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Journal of Nutritional Biochemistry 20 (2009) 115 – 124
Journal of Nutritional Biochemistry

Time-course regulation of survival pathways by epicatechin on HepG2 cells☆

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Received 1 June 2007; received in revised form 12 November 2007; accepted 10 December 2007

Abstract

Polyphenols, such as epicatechin, have been reported to exhibit a wide range of biological activities. The objective of the present study was to investigate the time-dependent regulation by epicatechin of survival/proliferation pathways in HepG2 cells. Treatment of HepG2 cells with 10 μmol/L epicatechin did not result in any cell damage up to 18 h, as evaluated by the lactate dehydrogenase assay. Moreover, the enhanced cell death evoked by an oxidative stress induced with *tert*-butyl hydroperoxide was prevented in the cells pretreated 4 or 18 h with epicatechin. Epicatechin-induced survival was a rapid event that was accompanied by early and sustained activation of major survival signaling proteins, such as AKT/phosphatidylinositol 3-kinase and extracellular-regulated kinase (activated from 5 min to 18 h), as well as protein kinase C (PKC)-α (30 min to 18 h), in concert with unaltered *c-jun* N-amino terminal kinase levels and early inactivation of key death-related signals like PKC-δ (5 min to 18 h). Additionally, reactive oxygen species generation was transiently reduced when cells were treated with 10 μmol/L epicatechin (15–240 min). These data suggest that epicatechin induces cellular survival through a tight regulation of survival/proliferation pathways that requires the integration of different signals and persists over time, the ultimate effect on HepG2 cells being regulated by the balance among these signals.

Keywords: Epicatechin; HepG2 cells; AKT/PI3K pathway; ERK; JNK; PKC

1. Introduction

Many natural dietary polyphenols are widely distributed in vegetables, fruits and beverages, and have been reported to possess different biological activities such as antioxidant, antiinflammatory, antithrombogenic, antiviral and anticarcinogenic [1,2]. Epicatechin is a flavonoid commonly found in the diet, particularly abundant in green tea and cocoa [3], which is extensively metabolized during absorption in the small intestine and in the liver [4]. It is well known that epicatechin possesses in vitro antioxidant activity, effectively scavenging free radicals and interfering with the oxidative/antioxidative potential of the cell [5,6]. Furthermore, it has been suggested that epicatechin might exert beneficial and/or cytotoxic actions by stimulating the antioxidant defense metabolism through the redox-regulated transcription factors and modulation of gene expression and signal cascades such as mitogen-activated protein kinases (MAPKs) [7–9], although the detailed molecular mechanisms of its action remain to be elucidated.

Regulation of cell viability by epicatechin alone through the analysis of cellular oxidative stress and signal transduction pathways related to cell death (caspases) and cell survival/proliferation [MAPKs, AKT/phosphatidylinositol (PI) 3-kinase (PI3K)] has been analyzed in a limited number of studies, since most reports using cell culture systems have been focused on the antioxidant effect of epicatechin after

Abbreviations: AKT/PKB, protein kinase B; EGCG, epigallocatechin-3-gallate; ERK, extracellular-regulated kinase; FBS, fetal bovine serum; JNK, *c-jun* N-amino-terminal kinase; MAPK, mitogen-activated protein kinase; PI, phosphatidylinositol; PI3K, phosphatidylinositol-3-kinase; PKC, protein kinase C; ROS, reactive oxygen species; SDS, sodium dodecyl sulfate; *t*-BOOH, *tert*-butyl hydroperoxide.

☆ This work was supported by the grants AGL2004-302 from the Spanish Ministry of Science and Technology (CICYT) and 200570M050 from the Comunidad de Madrid (CAM).

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doi:10.1016/j.jnutbio.2007.12.006

exposing cells to prooxidants [5,6,10]. Moreover, in certain circumstances, epicatechin has been considered as an inactive polyphenol in comparison to other green tea polyphenols, when its capacity to induce apoptosis was analyzed [11]. Thus, epicatechin protects cells from oxidative insults by modulating the cellular antioxidant defenses and reducing reactive oxygen species (ROS) production in the presence of stressors [5,6,10].

