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Molecular Mechanisms of (−)-Epicatechin and Chlorogenic Acid on the Regulation of the Apoptotic and Survival/Proliferation Pathways in a Human Hepatoma Cell Line

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Dietary polyphenols have been associated with reduced risk of chronic diseases, but the precise molecular mechanisms of protection remain unclear. This work was aimed at studying the effect of (−)-epicatechin (EC) and chlorogenic acid (CGA) on the regulation of apoptotic and survival/proliferation pathways in a human hepatoma cell line (HepG2). EC or CGA treatment for 18 h had a slight effect on cell viability and decreased reactive oxygen species formation, and EC alone promoted cell proliferation, whereas CGA increased glutathione levels. Phenols neither induced the caspase cascade for apoptosis nor affected expression levels of Bcl-x_L or Bax. A sustained activation of the major survival signals AKT/PI-3-kinase and ERK was shown in EC-treated cells, rather than in CGA-exposed cells. These data suggest that EC and CGA have no effect on apoptosis and enhance the intrinsic cellular tolerance against oxidative insults either by activating survival/proliferation pathways or by increasing antioxidant potential in HepG2.

KEYWORDS: Dietary phenols; HepG2 cells; cell viability; apoptosis; caspases; Bcl-2 family proteins; AKT/PI3-kinase pathway; ERK

INTRODUCTION

Numerous epidemiological and intervention studies in both humans and animals have shown that regular consumption of fruits, vegetables, and tea is associated with reduced risk of chronic and degenerative diseases, including cancer, Parkinson's, Alzheimer's, and cardiovascular diseases (*1*, *2*). Polyphenols are naturally occurring compounds, which are widely distributed in vegetables, fruits, and beverages (tea and wine), and possess different biological activities such as antioxidant, antiinflammatory, antithrombogenic, antiviral, and anticarcinogenic (*1*, *2*). Chlorogenic acid (CGA) and (−)-epicatechin (EC) are common dietary polyphenols, and they can be found in cherry, kiwi, and plum and in green tea and cocoa, respectively (*3*). Both phenolic compounds constitute an important part of the human diet and are extensively metabolized during absorption in the small intestine and in the liver (*4*). CGA and EC possess a well characterized in vitro antioxidant activity, and in this regard, recent studies have shown that both phenols are effective free radical scavengers and interfere with the oxidative/antioxidative potential of the cell (*5*−*7*). In addition, many studies have shown that tea polyphenols are potent chemopreventive agents protecting against many types of cancer. These effects are mediated through the inhibition of cell growth, cell cycle dysregulation, and induction of apoptosis (*8*, *9*). Furthermore, the preventive role of CGA in human colon cancer (*10*), oral tumor cell lines (*11*), and murine preadipocytes (*12*) has been demonstrated. However, the exact mechanism of the differential effects of EC and CGA is still unclear, and it has been suggested that these compounds might exert beneficial and/or cytotoxic actions through their ability to stimulate the antioxidant defense metabolism through the redox-regulated transcription factors and their modulation of signal cascades such as mitogen-activated protein kinases (MAPKs) (*13*).

Most studies using cell culture systems have been focused on the antioxidant effect of polyphenols after exposure of cells to pro-oxidants (*5*−*7*). However, there is little literature evaluating the effect of the outcome of these dietary phenolic compounds alone in a cell culture line, and even more limited is the number of references in which has been analyzed the regulation of oxidative stress and other molecular mechanisms such as modulation of signal transduction pathways related to cell death (caspases), cell survival/proliferation [MAPKs, protein kinase B (AKT)/phosphatidylinositol-3-kinase (PI-3-kinase)] and mitochondrial function, which contribute significantly to the regulation of cell viability. Accordingly, the protective effects of CGA and EC have not been studied intensely, and some

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authors have considered these phenols, in particular EC, as inactive compounds when compared to other green tea polyphenols because only the potential of EC for apoptosis induction was evaluated (*9*).

The aim of this study was to evaluate the potential modifications of cellular redox status and apoptotic and survival/proliferation pathways of a human hepatoma cell line (HepG2) after treatment with EC or CGA. Therefore, reactive oxygen species (ROS) production and intracellular glutathione (GSH) levels as markers for redox status were evaluated. Also, the mechanisms underlying the biological effects of EC and CGA on HepG2 were investigated by studying the balance of influence between pro-death pathways (caspase-3 and the Bcl-2 family members Bcl-x_S and Bax) and pro-survival pathways [Bcl-x_L, AKT/PI3K, and extracellular regulated kinase (ERK)].

