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Cocoa flavonoids up-regulate antioxidant enzyme activity via the ERK1/2 pathway to protect against oxidative stress-induced apoptosis in HepG2 cells☆

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Abstract

Oxidative stress is widely recognized as an important mediator of apoptosis in liver cells and plays a pivotal role in the pathogenesis of several diseases. Cocoa flavonoids have shown a powerful antioxidant activity providing protection against oxidation and helping prevent oxidative stress-related diseases. However, the molecular mechanisms responsible for this protection are not fully understood. Thus, in this study we investigated the protective effect of a cocoa polyphenolic extract (CPE) against *tert*-butyl hydroperoxide (t-BOOH)-induced apoptosis and the molecular mechanisms involved in this process. Incubation of HepG2 cells with t-BOOH induced apoptosis as evidenced by caspase-3 activation. This effect was accompanied by increased reactive oxygen species formation and by transient activation of the extracellular regulated kinases (ERKs) as well as sustained activation of the c-Jun N-terminal kinases (JNKs). On the contrary, pretreatment of HepG2 cells with CPE prevented apoptosis through the reduction of reactive oxygen species generation and the modulation of the apoptotic pathways activated by t-BOOH. CPE treatment also activated survival signaling proteins, such as protein kinase B (AKT) and ERKs, and increased the activities of two antioxidant enzymes, glutathione peroxidase (GPx) and glutathione reductase (GR). ERK's implication on GPx and GR induction and the protective effect of CPE against t-BOOH-induced oxidative stress and apoptosis were confirmed through experiments with selective inhibitors. These findings suggest that CPE is an effective inductor of GPx and GR activities via ERK activation and that this up-regulation seems to be required to attenuate t-BOOH-induced injury.

Keywords: Apoptosis; ROS; Signaling pathways; Glutathione peroxidase; Glutathione reductase; Polyphenols

1. Introduction

Overproduction of reactive oxygen species (ROS) is considered a major cause of molecular injury and has been implicated in the pathogenesis of several human diseases and age-related degenerative processes [1]. In particular, oxidative stress plays a crucial role in the induction and progression of hepatic diseases, since the liver is the main target organ of several cytotoxic agents that can cause ROS- and free radical-mediated apoptosis [2]. Numerous compounds with antioxidant properties have the ability to neutralize the conditions of oxidative stress that accompany these pathologies and therefore have been proposed as therapeutic agents to counteract liver damage [3]. Among these, natural polyphenolic compounds, especially flavonoids, have been largely studied for their strong antioxidant capacity [4]. Plant polyphenols are abundant in fruits and vegetables and in plant-derived beverages such as tea and wine, and they have been demonstrated to possess therapeutic potential in some human disorders, including cancer, atherosclerosis and liver diseases [5]. Supporting this, the antioxidant and chemoprotective properties of individual food flavonoids or polyphenolic extracts have been widely reported in cultured cells [6], animal models [7] and humans [8,9].

Many of the biological actions of dietary polyphenols have been generally related to their free radical scavenging and antioxidant capacity, but emerging findings seem to indicate that natural compounds may also act in increasing endogenous antioxidant defense potential [10]. Several studies have shown that tea polyphenols, such as epigallocatechin gallate and theaflavins [11], as well as the flavanol quercetin [12], induce a varied set of antioxidant enzymes in diverse organs or cultured cells. The protective effect of these proteins is related to their function in sequestering ROS and/or maintaining the cell and cellular components in their appropriate redox state [10]. Consequently, activation of several cytoprotective proteins, which have a variety of antioxidant actions, seems to represent a novel mechanism of the chemoprevention by natural polyphenols [11].

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Cocoa is an essential source of flavonoids; in fact, cocoa-derived products are commonly consumed in many countries in the European Union and the United States [13]. Thus, they can be considered as dietary antioxidants and as natural products with therapeutic properties. Many in vitro and in vivo studies have shown that cocoa and its flavonoids possess beneficial effects against oxidative stress-related diseases by directly scavenging free radicals [14] and by increasing the activities of antioxidant enzymes [15]. However, many details of the molecular mechanisms associated to this latter process remain to be further investigated. It is known that a number of cellular kinases, including the mitogen-activated protein kinases (MAPKs) and phosphatidylinositol-3-kinase (PI3K), are activated in response to treatments with dietary compounds [16]. MAPK and PI3K pathways transduce a multitude of extracellular stimuli by phosphorylating and activating downstream transcription factors, enabling the cell to respond to stresses by increasing or decreasing the expression of critical genes [16]. In fact, more recent studies have indicated that both green [11] and black [17] tea polyphenol extracts could stimulate the transcription of antioxidant/detoxificant enzymes through the activation of MAPK signaling pathways. To this date, comparable studies about the mechanisms underlying the chemopreventive effects exerted by cocoa polyphenols are more limited.

We have recently shown that a polyphenolic extract from cocoa powder composed mostly of epicatechin, catechin and dimeric procyanidins has the ability to protect human HepG2 liver cells against oxidative stress [18]. The aim of the present work was to evaluate the underlying mechanisms of the protective effect of this cocoa polyphenolic extract (CPE) against apoptosis induced by the potent pro-oxidant *tert*-butyl hydroperoxide (t-BOOH). To this end, generation of ROS, caspase-3 activation as a marker of apoptosis and the possible pathways activated during t-BOOH-induced apoptosis were investigated, as well as the effect of CPE treatment before the oxidative induction in all these processes. The impact of cocoa polyphenols on the activities of glutathione-related enzymes, the potential implication of upstream signaling kinases and their role in CPE-induced cytoprotection were also evaluated.

2. Materials and methods

2.1. Materials and chemicals

Gallic acid, t-BOOH, PD98059 [2-(2-amino-3-methoxyphenyl)-4*H*-1-benzopyran-4-one], wortmannin, gentamicin, penicillin G and streptomycin were purchased from Sigma Chemical (Madrid, Spain). The fluorescent probe 2′,7′-dichlorofluorescin (DCFH) diacetate was from Molecular Probes (Eugene, OR, USA). Anti-AKT and antiphospho-Ser473-AKT (p-AKT), anti-ERK1/2 (where ERK indicates extracellular regulated kinase) and antiphospho-ERK1/2 (p-ERK) recognizing phosphorylated Thr202/Thy204 of ERK1/2, anti-JNK1/2 (where JNK indicates c-Jun N-terminal kinase) and antiphospho-JNK1/2 (p-JNK) recognizing phosphorylated Thr183/Tyr185 of JNK1/2 and anti-β-actin were obtained from Cell Signaling Technology (9271, 9272, 9101, 9102, 9251, 9252 and 4697, respectively; Izasa, Madrid, Spain). Caspase-3 substrate [Ac-DEVD-AMC (*N*-acetyl-Asp-Glu-Val-Asp-7-amino-4-methylcoumarin)] was purchased from Pharmingen (San Diego, CA, USA). Materials and chemicals for electrophoresis and the Bradford reagent were from BioRad (BioRad Laboratories, Madrid, Spain). Cell culture dishes and cell culture medium were from Falcon (Cajal, Madrid, Spain) and Biowhittaker Europe (Innogenetics, Madrid, Spain), respectively.

2.2. Cocoa polyphenol extraction

Natural Forastero cocoa powder (Nutrexpa, Barcelona, Spain) was used for this study. Soluble polyphenols were extracted by sequentially washing 1 g of sample with 40 ml of 16 mM hydrochloric acid in 50% aqueous methanol (50:50, v/v, 1 h at room temperature, constant shaking) and 40 ml of acetone–water (70:30, v/v, 1 h at room temperature, constant shaking) in 50-ml centrifuge tubes [19]. After centrifugation (15 min, 3000×*g*), supernatants from each extraction step were combined and made up to 100 ml. The desiccated extract was dissolved in distilled water and kept frozen until assay. The total polyphenol content was determined by the Folin–Ciocalteau spectrophotometric method [19] using gallic acid as standard. A Beckman DU640 spectrophotometer (Beckman Instruments, Fullerton, CA, USA) was used. Analysis of the CPE by LC-MS showed that the extract is composed mostly of epicatechin (383.5 mg/100 g), catechin (116 mg/100 g), procyanidins (254.5 mg/100 g) and non-flavonoid compounds, such as theobromine [18].