Reactive oxygen species are constantly generated during intracellular metabolism and in response to environmental stimuli. Many evidences indicate that ROS play a central role in different intracellular signal transduction pathways key for a variety of cellular processes. Aberrant ROS signaling may result in physiological and pathological changes, such as apoptosis induction [12]. However, we have recently shown that epicatechin prevents ROS formation in HepG2 in a dose-dependent manner [9]. Additionally, epicatechin does not induce apoptotic HepG2 cell death, nor evokes caspase-3 activation or modifies the levels of Bcl-2 anti- and proapoptotic proteins [9,13], while it has been reported to inhibit H_2O_2-induced cell death in fibroblasts by suppressing caspase-3 activity [14].

Signaling pathways mediated by PI3K/protein kinase B (AKT), MAPKs such as extracellular-regulated kinase (ERK) or *c-jun* N-amino terminal kinase (JNK) and protein kinase C (PKC) play a main role in cell survival/proliferation, cell growth and apoptosis [15–17]. Epicatechin dose-dependently induced activation of AKT [9] and PI3K [18,19], which led to an increased cell survival/proliferation and blockage of apoptosis [9]. Furthermore, both activation and inhibition of ERK and/or JNK have been shown to contribute to the induction of cell survival/proliferation [20–23]. Epicatechin also competed with the phorbol ester phorbol 12-myristate 13-acetate in binding to the regulatory domain of recombinant human PKC-α in a dose-dependent manner and showed a slight inhibition of the kinase activity of PKC [24]. Nevertheless, most of these studies have been carried out after long-term treatments with the flavonoid, but potential variations of the observed effects could take place in a time-course manner during the activation of these pathways; therefore, it is of great importance to characterize the role of epicatechin in the regulation of survival/proliferation routes of HepG2 cells and the mechanisms involved.

HepG2 cells, a human hepatoma cell line, are considered a good model to study in vitro hepatic xenobiotic metabolism and toxicity, since they maintain many of the specialized functions that characterize normal human hepatocytes [25]. A variety of studies evaluating the cytoprotective effect of natural antioxidants have been carried out in HepG2 cells [5,10,26]. Therefore, the aim of this study was to evaluate the underlying mechanisms of the survival effect of epicatechin on HepG2 cells in a time-course manner. Cell integrity, generation of ROS as a marker for redox status and potential protective effect of the dietary compound against an oxidative stress chemically induced by the potent prooxidant *tert*-butyl hydroperoxide (*t*-BOOH) were investigated, as well as its influence on related key prosurvival pathways, namely, AKT/PI3K, MAPKs (ERK and JNK) and PKC (isoforms α and δ). The data presented demonstrate that the survival effect of epicatechin is due to the merged induction of critical prosurvival pathways as an early event.

2. Materials and methods

2.1. Materials and chemicals

Epicatechin, wortmannin, gentamicin, penicillin G, streptomycin and *t*-BOOH were purchased from Sigma Chemical, Madrid, 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, anti-JNK1/2 and anti-phospho-JNK1/2 recognizing phosphorylated Thr183/Tyr185 of JNK1/2 and anti-β-actin were obtained from Cell Signalling Technology (9271, 9272, 9101, 9102, 9251, 9252 and 4697, respectively; Izasa, Madrid, Spain). Anti-p110β (catalytic PI3K subunit), anti-PKC-α and anti-PKC-δ were purchased from Santa Cruz Biotechnology, Santa Cruz, CA (sc-7175, sc-208 and sc-937, respectively). Anti-p85α/β (regulatory PI3K subunit) was from Upstate Biotechnology, Lake Placid, NY. Materials and chemicals for electrophoresis were from BioRad Laboratories, Madrid, Spain. Cell culture dishes were from Falcon (Cajal, Madrid, Spain), and fetal bovine serum (FBS) and cell culture medium Dubelcco's Modified Eagle's Medium (DMEM)-F12 were from Biowhittaker Europe (Innogenetics, Madrid, Spain).

2.2. Cell culture and epicatechin treatment

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. For the time-course experiments, cells were treated with 10 μmol/L of epicatechin and then harvested at different times (5, 10, 15, 30, 60, 120, 240 and 1080 min).

For evaluating the protective effect of epicatechin, cells were pretreated with the flavanol (10 μmol/L) for 4 or 18 h before the incubation with the prooxidant *t*-BOOH (200 μmol/L) for 4 h.

In the experiments with epicatechin and the selective pharmacological inhibitor of PI3K wortmannin, some cells were preincubated with wortmannin (200 nmol/L) for 2 h

prior to epicatechin treatment with 10 μmol/L for 15 min or 18 h.