MATERIALS AND METHODS

Materials and Chemicals. (−)-Epicatechin (>95% of purity), chlorogenic acid (>95% of purity), Trypan Blue, *o*-phthalaldehyde (OPT), LY294002 [2-(4-morpholinyl)-8-phenyl-1(4*H*)-benzopyran-4-one hydrochloride], PD98059 [2-(2-amino-3-methoxyphenyl)-4*H*-1-benzopyran-4-one], gentamicin, penicillin G, and streptomycin were purchased from Sigma Chemical (Madrid, Spain). Cell proliferation ELISA 5-bromo-2′-deoxyuridine (BrdU) (colorimetric) assay kit was from Roche Diagnostics (Roche Molecular Biochemicals, Barcelona, Spain). The fluorescent probe 2′,7′-dichlorofluorescin diacetate (DCFH-DA) was from Molecular Probes (Eugene, OR). Anti-AKT and anti-phospho-Ser473-AKT, as well as anti-ERK1/2 and anti-phospho-ERK1/2 recognizing phosphorylated Thr202/Thy204 of ERK1/2, and anti-β-actin were obtained from Cell Signalling Technology (9271, 9272, 9101, 9102, and 4697, respectively; Izasa, Madrid, Spain). Anti-Bcl-x, anti-Bax, and anti-p110β (catalytic PI-3-kinase subunit) were purchased from Santa Cruz (sc-634, sc-526, and sc-7175, respectively; Santa Cruz Biotechnology Inc., Santa Cruz, CA). Anti-p85α/β (regulatory PI-3-kinase subunit) was from Upsate Biotechnology (Lake Placid, NY). Caspase-3 substrate (Ac-DEVD-AMC) was purchased from Pharmingen (San Diego, CA). Materials and chemicals for electrophoresis were from Bio-Rad Laboratories S.A. (Madrid, Spain). Cell culture dishes and cell culture medium were from Falcon (Cajal, Madrid, Spain) and Biowhittaker Europe (Innogenetics, Madrid, Spain), respectively.

Cell Culture and Phenolic Compound Treatments. Human hepatoma HepG2 cells were a gift from Dr. Paloma Martin-Sanz (Centro de Investigaciones Biologicas, Madrid, Spain). They were grown in DMEM-F12 medium supplemented with 2.5% FBS and the following antibiotics: gentamicin, penicillin, and streptomycin (50 mg/L). Cells were maintained at 37 °C in a humidified atmosphere of 5% CO_2.

Cells were seeded and routinely grown in DMEM-F12 medium and 2.5% FBS, but they were changed to serum-free medium 24 h before the assay. EC and CGA were dissolved in 50% methanol, sterilized by filtering, and then diluted to the appropriated concentration in culture media. Cells were treated with different concentrations of either EC or CGA (1, 10, 25, 50, 75, and 100 μM) during 18 h.

In the experiments with EC and the pharmacological inhibitors, cells were preincubated with the indicated concentrations of EC or EC in combination with 50 μM LY294002 and/or 50 μM PD98059 for 2 h prior to 18 h of EC treatment.

Cell Viability Assay. Cell viability was calculated by counting cells in a Neubauer chamber. An aliquot of the total cell suspension (1.5×10^5 cells) was mixed with an equal volume of Trypan Blue and incubated for 5 min at room temperature.

Cell viability was also determined by using the Crystal Violet assay (*14*). HepG2 cells were seeded at low density (10^4 cells per well) in 96-well plates, grown for 20 h, and incubated with Crystal Violet (0.2% in ethanol) for 20 min. Plates were rinsed with tap water and allowed to dry, and 1% sodium dodecyl sulfate (SDS) was added. The absorbance of each well was measured using a microplate reader at 570 nm.

Cell Proliferation Assay (BrdU Assay). A colorimetric immunoassay (ELISA) was used for the quantification of cell proliferation. This method is based on the measurement of BrdU incorporation into genomic DNA during DNA synthesis of proliferating cells. HepG2 cells were seeded (10^4 cells per well) in 96-well plates, grown for 20 h, and labeled by the addition of BrdU for 4 h. Then the anti-BrdU antibody was added, and the immune complexes were detected by the subsequent substrate (tetramethylbenzidine) reaction and quantified by measuring the absorbance at 620 nm in a microplate ELISA reader.

Determination of ROS. Cellular oxidative stress was quantified by the dichlorofluorescin (DCFH) assay using a microplate reader (*15*). After being oxidized by intracellular oxidants, DCFH becomes dichlorofluorescein (DCF) and emits fluorescence. By quantifying fluorescence, a fair estimation of the overall oxygen species generated under the different conditions was obtained. Briefly, 5 μM DCFH was added to the wells (2×10^5 cells per well) and incubated for 30 min at 37 °C. Then the cells were washed twice with serum-free medium, and multiwell plates were immediately measured in a fluorescent microplate reader (Bio-Tek, Winooski, VT) at an excitation wavelength of 485 nm and an emission wavelength of 530 nm.

Determination of Reduced Glutathione (GSH). GSH was quantified according to the fluorometric assay of Hissin and Hilf (*16*). The method is based on the reaction of GSH with *o*-phtalaldehyde (OPT) at pH 8.0. In brief, cells were homogenized by ultrasound with 5% trichloroacetic acid and 2 mM EDTA. Lysates were clarified by centrifugation at 7000*g* for 30 min at 4 °C. Fifty microliters of supernatant was mixed with 1 M NaOH, 0.1 M sodium phosphate buffer, and 10 μL of OPT (10 mg/mL). Fluorescence was measured at an excitation wavelength of 340 nm and an emission wavelength of 460 nm. A standard curve of GSH (5 ng−1 μg) was used.