2.3. Cell culture and cocoa treatment

Human HepG2 cells were grown in a humidified incubator containing 5% CO_2 and 95% air at 37°C. They were grown in DMEM F-12 medium from Biowhitaker (Lonza, Madrid, Spain) supplemented with 2.5% Biowhitaker fetal bovine serum (FBS) and 50 mg/L of each of the following antibiotics: gentamicin, penicillin and streptomycin. Plates were changed to an FBS-free medium on the day before the assay. The serum added to the medium favors growth of most cell lines but might interfere in the running of the assays and affect the results. Moreover, a fairly good growth of HepG2 cells has been observed.

For the cocoa treatment, different concentrations of CPE (0.05, 0.5, 5 and 50 μg/ml) diluted in serum-free culture medium and filtered through a 0.2-μm membrane were added to the cell plates for 20 h. After CPE treatment, the medium was discarded and fresh medium containing different concentrations of t-BOOH was added to evaluate the protective effect of CPE against an oxidative insult. In the experiments with the pharmacological inhibitors, cells were preincubated with 50 μM PD98059 (specific inhibitor of MAPK–ERK) or with 200 nM wortmannin (inhibitor of PI3K) for 2 h prior to 20 h of CPE treatment.

2.4. Cell viability and cytotoxicity

Cell viability/damage was determined by two alternative methods: gross detection of cell viability by using the crystal violet assay [20] and cytotoxicity by lactate dehydrogenase (LDH) leakage into the extracellular medium [21].

For the crystal violet assay, HepG2 cells were seeded at low density (10^4 cells per well) in 96-well plates, grown for 20 h with the different treatments and incubated with crystal violet (0.2% in ethanol) for 20 min. Plates were rinsed with distilled water and allowed to dry; 1% SDS was then added. The absorbance of each well was measured using a microplate reader at 570 nm (Bio-Tek, Winooski, VT, USA).

Cytotoxicity was evaluated by the LDH method collecting the culture medium, and the cells were scraped in phosphate-buffered saline (PBS) after the different treatments. Cells were first sonicated to ensure breaking down the cell membrane to release the total amount of LDH; then, after centrifugation (1000×*g*, 15 min) to clear up the cell sample, 10 μl was placed into a well of a 96-multiwell system for the assay. In the same manner, 10 μl of each culture medium was also deposited into a well of a 96-multiwell system. The LDH leakage was estimated from the ratio between the LDH activity in the culture medium and that of the whole cell content.

2.5. Apoptosis

Apoptosis was evaluated as activation of caspase 3 [22]. Cells were lysed in a buffer containing 5 mM Tris, pH 8, 20 mM EDTA and 0.5% Triton X-100. The reaction mixture contained 20 mM Hepes, pH 7, 10% glycerol, 2 mM dithiothreitol and 30 μg of protein per condition, as well as 20 μM Ac-DEVD-AMC as substrate. Enzymatic activity was determined by measuring fluorescence at an excitation wavelength of 380 nm and an emission wavelength of 440 nm (Bio-Tek).

2.6. ROS generation

Cellular ROS were quantified by the DCFH assay using a microplate reader. For the assay, cells were plated in 24-well multiwells at a rate of 2×10^5 cells per well and changed to an FBS-free medium and the different treatments the day after. Twenty hours later, 5 μM DCFH was added to the wells for 30 min at 37°C. Then, cells were washed twice with PBS, and 0.5 ml of serum-free medium with or without t-BOOH was added per well. Multiwell plates were immediately measured (time 0) in a fluorescent microplate reader at an excitation wavelength of 485 nm and an emission wavelength of 530 nm (Bio-Tek). After being oxidized by intracellular oxidants, DCFH will become dichlorofluorescin (DCF) and emit fluorescence. A fair estimation of the overall oxygen species generated under the different conditions was obtained by quantifying fluorescence over a determined period. This parameter gives a very good evaluation of the degree of cellular oxidative stress. The assay has been described elsewhere [21].

2.7. Determination of glutathione peroxidase (GPx) and glutathione reductase (GR) activities

Treated cells were collected in PBS and centrifuged at low speed (300×*g*) for 5 min to pellet cells to assay the activities of GPx and GR. Cell pellets were resuspended in 20 mM Tris containing 5 mM EDTA and 0.5 mM mercaptoethanol, sonicated and centrifuged at 3000×*g* for 15 min. Enzyme activities were measured in the supernatants. Determination of GPx activity was based on the oxidation of GSH by GPx, using t-BOOH as a substrate, coupled to the disappearance of NADPH by GR [23]. GR activity was determined by following the decrease in absorbance due to the oxidation of NADPH utilized in the reduction of oxidized glutathione [24]. The methods have been previously described [21]. Protein was measured by the Bradford reagent.

2.8. Preparation of cell lysates for Western blotting

Cells were lysed at 4°C in a buffer containing 25 mM Hepes, pH 7.5, 0.3 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM 1,4-dithiothreitol, 0.1% Triton X-100, 200 mM β-glycerol phosphate, 0.1 mM Na_3VO_4, 2 μg/ml of leupeptin and 1 mM phenylmethylsulfonyl fluoride to detect AKT, p-AKT, ERK1/2, p-ERKs, JNK1/2 and p-JNKs. The supernatants were collected, assayed for protein concentration by using the Bradford reagent, aliquoted and stored at −80°C until use for Western blot analyses.

2.9. Protein determination by Western blotting

Equal amounts of protein (100 μg) were separated by SDS-PAGE and transferred to polyvinylidene difluoride filters (Protein Sequencing Membrane, BioRad). Membranes were probed with the corresponding primary antibody followed by incubation with peroxide-conjugated antirabbit immunoglobulin (GE Healthcare, Madrid, Spain). Blots were developed with the use of an ECL system (GE Healthcare). Normalization of Western blot was ensured by β-actin, and bands were quantified by laser scanning densitometry (Molecular Dynamics, Sunnyvale, CA, USA).

2.10. Statistics

Statistical analysis of data was as follows: prior to analysis, the data were tested for homogeneity of variances by the test of Levene; for multiple comparisons, one-way analysis of variance was followed by a Bonferroni test when variances were homogeneous and by the Tamhane test when variances were not homogeneous. The level of significance was $P<.05$. SPSS version 12.0 was used.

3. Results

3.1. Effect of t-BOOH on cell viability and apoptosis in HepG2 cells

In order to study whether CPE was able to protect against an oxidative insult, we used t-BOOH, a chemical compound commonly used to induce oxidative stress and apoptosis in biological systems. The first goal was to determine the oxidative conditions leading to apoptosis and cell death in HepG2. Cells were treated for 3, 6, 12 and 24 h with different concentrations of t-BOOH, and cell viability was determined by using the crystal violet assay. Fig. 1A reveals that increasing concentrations of t-BOOH induced a dose-dependent increase in toxicity as shown by the decrease in cell viability. All the tested t-BOOH concentrations had lethal effects after 24 h of culture. The t-BOOH concentration of 100 μM provoked about 30% and 50% of cell death at 6 and 12 h respectively, whereas only about 30% of cells remained alive after 6 or 12 h of treatment with 200 μM t-BOOH. Therefore, 100 μM t-BOOH was the concentration chosen for the following experiments.