2.3. Cytotoxicity assay (lactate dehydrogenase leakage assay)

Lactate dehydrogenase (LDH) leakage was carried out as previously described [26]. In brief, culture medium was collected separately, and the cells were scraped. Cell suspension (1.5×10^6 cells) was sonicated to ensure breaking down the cell membrane to release the total amount of LDH. A mixture of 5 mmol/L pyruvate, 0.35 mmol/L NADH and 84 mmol/L Tris was added to the sample and read at 340 nm in a microplate ELISA reader (Bio-Rad, Madrid, Spain). Lactate dehydrogenase leakage was estimated as the ratio between LDH activity in the culture medium and that of the whole cell content.

2.4. Determination of ROS

Cellular oxidative stress was quantified by the DCFH assay using a microplate reader [9,26]. After being oxidized by intracellular oxidants, DCFH becomes dichlorofluorescein (DCF) and emits fluorescence. Briefly, 5 μmol/L DCFH was added to the wells (2 x 10^5 cells per well) and incubated for 30 min at 37 °C. Then the cells were incubated with 10 μmol/L epicatechin for the corresponding time, and by quantifying fluorescence at an excitation wavelength of 485 nm and an emission wavelength of 530 nm, a fair estimation of the overall oxygen species generated over the time was obtained.

2.5. Cell viability assay

Cell viability was determined by using the crystal violet assay [27]. 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.

2.6. Preparation of cell lysates

Cells were lysed at 4 °C in a buffer containing 25 mmol/L HEPES (pH 7.5), 0.3 mol/L NaCl, 1.5 mmol/L $MgCl_2$, 0.2 mmol/L EDTA, 0.5 mmol/L dithiothreitol, 0.1% Triton X-100, 200 mmol/L β-glycerophosphate, 0.1 mmol/L Na_3VO_4, 2 μg/ml leupeptin and 1 mmol/L phenylmethylsulfonyl fluoride. The supernatants were collected, assayed for protein concentration by using the Bio-Rad protein assay kit according to the manufacture's specifications, aliquoted and stored at −80 °C until used for Western blot analyses.

2.7. Western blot analysis

Equal amounts of proteins (100 μg) were separated by SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride filters (Protein Sequencing Membrane, Bio-Rad). Membranes were probed with the corresponding primary antibody followed by incubation with peroxide-conjugated anti-rabbit immunoglobulin (GE Healthcare, Madrid, Spain). Blots were developed with the ECL system (GE Healthcare). Normalization of Western blot was ensured by β-actin, and bands were quantified using a scanner and accompanying software.

2.8. Statistics

Data were tested prior to statistical analysis for homogeneity of variances by the test of Levene. In experiments when only one factor was studied (i.e., time), data were evaluated using one-way analysis of variance (ANOVA) followed by the Bonferroni test when variances were homogeneous or by the Tamhane test when variances were not homogeneous (only ROS for control cells). $P<.05$ was considered significant.

For LDH and ROS values, data were analyzed using two-way ANOVA with treatment (control or epicatechin-treated cells) and time as the two factors tested. Significant Treatment × Time interactions were only found in ROS generation at four time points or variables (30–240 min, $P<.05$), and subgroups were analyzed further by testing the effect of time within each group using one-way ANOVA as described above. A SPSS version 12.0 program was used.

3. Results

3.1. Cell toxicity (LDH) and intracellular generation of ROS

To determine the potential time-dependent cytotoxic effect of epicatechin in HepG2 cells, we evaluated the intracellular LDH released to the culture medium. In agreement with our previous studies [9], epicatechin did not induce membrane damage over time as LDH leakage after incubation with the flavonoid was comparable to control cells at all tested times (Table 1).