Western Blot Analysis. To analyze Bcl-x and Bax levels, cells were resuspended in 25 mM HEPES (pH 7.5), 2.5 nM EDTA, 0.1% Triton X-100, 5 μg/mL leupeptin, and 1 mM phenylmethanesulfonyl fluoride. To detect AKT, phospho-AKT, ERK1/2, and phospho-ERK1/2, as well as PI-3-kinase subunits (p85 and p110), 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 dithiothreitol, 0.1% Triton X-100, 200 mM β-glycerolphosphate, 0.1 mM Na_3VO_4, 2 μg/mL leupeptin, and 1 mM phenylmethanesulfonyl fluoride. Later, the protein concentration of cell lysates was analyzed, by using the Bio-Rad protein assay kit according to the manufacturer's specifications. Afterward, equal amounts of proteins (100 μg) were separated by SDS−polyacrylamide gel electrophoresis and transferred to poly(vinylidene difluoride) (PVDF) filters (Protein Sequencing Membrane, Bio-Rad). Membranes were probed with the corresponding primary antibody followed by incubation with peroxide-conjugated anti-rabbit immunoglobulin. Blots were developed with the ECL system (GE Healthcare, Spain). Normalization of Western blot was ensured by β-actin, and bands were quantified by laser scanning densitometry (Molecular Dynamics, Sunnyvale, CA).

Fluorometric Analysis of Caspase-3 Activity. Caspase-3 activitiy was measured as previously described (*17*). 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, 30 μg of protein/condition, and 20 μM Ac-DEVD-AMC (*N*-acetyl-Asp-Glu-Val-Asp-7-amino-4-methylcoumarin) as substrate. Enzymatic activity was measured at an excitation wavelength of 380 nm and an emission wavelength of 440 nm.

Statistics. Prior to statistical analysis, data were tested for homogeneity of variances by the test of Levene; for multiple comparisons, one-way ANOVA was followed by the Bonferroni test when variances were homogeneous or by the Tamhane test when variances were not homogeneous (only ROS for CGA). $P < 0.05$ was considered to be significant. The SPSS version 12.0 program was used.

RESULTS

Cell Viability and Proliferation. To determine the potential effects on cell viability of EC and CGA in a human hepatoma cell line (HepG2), cells were exposed to a range of concentrations (0−100 μM) of phenolic compounds for 18 h, because very slight changes related to cell viability and apoptosis had

Table 1. Effects of EC and CGA on Cell Viability and Proliferation[a]

	cell viability (% of viable cells)		
	Trypan Blue	Crystal Violet	cell proliferation (% of controls)
EC (μM)			
C	96.9 ± 1.1 a	100.0 ± 23.7 a	100.0 ± 7.7 a
1	96.3 ± 0.3 ab	98.2 ± 19.9 a	98.4 ± 8.5 a
10	94.6 ± 0.6 b	90.8 ± 16.6 a	104.5 ± 14.6 ab
25	91.2 ± 1.3 cd	93.4 ± 20.9 a	109.8 ± 9.3 abc
50	91.0 ± 1.2 c	126.5 ± 19.9 b	126.5 ± 17.6 cd
75	89.1 ± 1.2 c	123.9 ± 19.7 b	117.7 ± 14.3 bd
100	86.5 ± 2.2 d	117.1 ± 11.2 b	134.9 ± 7.9 d
CGA (μM)			
C	97.3 ± 0.7 a	100.0 ± 16.1 a	100.0 ± 3.3 a
1	97.4 ± 0.5 ab	97.42 ± 17.1a	92.4 ± 7.1 ab
10	96.6 ± 0.2 abc	95.3 ± 15.3 a	72.0 ± 7.2 c
25	96.9 ± 0.7 ab	96.8 ± 20.9 a	85.0 ± 9.9 bc
50	96.1 ± 0.3 bc	96.4 ± 11.8 a	89.5 ± 7.8 bc
75	95.1 ± 1.2 c	99.7 ± 15.5 a	81.6 ± 8.8 c
100	93.5 ± 1.0 d	93.8 ± 17.5 a	88.4 ± 6.6 bc

[a] Cell viability was determined either as the percentage of viable cells in the total number of cells counted by Trypan Blue assay or as relative percent of Crystal Violet stained control cells. Cell proliferation was calculated as percentage of the relative increase of BrdU incorporated into genomic DNA over the control values. Data represent the means (± SD) of 8–10 separate experiments. Means for each antioxidant within a column without a common letter differ, $P < 0.05$.

been previously reported at shorter incubation times (*18*). A slight reduction of cell viability (Trypan Blue assay) was observed only when cells were treated with the highest concentrations of EC and CGA (**Table 1**).

The study of cell viability analyzing relative density of cell adherents (Crystal Violet) and cell proliferation measuring BrdU incorporation into genomic DNA showed a slight arrest of HepG2 cell growth when cells were treated for 18 h with 10−100 μM CGA (**Table 1**); however, concentrations over 25 μM EC caused a significant increase of HepG2 cell growth (17−35%).

Intracellular ROS and GSH Levels. To test whether different concentrations of both phenolic compounds had an effect on the generation of oxygen radicals in HepG2 in culture, ROS production was assayed. Levels of intracellular ROS decreased after 18 h in the presence of either EC or CGA when compared to control cells (**Table 2**). Leakage of probe was not observed in cells throughout the assay, as determined in our laboratory in previous tests during method setup (*15*). Thus, any potential contribution of extracellularly oxidized DCF to the final fluorescence can be ruled out.

Additionally, the concentration of GSH was analyzed as an index of the intracellular nonenzymatic antioxidant defenses. After 18 h of treatment, 25−100 μM CGA evoked an increase in the intracellular concentration of GSH, whereas EC did not induce any change in the GSH levels at any of the concentrations assayed (**Table 2**).