HepG2 cells were exposed to 100 μM t-BOOH for 6 h and caspase-3 activity, as a biomarker of apoptosis, was analyzed at different times to assess whether this cytotoxic effect was to some extent due to apoptosis. As shown in Fig. 1B, the presence of t-BOOH induced the activation of caspase 3, which started at 2 h of incubation and reached an activation peak at 3 h. These results indicate that t-BOOH is able to activate caspase 3 and induces apoptosis in HepG2 cells.

3.2. Effect of t-BOOH on ROS generation and signaling pathways

To elucidate the molecular mechanism of t-BOOH-induced apoptosis and cell death in HepG2, we examined the production of ROS and the subsequent activation of intracellular signaling. As shown in Fig. 2A, t-BOOH treatment at a concentration that induces apoptosis (100 μM) caused a significant increase in ROS generation, as early as 15 min after treatment, with a further increase at 30 and 60 min.

Next, we investigated the effect of t-BOOH-induced oxidative stress on the expression and activity of two important constituents of the MAPK signaling cascade, ERK1/2 and JNK1/2. Accordingly, we examined protein expression of total and phosphorylated (active form) ERKs and JNKs at different times (0–180 min). As shown in Fig. 2B, phosphorylation of ERKs and that of JNKs were markedly induced in the presence of 100 μM t-BOOH. Increased p-ERKs were detectable

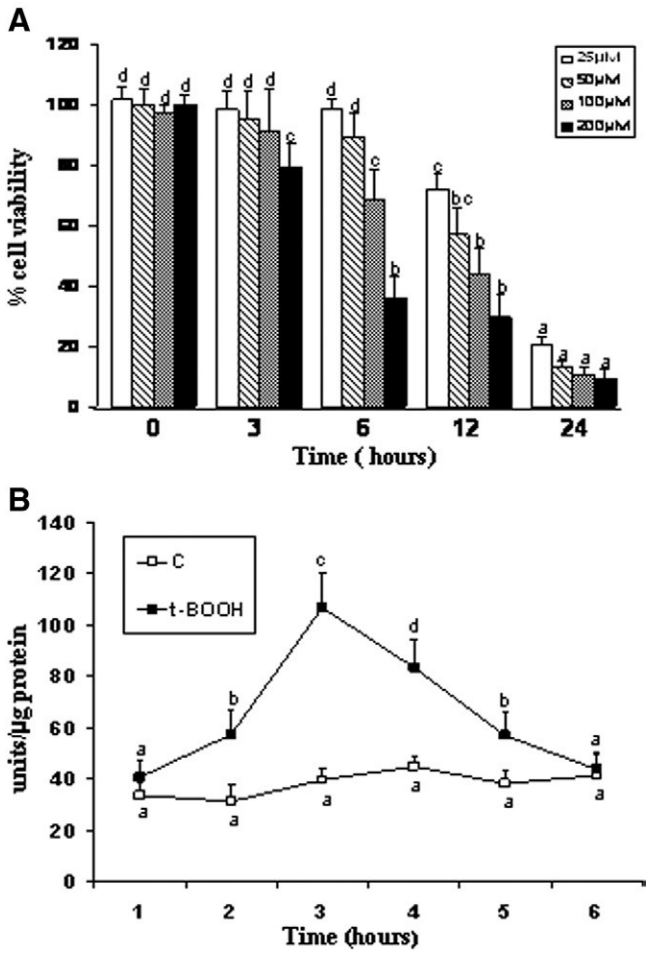

Fig. 1. Effect of t-BOOH on cell viability, and caspase-3 activity in HepG2 cells. (A) Cells were treated at 3, 6, 12 and 24 h with different concentrations of t-BOOH and cell viability was determined as percent of crystal violet-stained cells relative to untreated control. (B) HepG2 cells were incubated during increasing times with or without t-BOOH (100 μM) and caspase-3 activity (units per microgram of protein) was assayed as described in Materials and Methods. Data represent the mean±S.D. of 10–12 samples per condition. Different letters denote statistically significant differences, $P<.05$.

within 30 min and continued augmented after 60 min of treatment, decreasing at a longer incubation time (180 min). Phosphorylation of JNKs was evident after 60 min of treatment and remained active until 180 min. Total ERK and JNK protein levels did not change during t-BOOH exposure. These results indicate that t-BOOH-induced oxidative stress in HepG2 cells is accompanied by a quick but transient increase of p-ERKs and a sustained increase of p-JNKs.

3.3. CPE protects HepG2 cells from t-BOOH-induced apoptosis and cell death

Before testing the role of CPE on protection against t-BOOH-induced cell damage, we evaluated the direct effect of CPE on HepG2 cells. To this end, cells were treated for 20 h with CPE in a range of concentrations varying from 0.05 to 50 μg/ml. As shown in Fig. 3A, there was no difference on cell viability between CPE-treated and control cells, indicating that none of the tested concentrations of CPE induced cell injury in HepG2 cells.

HepG2 cells treated for 20 h with CPE were further exposed to 100 μM t-BOOH and apoptosis and cell death were evaluated to assess whether treatment with CPE repressed the cytotoxic effect induced by t-BOOH. Control and CPE-treated cells were exposed to t-BOOH for