Table 1
Time-course effect of epicatechin (10 μmol/L) on LDH leakage and on intracellular ROS generation

Time (min)	LDH leakage (% of LDH)		ROS (fluorescence units)	
	C	Epicatechin	C	Epicatechin
0	2.73 ± 0.68[a]	2.73 ± 0.68[a]	1397 ± 186[a]	1473 ± 110[a]
15	2.70 ± 0.44[a]	2.02 ± 0.79[a]	2429 ± 438[b]	2235 ± 42[b]
30	2.21 ± 0.85[a]	2.72 ± 0.39[a]	4080 ± 1012[c]	2648 ± 39*[b]
60	2.31 ± 0.15[a]	3.12 ± 0.77[a]	4041 ± 347[c]	3394 ± 28*[c]
120	2.59 ± 0.34[a]	3.42 ± 0.59[a]	5751 ± 551[d]	4666 ± 12*[d]
240	2.08 ± 0.56[a]	3.04 ± 0.39[a]	16 666 ± 1921[e]	6550 ± 81*[e]
1080	3.48 ± 0.87[a]	4.28 ± 0.76[a]	24 506 ± 3216[f]	23 299 ± 581[f]

Data represent the means ± S.D. of six to eight different samples per condition. There was Time × Treatment (control or epicatechin-treated cells) interaction only in ROS generation for four time points (30–240 min), and subgroups were analyzed further by testing the effect of treatment between groups using one-way ANOVA, $P<.05$, denoted with an asterisk. Means in a column without a common letter differ, $P<.05$.

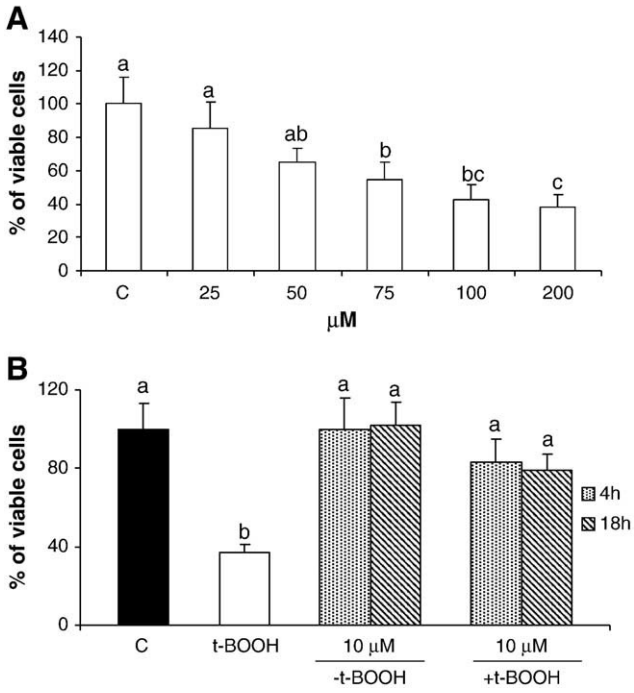

Fig. 1. Effect of epicatechin (10 μmol/L) on cell viability. (A). *t*-BOOH-induced cell death. HepG2 cells were incubated with the noted concentrations of *t*-BOOH for 4 h. (B) Protective effect of epicatechin against *t*-BOOH. HepG2 cells were pretreated with 10 μmol/L epicatechin for 4 and 18 h, then cultures (except untreated cells and cells treated with epicatechin alone for 4 and 18 h) were exposed to 200 μmol/L *t*-BOOH for 4 h. Cell viability was determined by crystal violet and expressed as relative percentage of control (untreated) cells staining. Data represent means ± S.D. of eight samples per condition. Different letters denote statistically significant differences, $P<.05$.

To test whether epicatechin affected generation of oxygen radicals in HepG2 cells, ROS production was determined. ROS generation by epicatechin-treated cells at 18 h was similar to that of control cells, but those treated with the flavonoid showed much lower ROS values than controls up to 4 h (Table 1). Interestingly, levels of intracellular ROS were significantly decreased in the presence of epicatechin at 15 min of treatment with the flavanol. Leakage of probe was not observed in cells throughout the assay, as determined in our laboratory in previous tests during method setup [9,26]. Thus, any potential contribution of extracellularly oxidized DCF to the final fluorescence can be ruled out.

3.2. Protective effect of epicatechin against *t*-BOOH-induced cell death

Since the flavanol did not show a cytotoxic effect at any tested time but might induce cell survival/proliferation, the possible protective effect of epicatechin against *t*-BOOH-induced cell death was evaluated in HepG2 cells. To select the concentration of *t*-BOOH that was able to induce cell death in HepG2 cells, we evaluated the cytotoxicity of *t*-BOOH with different doses after 4 h of incubation. Increasing concentrations of *t*-BOOH induced a dose-dependent decrease in cell viability (Fig. 1A). Concentration of 200 μmol/L *t*-BOOH was chosen to test the potential protective effect of epicatechin against the prooxidant. Cell viability was markedly reduced by a 4-h treatment with *t*-BOOH (200 μmol/L), whereas cell pretreatment for 4 or 18 h with epicatechin (10 μmol/L) before being submitted to *t*-BOOH protects cells against the prooxidant cytotoxicity (Fig. 1B).