Apoptosis Pathway. The effect of EC and CGA on the enzymatic activity of caspase-3, which is a crucial effector in the terminal or execution phase of the apoptotic pathway, was evaluated by using a fluorogenic peptide substrate. The proteolytic caspase-3 activity remained unchanged after 18 h of incubation with all EC and CGA concentrations (**Table 3**). Moreover, the cleaved form of caspase-3 could not be detected by Western blot (data not shown). These results, together the slight effect on cell viability or even the induction of cell proliferation reported above, suggest that 18 h of treatment with

Table 2. Effect of EC and CGA on Intracellular Reactive Oxygen Species (ROS) Generation and Intracellular Concentration of Reduced Glutathione[a]

	ROS (fluorescence units)	GSH (ng/mg of protein)
EC (μM)		
C	3843 ± 97 a	33.8 ± 2.4 a
1	3809 ± 231 a	33.9 ± 3.6 a
10	3654 ± 91 a	30.2 ± 6.8 a
25	3222 ± 43 b	27.9 ± 6.9 a
50	3067 ± 330 b	34.7 ± 3.0 a
75	2950 ± 122 bc	35.5 ± 7.4 a
100	2655 ± 82 c	34.8 ± 6.9 a
CGA (μM)		
C	3817 ± 112 a	34.3 ± 2.4 ab
1	3530 ± 327 ab	30.4 ± 2.3 a
10	3184 ± 106 b	36.7 ± 3.1 bc
25	3135 ± 189 b	39.7 ± 3.4 cd
50	2793 ± 403 b	41.0 ± 2.1 d
75	1097 ± 53 c	38.5 ± 3.0 cd
100	1056 ± 34 c	39.1 ± 3.3 cd

[a] Data represent the means (± SD) of 6–10 separate experiments. Means for each antioxidant within a column without a common letter differ, $P < 0.05$.

Table 3. Effects of EC and CGA on Caspase-3 Activity[a]

	caspase-3 activity (units/mg of protein)	
	EC	CGA
C	25.8 ± 3.7 a	29.4 ± 0.5 a
1 μM	24.9 ± 2.1 a	26.3 ± 2.4 a
10 μM	25.0 ± 2.3 a	26.6 ± 1.6 a
25 μM	25.3 ± 2.2 a	26.7 ± 0.5 a
50 μM	26.7 ± 2.1 a	28.1 ± 2.7 a
75 μM	26.8 ± 1.5 a	26.0 ± 1.8 a
100 μM	26.3 ± 2.4 a	30.0 ± 3.8 a

[a] Data represent means ± SD of 6–10 separate experiments. Means for each antioxidant without a common letter differ, $P < 0.05$.

both phenolic compounds has neither a prominent cytotoxic effect nor a pro-apoptotic action, which agrees with our previous results (*18*).

Antiapoptotic and Proapoptotic Bcl-2 Family Members. An imbalance in the expression of anti- and proapoptotic proteins is one of the major mechanisms underlying the ultimate fate of cells in the survival/apoptotic process. It is known that different members of the *bcl-2* gene family regulate survival or death in mammalian cells (*19*). Therefore, the possible modulation of expression of anti- or proapoptotic members of this protein family (Bcl-x and Bax, respectively) by EC and CGA was examined.

Bcl-x_L has been described to be a caspase substrate, and the product of Bcl-x_L cleavage, Bcl-x_S, has a proapoptotic function. This proteolytic fragment (Bcl-x_S) was not detected when the cells were incubated either with EC or with CGA for 18 h. Bcl-x_L levels remained unchanged with all tested concentrations of both phenolic compounds after 18 h of exposure (**Figure 1**).

Bax is a pro-apoptotic *bcl-2* family member, and it has been shown to oligomerize to form pores in the mitochondria and mediate the apoptotic death (*19*). After treatment either with EC or with CGA for 18 h, Bax remained unchanged at all concentrations studied (**Figure 1**). These results indicate that the expression levels of Bcl-x_L/Bax might not be involved in the modulation of the potential proliferative/survival effect induced by EC or CGA in HepG2 cells.

AKT Phosphorylation. AKT phosphorylation is associated with the activation of this kinase, which prevents cells from apoptosis and induces cell survival and proliferation (*20*). To

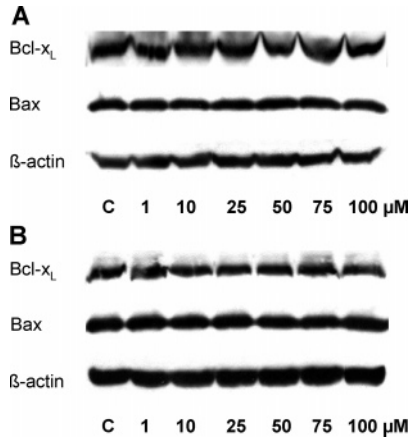

Figure 1. Effects of (**A**) EC and (**B**) CGA on the levels of Bcl-x_L and Bax in HepG2 cells. The same blots were reprobed with β-actin.

analyze whether AKT phosphorylation was affected by EC or CGA, total and phosphorylated levels of this protein were determined. EC treatment did not change total AKT protein expression, whereas phosphorylated AKT levels were differently modified depending on the concentration (**Figure 2A**). Thus, 10 μM EC induced a sharply increased phosphorylation of AKT, whereas higher concentrations (50−100 μM) evoked a decrease in AKT phosphorylation. On the other hand, no enhanced AKT phosphorylation was detected after 18 h of treatment with CGA (**Figure 2B**).

