involved in DEN biotransformation when it is administered both on its own and in the presence of DEN. CYPs are well-known to have distinct but often overlapping substrate specificity, and more than one CYP isoform may be involved in the metabolism of a given chemical. Therefore, if different CYPs are involved in the biotransformation of DEN, any change in the expression of CYP isoforms due to CAPE exposure may both modify the proportions of primary metabolites and alter the bioactivation of DEN and its downstream effects. Further studies are needed to characterize the hepatic CYP isoforms involved in the bioactivation of DEN.

Experimental evidence about the effect of CAPE on the regulation of liver CYP isoforms is very limited. The present study is the first report of the effects of CAPE treatment on the regulation of CYP1A1/2, CYP2B1/2, and CYP2E1 in the liver. An alcoholic extract of propolis containing a very high level of CAPE has been reported to decrease the enzyme activity of CYP1A1 and CYP1A2 and, consequently, reduce carcinogen bioactivation (Jen *et al.*, 2000). In the same way, dietary garlic powder has been shown to act as an anticarcinogen by modifying the expression of CYP2E1 (Park *et al.*, 2002). Bicyclol has been shown to act as an anticarcinogen by increasing CYP2B1 enzyme activity and, in particular, enhancing the denitrosation of DEN (Zhu *et al.*, 2006). Therefore, the ability of CAPE to modify the expression of several CYP isoforms evidences an additional mechanism for explaining the chemopreventive activity of CAPE in the initiation stage of chemical carcinogenesis.

It is an oversimplification to hypothesize that only one CYP isoform participates in a given reaction *in vivo*. DEN is a carcinogen used in several models, but it is not clear which of several CYP isoforms participate in its bioactivation in our model in particular. The measurements of enzymatic activity reported here are insufficient to conclude definitively that the chemoprotective activity of CAPE is related to CYP. Nevertheless, our results strongly suggest that CAPE acts through the inhibition of carcinogen metabolism, and they warrant further study in search of a definitive answer.

In conclusion, CAPE may modify the enzyme activity of CYP isoforms involved in DEN activation. The modification of CYP-dependent metabolism in the liver may constitute an alternative mechanism for understanding CAPE's chemoprotective effect in a hepatocarcinogenesis model.

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