3.3. AKT phosphorylation

AKT phosphorylation activates this kinase, which protects cells from apoptosis and induces cell survival and proliferation [16]. To test whether the epicatechin-induced effect on cellular survival/proliferation routes is related to AKT phosphorylation, we treated HepG2 cells with a fixed concentration of flavonoid (10 μmol/L) at different times, and total and phosphorylated levels of the protein were analyzed. Total AKT protein levels did not change during the incubation with epicatechin, whereas the p-AKT/AKT ratio was altered in a time-dependent manner (Fig. 2). AKT activation through phosphorylation was evident as early as after 5 min of incubation, and it was maintained up to 18 h of treatment (Fig. 2).

3.4. PI3K protein levels

PI3K/AKT signaling transduction pathway modulates cellular survival/proliferation, is regulated by several growth factors and plays a protective role in induced apoptosis

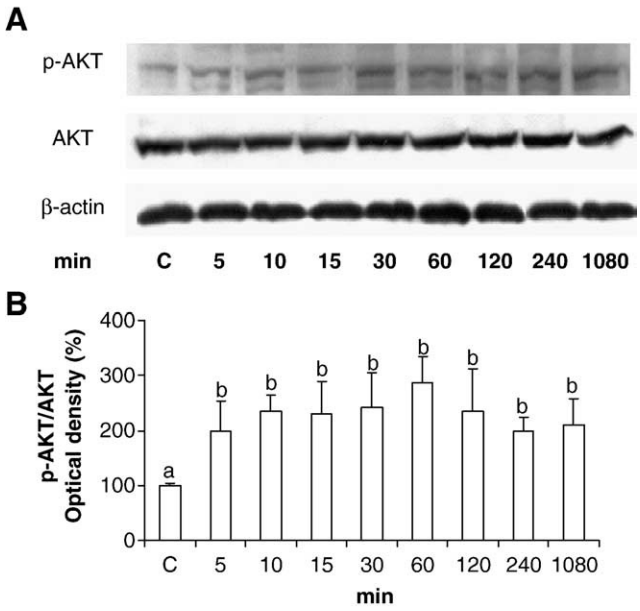

Fig. 2. Time-dependent effect of epicatechin on basal levels of phosphorylated AKT (Ser473) and total AKT. (A) Bands of a representative experiment. (B) Values (means ± S.D., $n=6$) represent the relative percentage increase of the p-AKT/AKT ratio over the control condition. Normalization of Western blots was ensured by β-actin. Different letters denote statistically significant differences, $P<.05$.

[15,16,28]. Therefore, the effects of epicatechin on levels of both PI3K subunits, p85 regulatory subunit and p110 catalytic subunit, were examined. As shown in Fig. 3, the expression of p85 subunit was not altered by the flavonoid treatment, and p110 subunit expression levels were only increased at the longest incubation time (18 h).

3.5. AKT/PI3K activation on the survival/proliferative effect

To address whether the increased AKT phosphorylation induced by epicatechin was mediated by an enhanced PI3K activity, the effect of specific blockade of PI3K using a selective inhibitor was determined. Treatment of HepG2 cells with wortmannin followed by addition of epicatechin (10 μmol/L) markedly inhibited the epicatechin-induced AKT activity, since the phosphorylated protein levels decreased at both short and long incubation times (15 and 1080 min, respectively) (Fig. 4). Epicatechin-induced AKT activation was inhibited by wortmannin as early as after 15 min of incubation, leading to AKT phosphorylation levels similar to those of control cells (Fig. 4A and B). In addition, these reduced AKT phosphorylated levels induced by the selective blockade of the PI3K pathway were also observed after 18 h of treatment, showing lower levels than controls at the longest incubation time (Fig. 4A and C).