PI-3-Kinase Protein Levels. The PI-3-kinase pathway is regulated by a variety of growth factors, and the activation of the PI-3-kinase/AKT signaling pathway is strongly implicated in the regulation of survival and/or protection of cells (*20*). Thus, the effect of the two studied phenols on the levels of the p85 regulatory subunit and the p110 catalytic subunit of PI-3-kinase was examined. Expression of the p85 subunit remained unchanged after EC treatment, whereas levels of the p110 subunit were significantly increased in the presence of this flavonoid (**Figure 3A**). On the contrary, **Figure 3B** shows that the protein levels of both subunits were not altered following CGA treatment.

ERK Phosphorylation. The ERK pathway is linked to cellular death/survival signaling, and it could be activated in response to certain situations of cellular stress (*21*). Therefore, it was of relevance to study whether the effect induced by the selected phenols on HepG2 cells was related to ERKs. To this end, total and phosphorylated (active form) protein expressions of ERK1/2 were analyzed. Treatment of HepG2 cells for 18 h with EC resulted in an enhanced phosphorylation of ERK1/2 at 1−75 μM and no changes at 100 μM (**Figure 4A**). As in the case of AKT, the highest level of activation (phosphorylation) of ERK1/2 was elicited by 10 μM EC. On the other hand, CGA did not affect the levels of the two bands corresponding to ERK1 (44 kDa) and ERK2 (42 kDa) at any of the tested concentrations (**Figure 4B**). Total ERK1/2 protein levels did not change either during the treatment with EC or during CGA exposure, similarly to what was observed for total AKT protein expression.

AKT/PI-3-Kinase and ERK Activation on the Survival/Proliferative Effect. To address the role of AKT/PI-3-kinase and ERK activation in EC-induced survival/proliferation, the effect of a specific blockade of PI-3-kinase and ERKs using selective inhibitors was determined, and the selected concentration was the dose that showed the highest effect on the activation of both proteins, 10 μM. Treatment of HepG2 cells with LY294002 followed by the addition of EC (10 μM) markedly reduced cell viability and inhibited the EC-induced AKT activity because AKT phosphorylation decreased (**Figure 5A**,**B**). On the other hand, treatment with the ERK selective inhibitor PD98059 significantly reduced ERK phosphorylated levels, but it did not alter cell viability (**Figure 5A**,**C**). Combined treatment with PI-3-kinase and ERK inhibitors highly reduced cell viability, similarly to what was observed for the specific inhibitor of AKT alone (**Figure 5A**).

DISCUSSION

Polyphenols have been shown to be useful as antitumor, antithrombogenic, antinflammatory, and antioxidant agents (*1*, *2*). Their utilization as antitumor agents has turned out to be relevant because phenolic compounds seem to possess discriminating effects in normal and cancer cells (*22*). Differences in cellular oxidative stress, antioxidant metabolism, cell cycle regulation, induction and/or suppression of apoptosis, and cell signaling are considered to be involved in such selective features of polyphenols (*13*). Nevertheless, how dietary phenols protect cells and tissues from oxidative damage and their potential antiapoptotic or prosurvival actions are not yet defined.

In the present work we have shown the differential effects of EC and CGA on cell viability, cell proliferation, cellular redox state (ROS production and GSH levels), pro- and antiapoptotic members of the programmed cell death pathway, and key elements of cell survival/proliferation signaling cascades in HepG2 cells. These studies were carried out at a single incubation time point of 18 h with the phenols. Nevertheless, it should be mentioned that potential variations of the observed effects could take place in a time course manner during the activation of the pathways (research currently in progress) (*23*). However, previous studies with these polyphenols showed only minor changes related to cell viability and apoptosis at shorter incubation times (*18*).

The lack of or irrelevant cytotoxic effects of EC and CGA on HepG2 are demonstrated by a slight decrease in cell viability, as previously reported for both dietary phenolic compounds in other cell cultures such as human prostatic (*9*), oral (*11*), and colon (*10*) cancer cells and in mouse epidermal cells (*24*). Moreover, it has been described that the cytotoxic effect of EC was more pronounced in cancer human oral cavity cells than in their normal counterparts (*22*).

ROS are highly reactive metabolites generated during normal cell metabolism and could act as signaling factors. There is growing evidence that elevated intracellular ROS could be sufficient to trigger apoptosis, may cause cell death via MAPKs in certain oxidative conditions (*25*), and may be directly related to the depletion of total GSH, a key cellular defense mechanism against oxidative injury that correlates with cell survival. Our study shows a decrease of ROS production by EC and CGA and the induction of the antioxidant defense system by CGA, namely, increased GSH levels. There is evidence that both dietary phenols possess a high antioxidant activity and enhance cellular antioxidant defenses. In this regard, it has been shown that EC protects cells from oxidative insults by preventing hydroxyl radical formation in the presence of copper (*6*) and plays a role in modulating oxidative stress in lead-exposed HepG2 cells (*5*), showing a hepatoprotective activity (*26*). The observed beneficial effects of EC may also reflect its ability to modulate the cellular antioxidant defenses (*9*), but not by increasing GSH levels or inducing glutathione-*S*-transferase (GST) activity (*27*). Concerning CGA, it has been reported that this phenolic acid prevented cellular damage induced by different oxidative stressors (*7*). Additionally, CGA induced the