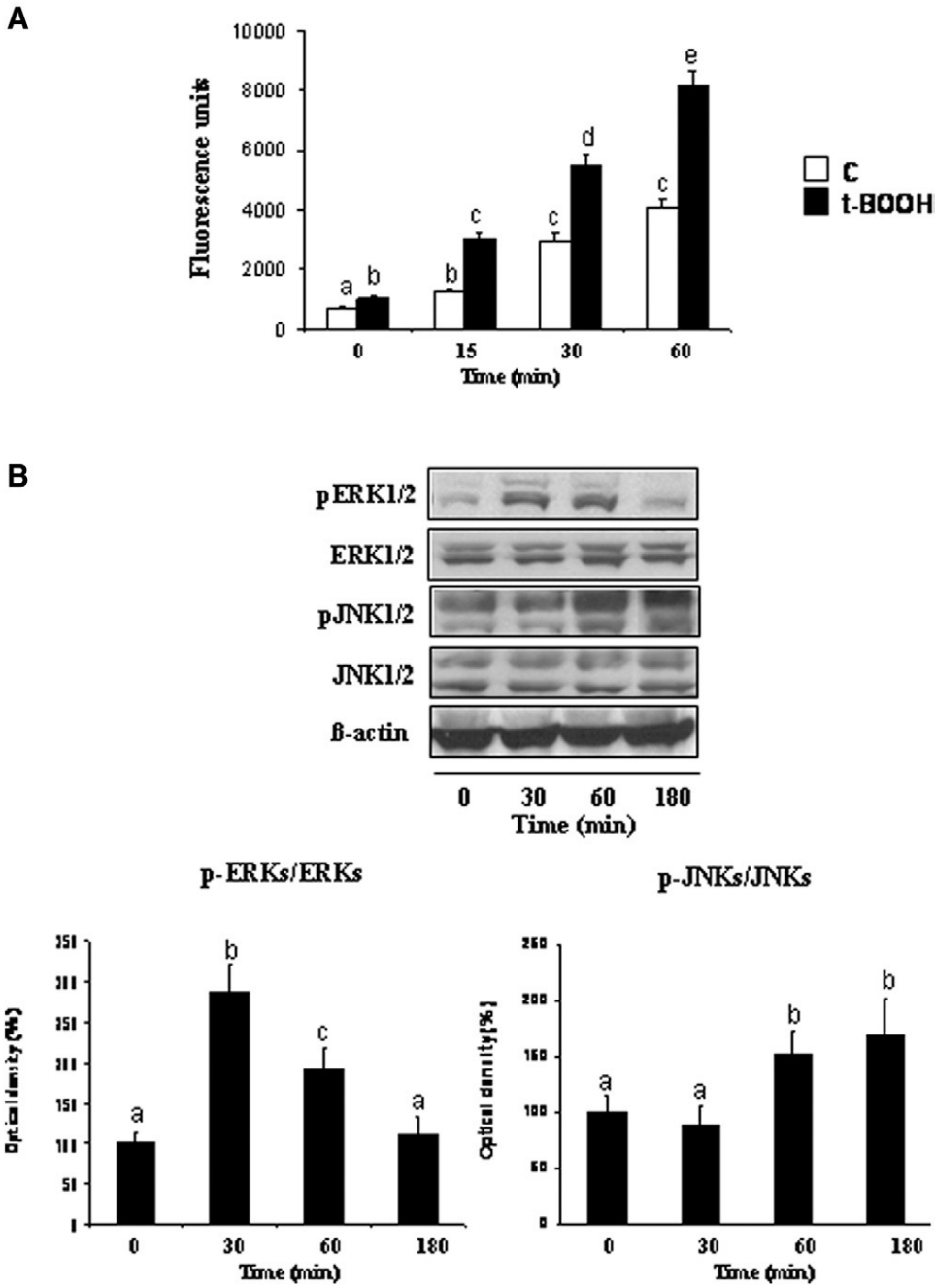

Fig. 2. Effect of t-BOOH on ROS generation and levels of phosphorylated ERK1/2 and JNK1/2, and total ERKs and JNKs. (A) HepG2 cells were exposed to 100 μM t-BOOH and intracellular ROS production was evaluated at 0, 15, 30 and 60 min. Values are the mean±S.D. of 10–12 samples per condition. (B) HepG2 cells exposed to 100 μM t-BOOH for the indicated times were subjected to Western blot analysis using phospho-specific antibodies to ERKs or JNKs. The same cell lysates were subjected to Western blot analysis using corresponding non-phospho-specific antibodies to detect total ERKs or JNKs. Bands are representative of four to six experiments. The percentage values of the p-ERK/ERK and p-JNK/JNK ratios relative to the control condition (mean±S.D.) are shown. Normalization of Western blots was ensured by β-actin. Means without a common letter differ, $P<.05$.

3 h and caspase-3 activity was assayed. Fig. 3B shows that treatment of cells for 20 h with CPE was able to prevent the appearance of t-BOOH-induced apoptosis at all the concentrations tested. Similarly, cell toxicity was measured by the LDH assay after 6 h of t-BOOH exposure in CPE-treated and control cells. As shown in Fig. 3C, cell treatment with different concentrations of CPE significantly suppressed the damage triggered by t-BOOH in a dose-dependent manner. Altogether, these results show that treatment of cells with 0.05 to 50 μg/ml of CPE protects against t-BOOH-induced cell death and apoptosis. Since 5 μg/ml of CPE is considered a realistic physiological concentration and was able to evoke a significant protection in HepG2 cells, all subsequent experiments were performed with 5 μg/ml of CPE.

3.4. CPE treatment induces phosphorylation of ERKs and AKT

Since polyphenols can modulate a series of signaling pathways that play an important role in cell survival and apoptosis, such as MAPKs and PI3K/AKT, we decided to investigate whether cell treatment with CPE for 20 h is able to activate key proteins of these intracellular signaling cascades. To this end, HepG2 cells were exposed to 5 μg/ml of CPE for 20 h and immunoblots were

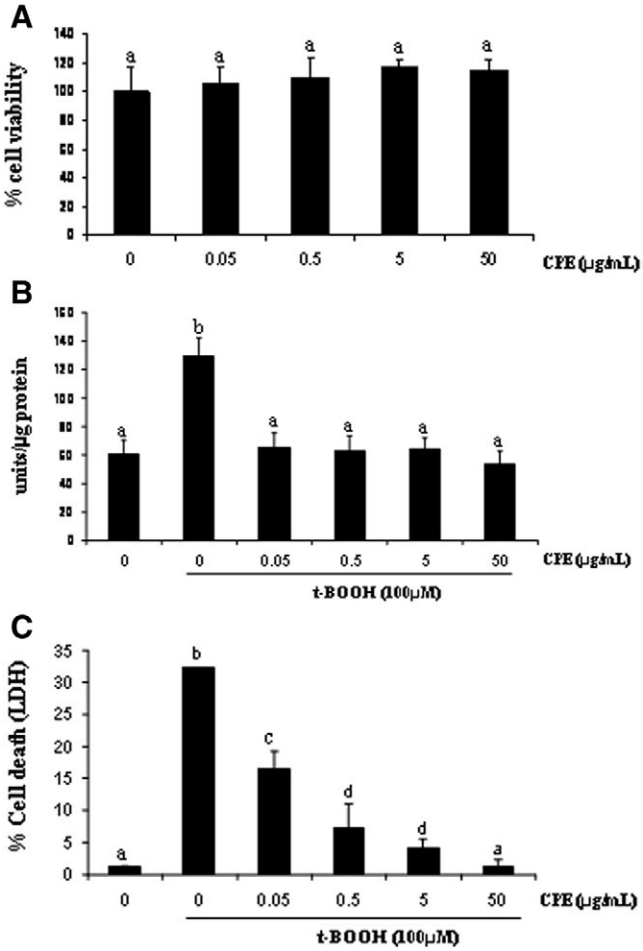

Fig. 3. Protective effect of the CPE against t-BOOH-induced apoptosis and cell death. (A) HepG2 cells were treated with the noted concentrations of CPE for 20 h and cell viability was determined by crystal violet and expressed as the relative percentage of control cell staining. (B) Control and CPE-treated cells were further exposed to 100 μM t-BOOH for 3 h and caspase-3 activity was measured. (C) Control and CPE-treated cells were exposed to 100 μM t-BOOH for 6 h and cell death as LDH leakage was evaluated. Data represent the mean±S.D. of 10–12 samples per condition. Different letters denote statistically significant differences, $P<.05$.

then performed using anti-AKT, anti-p-AKT, anti-ERKs, anti-p-ERKs, anti-JNKs and anti-p-JNKs. Fig. 4 illustrates that, after 20 h of treatment, CPE-treated cells showed a significant increase in the levels of phosphorylated AKT and ERK proteins but not in those of JNKs. Likewise, there was no difference in the total levels of AKT, ERKs and JNKs.

3.5. Effect of CPE treatment on ROS overproduction and signaling pathways induced by t-BOOH

Next, to gain further insight into the molecular mechanisms involved in cell protection by CPE, we studied whether the CPE-induced ERK and AKT activation is able to modulate the apoptotic pathways activated by t-BOOH. Accordingly, HepG2 cells were treated with 5 μg/ml of CPE for 20 h and then exposed to 100 μM t-BOOH. As shown in Fig. 5A, DCF fluorescence intensity caused by t-BOOH dropped significantly in cells treated with CPE for 20 h. The decrease in ROS production was observed as early as after 15 min of incubation and continued decreased up to 60 min.

Similarly, we examined total and phosphorylated ERKs and JNKs in cells treated for 20 h with CPE and then exposed to t-BOOH for different times (0–180 min). There was no change in total and p-ERK and p-JNK levels in CPE-treated cells in the presence of t-BOOH (Fig. 5B). As shown above, p-ERK levels before the t-BOOH insult were increased in CPE-treated cells as compared with untreated cells and, interestingly, remained activated in the presence of t-BOOH. These results indicate that CPE attenuated the generation of intracellular ROS induced by t-BOOH and prevented both the decrease in ERK activation and the increase of p-JNK levels.