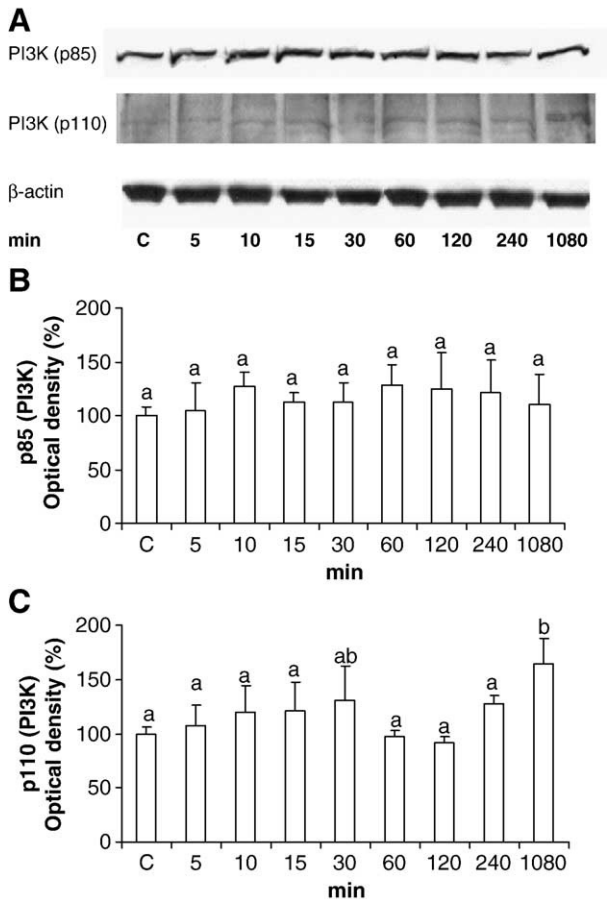

Fig. 3. Time-dependent effects of epicatechin on basal levels of p85 and p110 subunits of PI3K. (A) Representative blots of both PI3K subunits. Values of (B) p85 and (C) p110 PI3K subunits are shown as a percentage relative to the control condition (means ± S.D., n=5). Normalization of Western blots was ensured by β-actin. Means without a common letter differ, P<.05.

3.6. Extracellular-regulated kinase phosphorylation

Extracellular-regulated kinase signaling pathway is implicated in cellular death/survival signaling and is activated in response to certain situations of cellular stress [15,28]. Therefore, it was important to study the effect induced by epicatechin on HepG2 cells. Similarly to AKT regulation, total ERK1/2 protein levels did not change during the treatment with epicatechin (Fig. 5A and B). However, epicatechin incubation resulted in a time-dependent activation of ERKs, evoking an increase of ERK phosphorylation as early as after 5 min of incubation. Active protein levels, continued to increase after 15 to 60 min of treatment, decreasing at longer incubation times (120–1080 min) to values similar to those observed after 10 min of treatment (Fig. 5A and B). Thus, although not uniformly, epicatechin-induced ERK phosphorylation was sustained over time.

3.7. c-jun amino-terminal kinase phosphorylation

c-jun N-amino-terminal kinase activation is connected to cell death, although in certain circumstances inhibition of the protein has also been reported in apoptosis [22]. Thus, it was relevant to analyze whether epicatechin-induced effects on survival/proliferation pathways were related to JNKs. Total and phosphorylated JNK1/2 protein levels were investigated. Treatment of HepG2 cells with epicatechin did not alter the total levels of the two bands corresponding to JNK1 and JNK2 (Fig. 5A and C), similarly to what was observed for total AKT and ERKs protein expressions. Correspondingly, phosphorylated JNK1/2 protein expression levels were not affected during epicatechin exposure at any tested times.

3.8. PKC-α and PKC-δ expression levels

Individual PKC isoforms are important signal transducers in a number of cellular responses, including mitogenesis, differentiation and apoptosis [17,24]. To address the role of PKC-α and PKC-δ in epicatechin-induced effect on survival/proliferation pathways, we analyzed their expression levels in the time-course study. Treatment of HepG2 cells with epicatechin enhanced PKC-α levels after 30 min, and protein levels continued to increase up to 18 h (Fig. 6A and B). Epicatechin treatment also significantly raised cytosolic PKC-δ levels after 5 min of incubation, which remained increased up to 18 h (Fig. 6A and C).