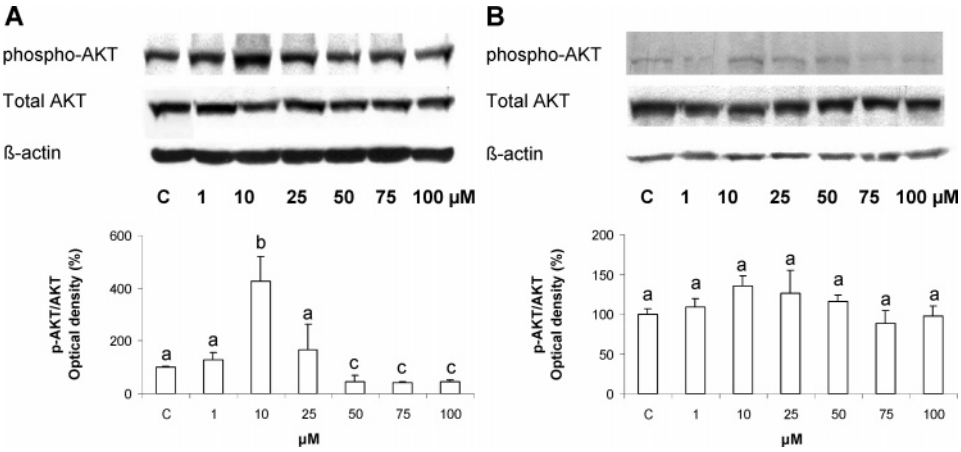

Figure 2. Effects of (**A**) EC and (**B**) CGA on the basal levels of phosphorylated AKT (Ser473) and total AKT. Values (means ± SD, $n = 6$) represent the relative percent increase of the p-AKT/AKT ratio over the control condition. Normalization of Western blots was ensured by β-actin. Means for each antioxidant without a common letter differ, $P < 0.05$.

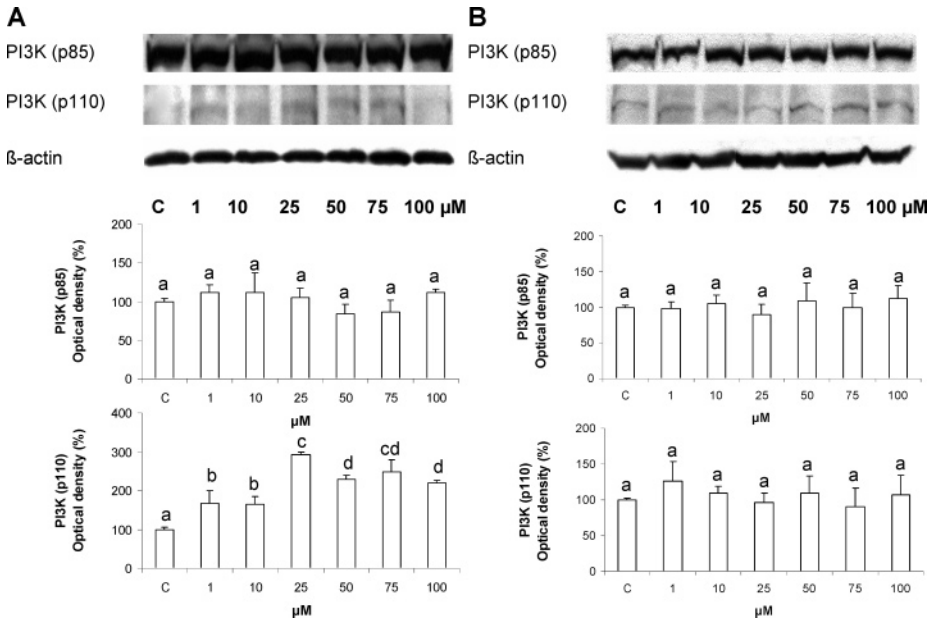

Figure 3. Effects of (**A**) EC and (**B**) CGA on the basal levels of p85 and p110 subunits of PI-3-kinase. Values (means ± SD, $n = 5$) show the relative increase of each PI-3-kinase subunit (p85 and p110) as a percent over the control condition. Normalization of Western blots was ensured by β-actin. Means for each antioxidant without a common letter differ, $P < 0.05$.

activity of the phase II detoxifying enzymes GST and NADPH quinone oxidoreductase (*24*), as well as prevented GSH depletion and ROS formation after an oxidative injury caused by different pro-oxidants (*7*).