3.6. CPE treatment increases antioxidant enzyme activity

A major protective strategy against oxidizing substances able to damage cells is the induction of antioxidant enzymes. Therefore, we examined whether CPE may activate two major antioxidant enzymes, the glutathione-related enzymes GPx and GR. To this end, HepG2 cells were treated with 5 μg/ml of CPE for 20 h and the activities of GPx and GR were evaluated. As shown in Fig. 6, the activation of both enzymes was significantly increased by treating cells with CPE.

3.7. ERK activation is implicated in the induction of GPx and GR activities by CPE

Studies on antioxidant enzyme induction by oxidative stress stimuli have shown that PI3K and MAPK pathways seem to be involved in the transduction of signals initiating gene activation. To determine whether a similar signal mechanism is responsible for the up-regulation of the activities of antioxidant enzymes by CPE, we analyzed the effect of PI3K- and ERK-specific inhibitors on the increase in GPx and GR activities. Following 2-h pretreatments with either PD98059 as a specific inhibitor of ERKs or wortmannin to inhibit PI3K, the activities of GPx and GR after 20 h of CPE treatment were studied. As shown in Fig. 7, pretreatment with PD98059 blocked CPE-induced activation of GPx and GR to levels similar to those in untreated control cells. However, the PI3K inhibitor only partially inhibited the activation of GR. These results show that the ERK pathway is directly involved in the CPE-induced activation of GPx and GR, whereas the AKT pathway is only partly implicated in GR activity.

3.8. Role of ERK pathway in the antiapoptotic and cytoprotective effect of CPE

Finally, we investigated whether the activation of AKT and ERKs induced by CPE could be implicated in the cytoprotective effect of CPE from t-BOOH-induced injury. In this regard, cells were pretreated with PI3K and ERK inhibitors for 2 h and treated with CPE for 20 h. Then, the CPE-treated and control cells were exposed to 100 μM t-BOOH, and ROS generation (0–60 min), caspase-3 activation at 3 h and cell death at 6 h were evaluated.

As expected, treatment of cells with 5 μg/ml of CPE for 20 h significantly reduced the generation of oxidative radicals caused by t-BOOH (Table 1). This reduction was completely blocked by PD98059 (a specific inhibitor of ERKs), whereas wortmannin (a specific inhibitor of AKT) had no significant effect. In other words, the protective ability of CPE against oxidative damage caused by reactive oxygen intermediates was eliminated in the presence of ERK inhibitors. Similarly, inhibition of ERKs allowed the activation of caspase 3 provoked by t-BOOH (Fig. 8A) and significantly blocked the cytoprotective effect of CPE against cell death induced by this pro-oxidant (Fig. 8B). On the contrary, treatment with wortmannin did not have any effect on caspase-3 activation (Fig. 8A) but partially blocked the protective effect of CPE in cell death (Fig. 8B). Taken together, these results indicate that activation of the ERK pathway seems to be required for the cytoprotective

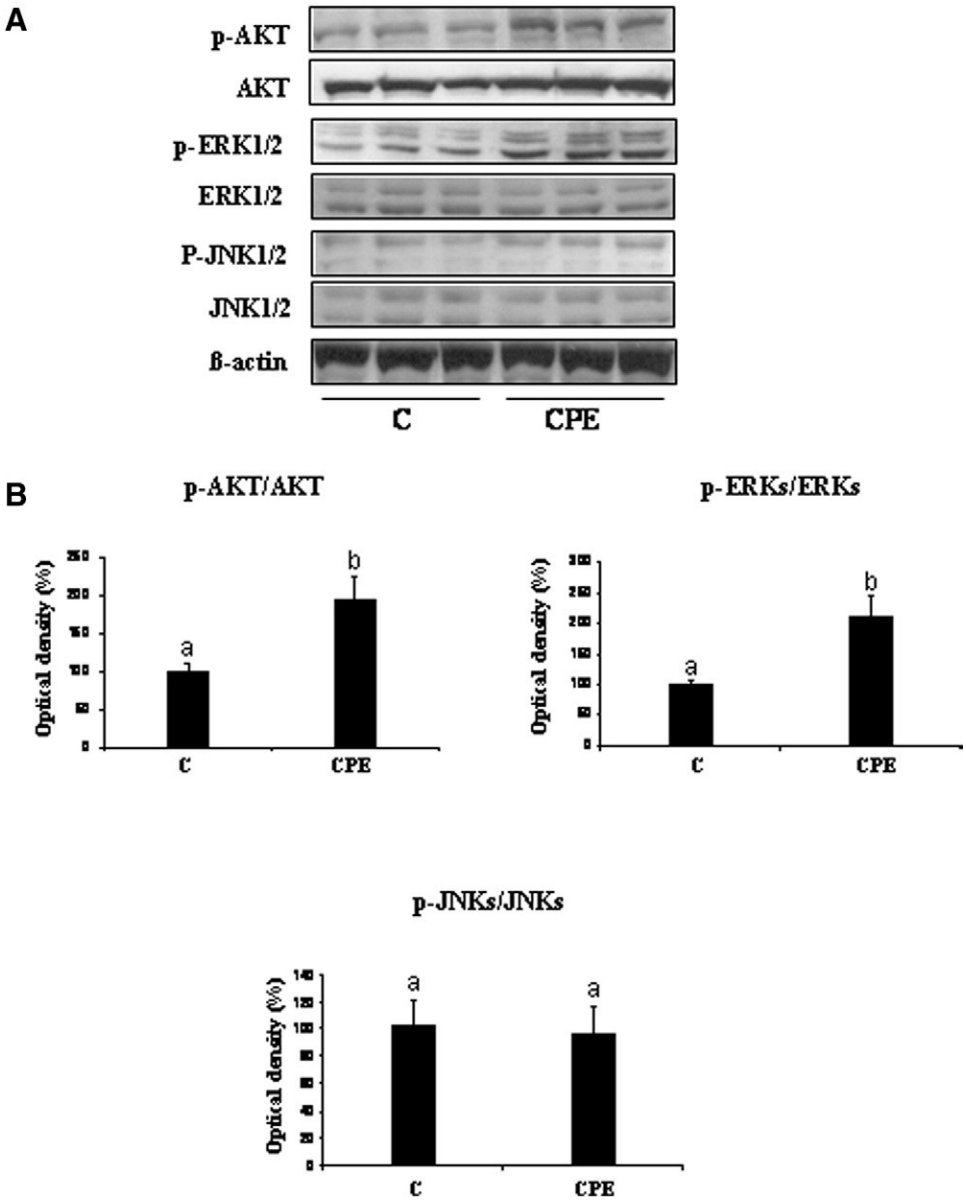

Fig. 4. Effect of CPE on basal levels of phosphorylated and total levels of AKT, ERKs and JNKs. (A) Control cells and cells treated with 5 μg/ml of CPE for 20 h (CPE) were subjected to Western blot analysis using phospho-specific antibodies to AKT, ERKs or JNKs. The same cell lysates were subjected to Western blot analysis using corresponding non-phospho-specific antibodies to detect total AKT, ERKs or JNKs. Bands are representative of four to six experiments. (B) Percentage values of the p-AKT/AKT, p-ERK/ERK and p-JNK/JNK ratios relative to the control condition (mean±S.D.). Normalization of Western blots was ensured by β-actin. Means without a common letter differ, $P<.05$.

effect exhibited by CPE against oxidative stress and apoptosis induced by t-BOOH.