4. Discussion

Polyphenols have been shown to possess antitumoral, antiinflammatory, antithrombogenic and antioxidant activities [1,2]. More recently, much attention has been focused

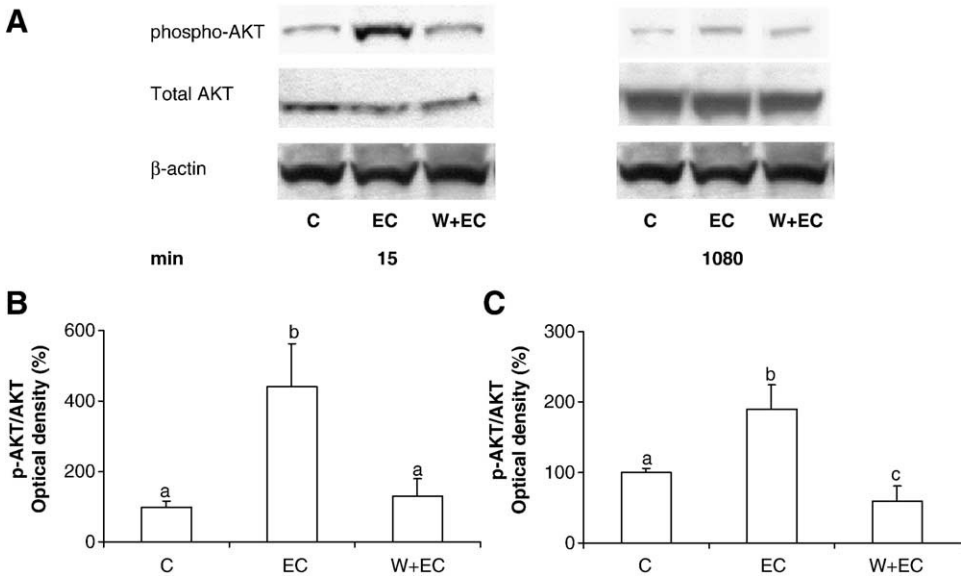

Fig. 4. Time-dependent effect of epicatechin (EC) and wortmannin (W) plus EC on the AKT/PI3K pathway. HepG2 cells were incubated with 10 μmol/L EC for either 15 min or 18 h in the presence or absence of 200 nmol/L W. Cell lysates were analyzed with antibodies to phosphorylated AKT (Ser473) and total AKT. (A) Bands of a representative experiment. Values (means ± S.D., n=6) represent the relative percentage increase of the p-AKT/AKT ratio over the control condition after (B) 15 min or (C) 1080 min of incubation with epicatechin. The same blots were reprobed with β-actin. Different letters denote statistically significant differences, P<.05.

on analyzing these biological properties, as polyphenolic compounds seem to act on cellular oxidative stress, antioxidant metabolism, cell cycle regulation, induction and/or suppression of apoptosis and cell signaling [7,8]. However, the molecular mechanisms by which dietary polyphenols and, in particular, epicatechin exert their biological actions as antiapoptotic and/or prosurvival agents in HepG2 cells have not been fully explained. We have recently reported that after 18 h of treatment with 10 μmol/L epicatechin, the induced prosurvival effect elicited by this flavonoid is enhanced by the activation of AKT/PI3K and ERK1/2 pathways. At this concentration, epicatechin did not induce cell proliferation and cytotoxicity, or had any effect on the cellular redox status, apoptotic death cascades or expression levels of different Bcl-2 protein family members [9,13].

Potential variations of the observed effects could occur on signaling transduction pathways in a time-dependent manner. An initial and fast effect (activation or inactivation) of a particular pathway could often be enough to regulate the activity of different proteins, which makes long-term treatments not always appropriate to determine the potential regulation of these routes [28]; however, in some cases, the modulatory effect on a particular protein could be sustained [28]. In the present work, we studied the molecular mechanisms underlying the biological effects of epicatechin by focusing on its time-course regulation of cell survival/proliferation pathways. We show that epicatechin is an effective activator of PI3K/AKT, ERK and PKC pathways, which are critical for the epicatechin-induced effect on cell proliferation/survival routes.

Epicatechin has been shown to induce minor alterations on cell viability and apoptosis at short incubation times [13], and neither cytotoxic nor proliferative effects of the flavonoid have been observed even after longer treatments (18 h) with an epicatechin concentration of 10 μmol/L in HepG2 cells [9]. In agreement with these findings, slight effects of this flavonoid on cell viability in human hepatic [29], prostatic [11], colonic [30,31], T lymphocytic [32] and oral cavity [33] cells have also been reported. Importantly, although this flavonoid is the least toxic when compared to other polyphenols in tea, its cytotoxic effects are more pronounced in cancer cells than in the normal counterparts [33], and it has also been shown that the response of the cancer cells to a catechin treatment can also depend on the length of the exposure [34].