Antioxidant effects of both dietary phenolic compounds could attenuate ROS-dependent death signaling cascades and determine the outcome in terms of cell survival/proliferation. In this regard, neither EC nor CGA activated caspase-3, a key effector in apoptotic signaling pathways, which agrees with our previous results (*18*). Although just a few studies have evaluated the outcome of EC treatment alone, to our knowledge the effect of the flavanol on caspase processing has never been reported previously, and only a few studies have demonstrated the lack of apoptotic effect for this catechin (*9*); moreover, Spencer and co-workers (*28*) have described the inhibition of H_2O_2-induced cell death through suppression of caspase-3 activity by EC and its metabolite 3′-*O*-methyl EC. As for CGA, few references have been found in the literature concerning its effects in cell culture, although its chemopreventive role and its limited effect in modulating cell viability and apoptosis have been demonstrated (*10*, *29*). On the other hand, an activation of apoptosis by EC has been reported at very high doses compared to the ones used in this study as a result of cell cycle arrest in colon cancer cells (*8*) or growth suppression in human prostate cancer (*9*); similarly, CGA induced programmed cell death by induction of oxidative stress (*11*, *29*), by arresting cell cycle in murine preadipocytes (*12*), and by caspase activation at millimolar concentrations in human squamous carcinoma cells (*11*).

It is well-known that the Bcl-2 protein family includes pro- and antiapoptotic members, and the ratio between both types of proteins determines in part the susceptibility of cells to a death or survival/proliferation signal (*17*, *19*). In this study, the expression levels of the proapoptotic Bax and the antiapoptotic Bcl-x_L remained unchanged after treatment with CGA or EC; moreover, the proapoptotic Bcl-x_S resulting from Bcl-x cleavage as an indication of apoptotic cell death was not detected. Similarly, unchanged levels of Bcl-2, Bcl-x_L, and Bax have been described in a study carried out with epigallocatechin-3-gallate (EGCG) in prostatic cancer cells (*9*). These results suggest that

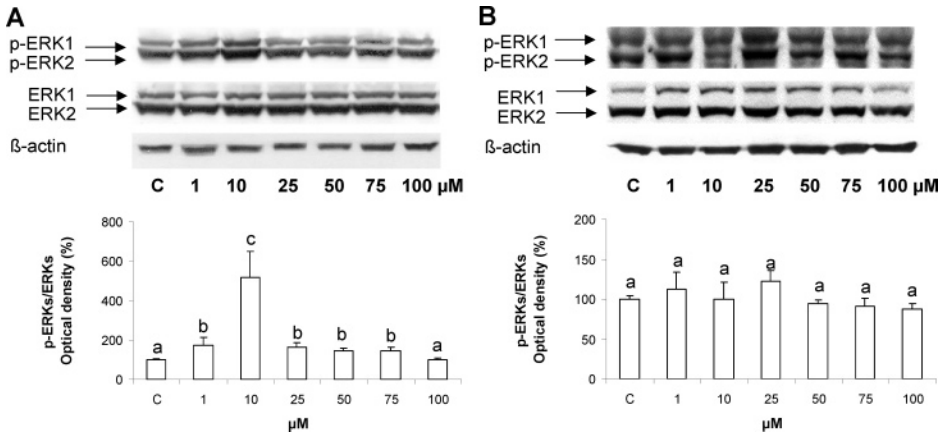

Figure 4. Effects of (**A**) EC and (**B**) CGA on the basal levels of phosphorylated ERK1/2 (Thr202/Tyr204) and total ERK1/2 in HepG2. Data represent means ± SD ($n = 6$) expressed as the relative increase in percentage of the p-ERK/ERK ratio over controls. Normalization of Western blots was ensured by β-actin. Means for each antioxidant without a common letter differ, $P < 0.05$.

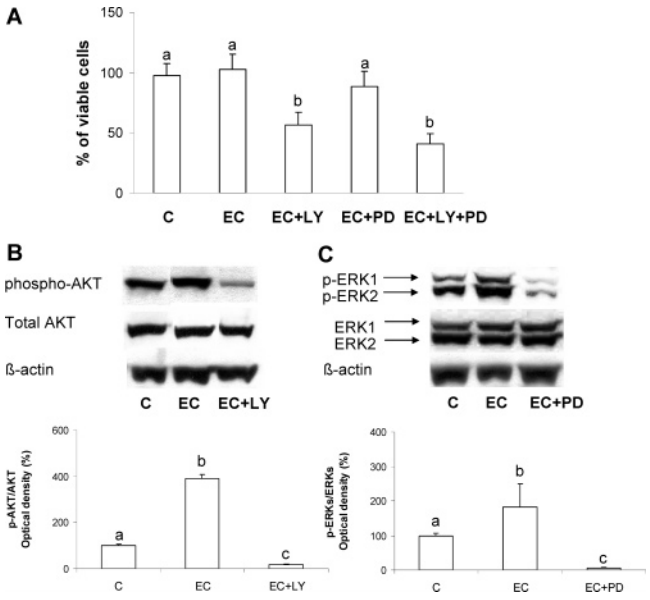

Figure 5. Effects of EC and selective inhibitors LY294002 (LY) and PD98059 (PD) on cell viability (**A**) and on the AKT/PI-3-kinase pathway (**B**) and ERKs (**C**). HepG2 cells were incubated with or without 10 μM EC for 18 h in the presence or absence of 50 μM LY and/or 50 μM PD. Cell viability was determined by Crystal Violet and expressed as relative percentage of control cells staining. Data represent means ± SD of 10 separate experiments. Cell lysates were analyzed with antibodies to phosphorylated AKT (Ser473), total AKT, phosphorylated ERK1/2 (Thr202/Tyr204), and total ERK1/2. Values (means ± SD, $n = 6$) represent the relative percent increase of the phosphorylated protein/total protein ratio over the control condition. The same blots were reprobed with β-actin. Different letters denote stastically significant differences, $P < 0.05$.