4. Discussion

Natural antioxidants that can inhibit free radical generation are considered essential in terms of protecting the liver from chemical-induced damage. Consequently, a high number of recent investigations have focused on natural dietary compounds that are involved in chemoprevention and on the mechanisms of their action [10]. In this regard, cocoa bean and its derived products have been shown to contain important antioxidants of a polyphenolic nature that possess different biological activities, such as the inhibition of different phases of tumor process and antioxidant and anti-inflammatory effects [14]. All these biological properties point to cocoa polyphenols as interesting candidates for cellular chemoprotection. In this study, we show that a CPE containing monomeric flavanols as well as procyanidin dimers and trimers [18] protects human liver cells against oxidative stress-induced apoptosis by reducing ROS production in the presence of a stressor and by modulating the activities of antioxidant enzymes, such as GPx and GR. In addition, we establish that the ability to modulate important proteins of cell signaling cascades by CPE is directly involved in its protective mechanism.

Previous studies demonstrated that t-BOOH-induced oxidative stress in HepG2 cells is a useful model for evaluating the cytoprotective effect of natural antioxidants [21,25–27]. Alternatively, t-BOOH at concentrations lower than 0.4 mM has also been considered a proapoptotic agent that is able to induce activation of caspases 9 and 3, DNA fragmentation and, eventually, HepG2 cell death [28]. Accordingly, here we show that HepG2 cells incubated in the presence of 100 μM t-BOOH undergo apoptosis as evidenced by activation of caspase 3, which is considered a very specific and sensitive apoptotic marker. Furthermore, caspase-3 activation was

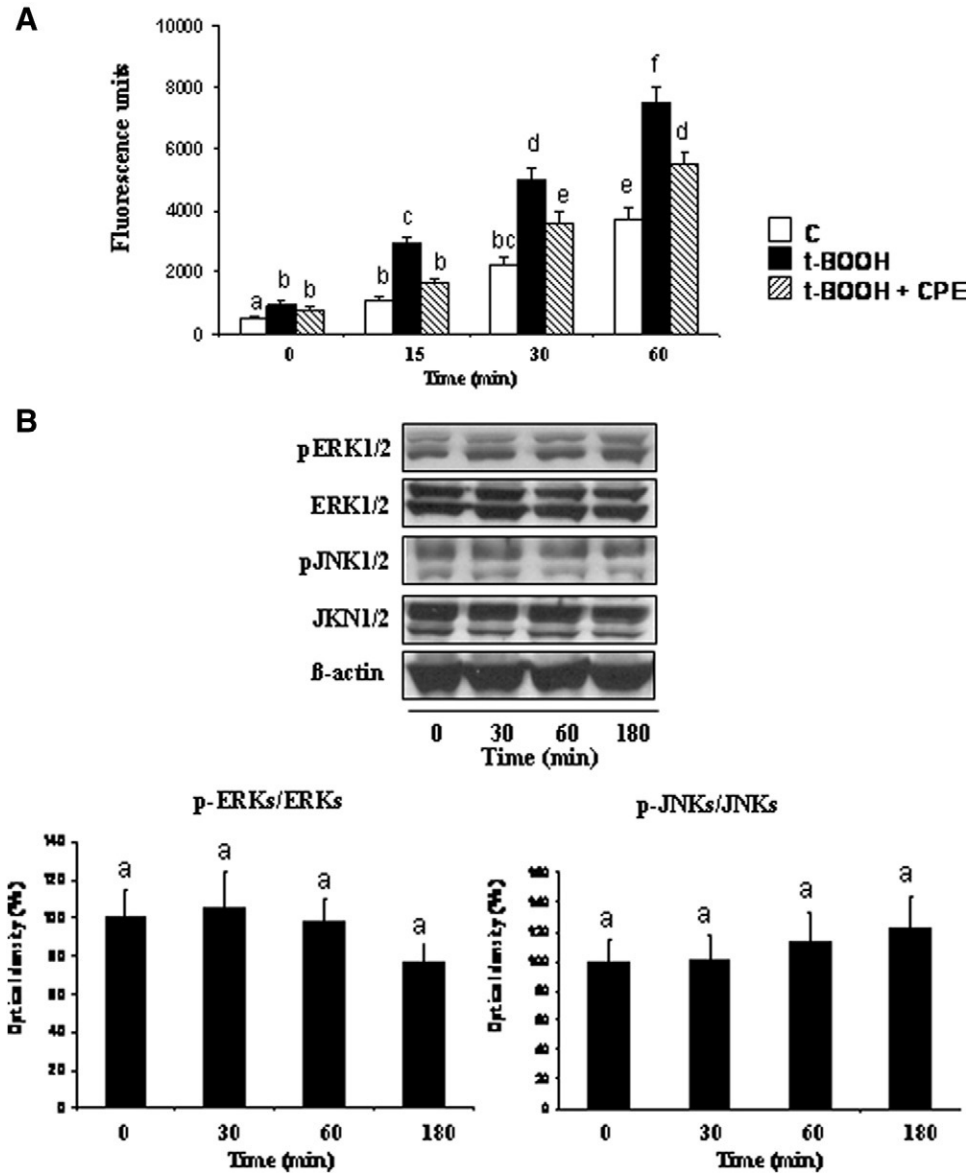

Fig. 5. Protective effect of CPE on the enhanced ROS generation and ERK and JNK activation induced by t-BOOH. (A) Control cells and cells treated with 5 μg/ml of CPE for 20 h were exposed to 100 μM t-BOOH. Then, intracellular ROS production was evaluated at 0, 15, 30 and 60 min. Values are the mean±S.D. of 10–12 samples per condition. (B) Cells treated with 5 μg/ml of CPE for 20 h were subsequently exposed to 100 μM t-BOOH for the indicated times and subjected to Western blot analysis using phospho-specific antibodies to ERKs or JNKs. The same cell lysates were subjected to Western blot analysis using the corresponding non-phospho-specific antibodies to detect total ERKs or JNKs. Bands are representative of four or five experiments. The percentage values of the p-ERK/ERK and p-JNK/JNK ratios relative to the control condition (mean±S.D.) are shown. Normalization of Western blots was ensured by β-actin. Means without a common letter differ, $P<.05$.

preceded by a rapid increase in intracellular ROS, supporting the role of ROS generation in t-BOOH-induced apoptosis [29].

Oxidative stress-induced injury results not only from direct chemical interactions by altering cellular macromolecules, including DNA, proteins and lipids, but also from profound alterations in signal transduction pathways [30]. Signaling cascades involving the MAPK–JNK pathways are key mediators of stress signals and seem to be mainly responsible for protective responses and stress-dependent apoptosis reactions [31–33]. In this regard, ROS generation has been described as a critical upstream activator of JNKs [34], and the persistent activation of JNKs has been directly involved in the development of apoptosis in hepatocytes and nonhepatic cells [34,35]. Conversely, sustained activation of the ERKs has been shown to confer hepatocyte resistance to death [33]. In this study, we show for the first time that t-BOOH is able to induce an increase in the phosphorylated levels of ERK and JNK proteins in HepG2 cells and that these events occur after increased ROS generation and prior to caspase-3 activation and cell death. In addition, the increase in the levels of p-ERKs was quick and transient, whereas the increase in those of p-JNKs was sustained over time. Therefore, as previously shown with other stressors [33], the decline after early activation of p-ERKs, together with persistent activation of JNKs, seems to be involved in t-BOOH-induced ROS-mediated apoptosis in HepG2 cells.