Bcl-2 proteins might not be directly involved in the molecular signaling pathways activated by the studied dietary phenols.

AKT is a protein kinase that promotes cell survival by preventing apoptosis (*20*, *21*). In our study, CGA did not affect AKT levels, but 10 μM EC induced activation of AKT by increasing the level of phosphorylated active AKT, which might promote cell proliferation/survival as discussed above (*20*, *21*). Similarly, EGCG has been demonstrated to cause phosphorylation and activation of AKT, which prevented the induced apoptosis and suggested AKT as a prime mediator of the cytoprotective effect of the flavonoid (*30*).

CGA did not affect PI-3-kinase protein levels (neither p85 nor p110 levels), in line with previous in vitro studies reporting that this compound exclusively inhibited inositol polyphosphate multikinase but not PI-3-kinase (*31*). However, induction of p110 expression observed at all concentrations of EC assayed has not been previously reported in other studies. AKT is a downstream target of PI-3-kinase; therefore, the increased p110 subunit levels might lead to membrane translocation of AKT evoking its observed activation and, thus, increasing cell survival/proliferation and protection against apoptosis. In this regard, other authors have reported an enhanced PI-3-kinase activity for this compound and other catechins (*30*, *32*). However, it should be mentioned that the increased levels of p110 might not be correlated with an increased PI-3-kinase activity.

As in the case of AKT/PKB signaling, ERK1/2 is reported to be required for cell survival (*20*, *21*). In many cells the survival-signaling cascade involves PI-3-kinase, AKT, and also the cross-talk between PI-3-kinase and ERKs (*21*). As previously reported by other authors (*21*), our study shows that a sustained activation of ERK1/2 is essential for HepG2 survival and proliferation as can be observed in control cells. In addition, an enhancement of ERK1/2 phosphorylation by EC together with an increased AKT phosphorylation and BrdU incorporation supports the role of this flavanol on the activation of the cell survival/proliferation pathway. Moreover, selective blockade of the PI-3-kinase pathway by a pharmacological inhibitor prevented activation of this kinase and inhibited EC-induced survival, whereas inhibition of ERKs prevented their phosphorylation without affecting cellular viability. In this regard, HepG2 cellular survival seemed to depend on the induction of the PI-3-kinase/AKT pathway. Taken together, it could be suggested that the activation of AKT/PI-3-kinase and ERK signaling cascades could independently contribute to the effect of EC on cell survival pathways as previously reported in hepatoma cells (*23*). Similarly, Li and co-workers (*34*) have demonstrated that a green tea catechin exerted EGFR downstream events [stimulation of ERKs, PI3K, AKT, mTOR, and p70(S6K)] and suppressed ROS generation without affecting cell viability. Additionally, when cells were treated with NAC (10 mM) alone, p38 and JNK1 activations were eliminated, because they are ROS dependent, but ERK1/2 activation was not, indicating that this activation is ROS independent and that the MAPK signaling pathway plays an important role in EGCG-mediated cytoprotective effects. Furthermore, the lack of parallelism between cell proliferation and AKT and ERK activation at high concentra-

tions of EC might be indicative of the involvement of other signals related to cell cycle and/or cell survival such as nuclear factor kappa B (NFκB) (*33*). Accordingly, EGCG, which induced apoptosis in cancer but not in normal cells, has been demonstrated to affect the important survival factor NFκB by inhibition in epidermal cancer cells and activation in its normal counterparts (*35*). In this regard, it has also been observed that CGA suppressed cell proliferation, up-regulated cellular antioxidant enzymes, and suppressed ROS generation mediated by NFκB (*24*).

In summary, our studies provide new insights into the relative contributions of ROS, GSH, caspases, Bcl-2 proteins, AKT/PI-3-kinase, and ERK1/2 on the effects of EC and CGA on cell survival/proliferation. EC and CGA appeared to have no effect on apoptotic death cascades as seen by the lack of outcome on caspase-3 activation. Most likely these phenols might act through limiting apoptosis related to oxidative stress by a reduced ROS production and, in the case of CGA, also by an increase in cellular antioxidant potential (GSH). Thus, it may be concluded that EC plays a role in cell survival partially mediated by induction of AKT/PI-3-kinase and ERK1/2 pathways at low concentrations, whereas other mechanisms might be involved at higher doses. Consequently, interventions with these antioxidants could be promising in the design and development of new treatments for limiting cellular oxidative damage.

ABBREVIATIONS USED

AKT/PKB, protein kinase B; BrdU, 5-bromo-2′-deoxyuridine; CGA, chlorogenic acid; EC, (−)-epicatechin; EGCG, epigallocatechin-3-gallate; EGFR, epidermal growth factor receptor; ERK, extracellular regulated kinase; FBS, fetal bovine serum; GSH, reduced glutathione; GST, glutathione-*S*-transferase; MAPK, mitogen-activated protein kinase; mTOR, mammalian target of rapamycin; NAC, *N*-acetyl-L-cysteine; NFκB, nuclear factor kappa B; OPT, *o*-phthalaldehyde; PI-3-kinase, phosphatidylinositol-3-kinase; ROS, reactive oxygen species; SDS, sodium dodecyl sulfate.

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