Pretreatment of cells with natural antioxidants prevented the cytotoxicity induced by oxidative stress inducers through the ability of these compounds to inhibit the increase of ROS levels and the subsequent activation of caspase 3, leading to apoptosis [36]. Consistent with these findings, we show that flavonoids from a cocoa extract effectively suppressed the apoptotic effects exerted by t-BOOH. Pretreatment of HepG2 cells with different concentrations of

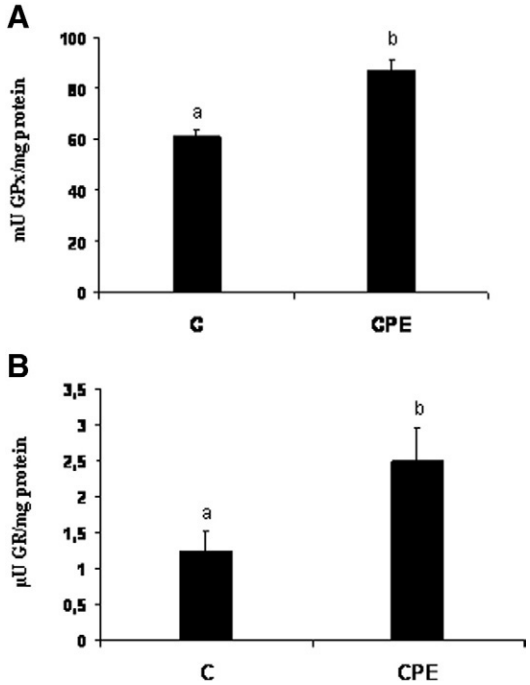

Fig. 6. Induction of GPx and GR activities by CPE. HepG2 cells were treated with 5 μg/ml of CPE for 20 h and GPx and GR activities were measured as described in Materials and Methods. (A) GPx activity in control and CPE-treated cells. (B) GR activity in control and CPE-treated cells. Values are the mean of six to eight samples per condition. Means without a common letter differ, $P<.05$.

CPE before the oxidative insult blocked caspase-3 activation induced by t-BOOH and protected cells from its cytotoxic effect as shown by decreased LDH leakage. In addition, CPE treatment attenuated the generation of intracellular ROS induced by t-BOOH and prevented both the decrease in ERK activation and the increase of p-JNK levels that preceded cell apoptosis and death. Accordingly, the protective effect of CPE on oxidative stress-induced apoptosis seems to be mediated through blunting the ROS-induced activation of JNKs. Furthermore, as previously indicated by others [33,34], our results show that the increase in the levels of p-ERKs after CPE treatment could be essential to avoid a prolonged and therefore lethal JNK activation and provides evidence for an antiapoptotic role for ERKs and a proapoptotic role for JNKs–MAPKs in hepatic cells.

Flavonoids possess in vitro antioxidant activity by directly scavenging intracellular ROS; however, more recently, it has been shown that they may also offer indirect protection by enhancing the activities of a number of protective enzymes [10]. In this regard, numerous studies have demonstrated the tight interrelation between dietary polyphenols and endogenous antioxidants, such as GSH and its related enzymes, GPx and GR [37]. GPx catalyzes the reduction of peroxides and is suggested to act as a barrier against hydroperoxide attack [38], whereas GR is implicated in recycling oxidized glutathione back to reduced glutathione [39]. Therefore, the presence of glutathione-dependent enzymes, which participate in the defense against hydrogen peroxides and superoxides, seems to be essential to prevent the cytotoxicity of ROS. In accordance with that, we demonstrate that 20 h of treatment of human HepG2 cells with CPE induced a significant increase in GPx and GR activities. This outcome pointed out that CPE-treated cells were in better conditions to face the increasing generation of ROS induced by the potent pro-oxidant t-BOOH and consequently to escape apoptosis. Supporting this, it has been shown that primary hepatocytes isolated from GPx knockout mice were more susceptible to ROS-induced apoptosis [40], whereas increased GPx activity was associated with the inhibition of apoptosis in wild-type mice by decreased activation of caspase 3 and JNK [41,42]. Based on our findings, we suggest that one of the mechanisms by which CPE inhibited oxidative stress-induced apoptosis was preventing ROS accumulation through the improvement of

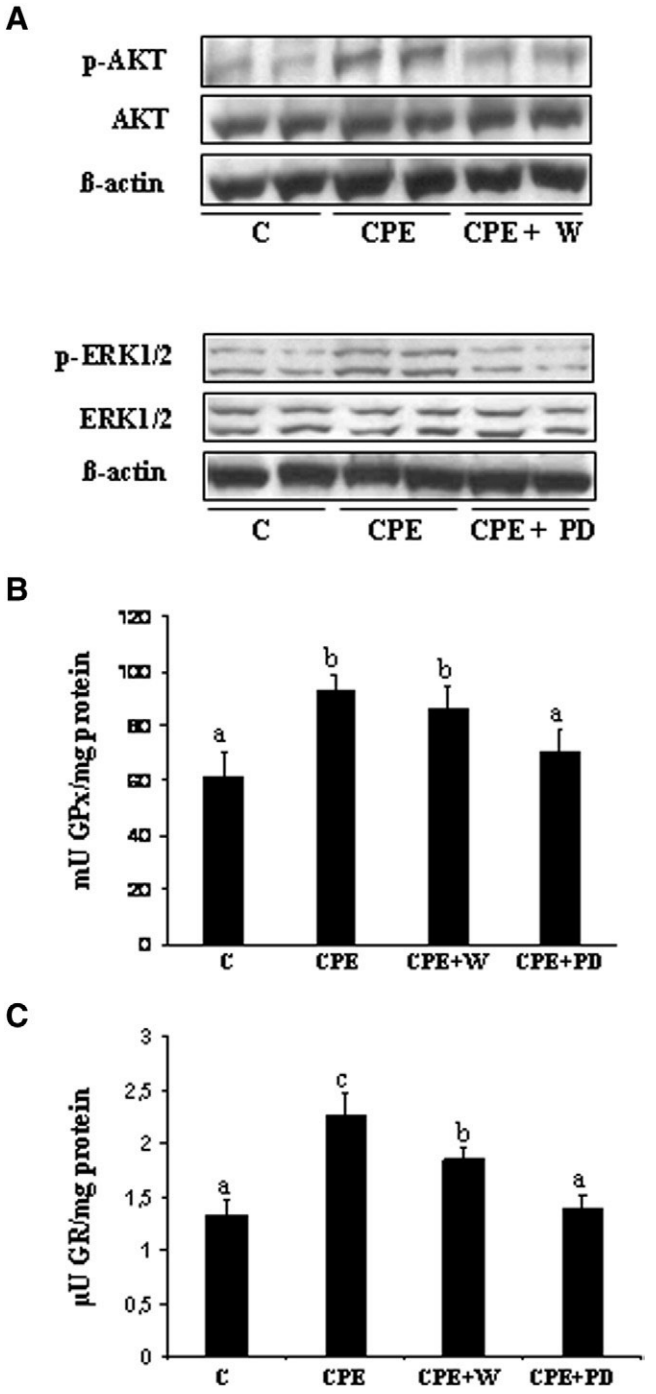

Fig. 7. Effects of wortmannin (W) and PD98059 (PD) on AKT and ERK pathways and on the increased GPx and GR activities induced by CPE. HepG2 cells were incubated with 5 μg/ml of CPE for 20 h in the presence or absence of 200 nM W or 50 μM PD. (A) Cell lysates were subjected to Western blot analysis using phospho-specific antibodies to AKT or ERKs. The same cell lysates were subjected to Western blot analysis using the corresponding non-phospho-specific antibodies to detect total AKT or ERKs. Bands are representative of four or five experiments. Normalization of Western blots was ensured by β-actin. (B) GPx and GR activities were measured as described in Materials and Methods. Values are the mean of six to eight samples per condition. Means without a common letter differ, $P<.05$.

Table 1
Effects of pretreatment with CPE (5 μg/ml), CPE plus wortmannin (CPE+W) and CPE plus PD98059 (CPE+PD) on intracellular ROS generation induced by t-BOOH (T)

	ROS (fluorescence units)			
	0 min	15 min	30 min	60 min
C	614±14[a]	1273±45[a]	3037±64[a]	4534±123[a]
C+T	938±22[c]	2855±94[c]	5333±192[c]	8447±406[d]
CPE+T	836±54[b]	1621±70[b]	3819±330[b]	5950±360[b]
(CPE+W)+T	789±55[b]	2043±109[b]	3894±139[b]	5946±195[b]
(CPE+PD)+T	795±80[b]	2707±118[c]	4748±191[c]	6767±166[c]

Data represent the mean±S.D. of 10–12 samples per condition. There was a significant increase in ROS generation through time within every condition. The Time×Condition interaction was further analyzed using one-way analysis of variance followed by the appropriate test. Means in a column with different letters differ, $P<.05$.

endogenous antioxidant defense. Interestingly, increased antioxidant enzyme activity could be a potential therapeutic strategy in liver diseases caused by oxidative stress, as shown in studies in which overexpression of other antioxidant enzymes (superoxide dismutases) inhibits apoptosis in alcohol-induced liver injury in rats [43,44].

Although activation of GSH-related enzymes represents an essential instrument of the polyphenolic antioxidative effect [45], the mechanism of action underlying this process is not fully understood. Recent studies have indicated that the effects of flavonoids on antioxidant enzyme expression and activity could be mediated by modification of signal transduction pathways [15]. Dietary compounds can activate a number of cellular kinases, including MAPKs and PI3K [16], and both pathways have been recently implicated in the up-regulation of several antioxidant/detoxificant enzyme activities [11]. In this work, we found that, compared with untreated HepG2 control cells, CPE-treated cells had higher levels of p-ERKs and p-AKT, while the levels of p-JNKs were not changed. Furthermore, up-regulation of GPx and GR activities was completely blocked by ERK inhibitors, whereas inhibition of AKT did not fully prevent the CPE-induced activation of these antioxidant enzymes. Therefore, AKT and ERK pathways were involved in the induction of GR activity by CPE, while only ERKs participated in GPx activation in HepG2 cells. Moreover, since a direct effect of biophenols on both the activity and the DNA transcription of GSH-related enzymes has been recently shown [45], we can also suggest that the increased activity found in CPE-treated cells could reflect an increase in the gene expression of GPx and GR. Both PI3K/AKT and MAPK pathways have been recently implicated in the mechanism of activation of the transcription factor Nrf2, which acts through the antioxidant-response element to initiate gene transcription [16]. Supporting this, recent studies have demonstrated that several dietary polyphenols could stimulate the transcription of antioxidant/detoxificant enzymes through the activation of these signaling pathways [16,46].

Finally, to elucidate the signaling pathway involved in the cytoprotective action of CPE, we explored the PI3K/AKT and MAPK pathways using the appropriate inhibitors. The results showed that ERK inhibitors abolished the effect of CPE, reducing the amplified ROS induced by t-BOOH, and suppressed the protective effect of CPE on t-BOOH-induced cell death and apoptosis. On the other hand, although AKT is activated by CPE in HepG2 cells, it does not participate in the prevention of the increased ROS generation and activation of caspase 3 induced by t-BOOH, and thus the inhibition of PI3K/AKT did not fully suppress the protection of CPE. These data suggest that ERK is a major pathway mediating the protective effect by CPE against oxidative stress-induced apoptosis. Moreover, since ERKs are also implicated in the increased activities of GPx and GR in CPE-treated HepG2 cells, these observations also suggest that intracellular increased activities of these enzymes could be an important factor in CPE-mediated cytoprotection against t-BOOH-induced apoptosis in HepG2 cells. Furthermore, it is worth mentioning that, besides their contribution to the induction of antioxidant enzymes, MAPK and PI3K cascades are also involved in the regulation of cell proliferation and cell survival machineries. Therefore, as we have recently demonstrated with epicatechin, the most abundant flavanol in cocoa [25], AKT and ERK activation probably plays an indirect protective role through the promotion of cell proliferation and survival signaling.

In this study, the ability of CPE to modulate the activities of the antioxidant enzymes was observed at concentrations considered realistic both in vivo and in vitro [18]. Biological properties of cocoa flavonoids are conditioned by their bioavailability; in this line, several in vivo studies have confirmed the absorption of catechin, epicatechin and dimeric procyanidins after the intake of different cocoa by-products by animals and humans [14]. In vitro, human HepG2 cells show very active absorption and metabolism of phenolic compounds [47,48] that clearly suggest a relevant uptake of cocoa polyphenols that coexist with their metabolites in the culture. The extent of the flavanol uptake and metabolism by HepG2 remains to be elucidated.

In summary, we showed that cocoa polyphenols exerted an antiapoptotic effect protecting HepG2 cells against both t-BOOH-induced cellular death and apoptosis. This antiapoptotic effect was associated with reduced ROS generation, avoidance of ERK deactivation and JNK activation, and prevention of caspase-3 activation in HepG2 cells. Moreover, pretreatment of HepG2 with CPE increases the activities of the glutathione-related enzymes GPx and GR and thus protects cells against oxidative stress-induced apoptosis by directly

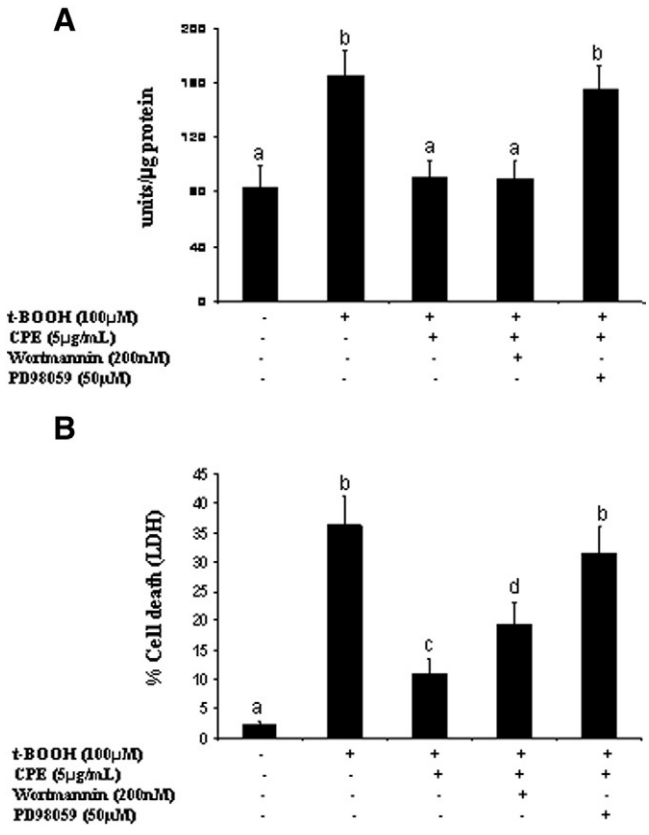

Fig. 8. Effects of CPE and selective inhibitors wortmannin (W) and PD98059 (PD) on caspase-3 activity and cell death. HepG2 cells were incubated with 5 μg/ml of CPE for 20 h in the presence or absence of 200 nM W or 50 μM PD. (A) Untreated and CPE-treated cells with or without inhibitors were exposed to 100 μM t-BOOH for 3 h and caspase-3 activity was assayed as described in Materials and Methods. (B) Control and CPE-treated cells with or without inhibitors were exposed to 100 μM t-BOOH for 6 h and cell death was determined by LDH leakage. Data represent the mean±S.D. of 10 experiments. Different letters denote statistically significant differences, $P<.05$.

counteracting free radicals and by activating the antioxidant defense system. Interestingly, our results provide evidence that ERKs act as mediators of the effects of CPE upon both the increased antioxidant enzyme activities and the CPE-induced cytoprotection against oxidative stress-induced apoptosis. These results provide new insights into the antioxidative mechanisms of cocoa flavonoids and point toward their antiapoptotic effect as an additional mechanism of action of these compounds.

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