ROS are highly reactive metabolites generated during normal cell metabolism that could act as signaling factors [12]. Epicatechin possesses a high antioxidant activity, and it has been demonstrated that it protects cells from oxidative insults by preventing cell death and free radical formation in the presence of metals [5,6] or t-BOOH [35], showing a cytoprotective activity in hepatic cells [10,36]. Moreover, treatment of cells with epicatechin alone resulted in unchanged or slightly decreased levels of ROS formation after different incubation times [9,11,32]. Our study shows a decrease of ROS production by epicatechin at short incubation times (up to 240 min), recovering then to control values after 18 h of incubation (Table 1). Therefore, it could be suggested that epicatechin might require more features than a reduced ROS production for the induced cytoprotective/survival effect, as described for epigallocatechin-3-

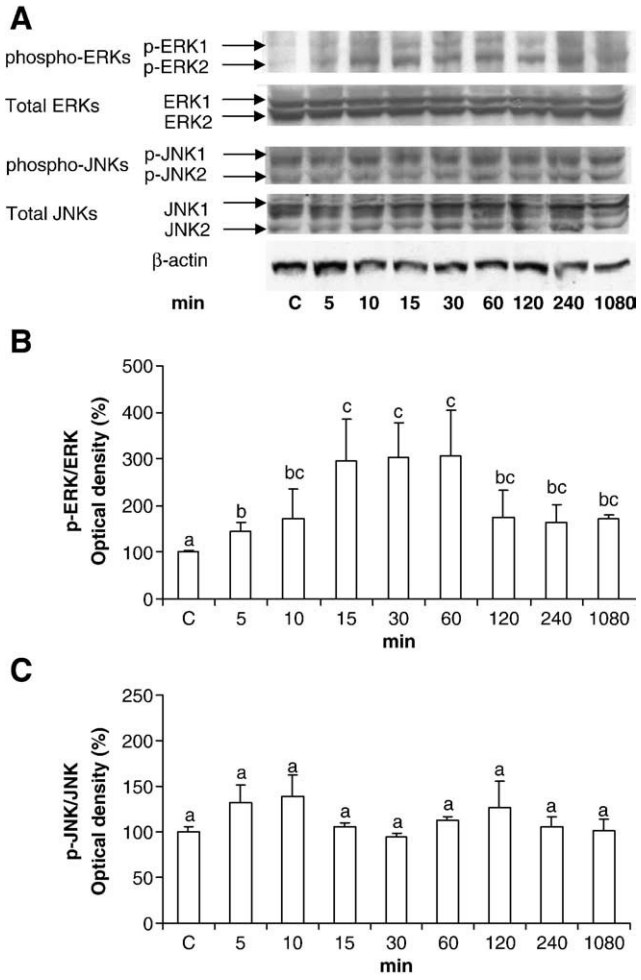

Fig. 5. Time-dependent effects of epicatechin on the basal levels of phosphorylated ERK1/2 (Thr202/Tyr204), total ERK1/2, phosphorylated JNK1/2 (Thr183/Tyr185) and total JNK. (A) Representative blots of both MAPKs. Values of (B) p-ERK/ERK and (C) p-JNK/JNK ratios are shown as the relative increase in percentage over controls (means ± S.D., n=6). Normalization of Western blots was ensured by β-actin. Means without a common letter differ, P<.05.

gallate (EGCG) [37]. In this regard, it has been reported that a higher concentration of epicatechin (100 μmol/L) not only modulated different genes involved in oxidative stress but also related to cell signaling, transcription, cell receptors, etc. [8].

Prosurvival effects of PI3K are due to its activation of the serine/threonine protein kinase AKT, which blocks apoptosis through different mechanisms such as direct participation in the apoptotic cascade or by regulating the transcription of pro- and antiapoptotic genes [16]. Epicatechin treatment results in a rapid activation of AKT, which agrees with previous studies showing the main role of AKT in the promotion of cell proliferation/survival [15,16] and as the prime mediator of a cytoprotective effect by preventing apoptosis [18]. In contrast to the marked effects of epicatechin on AKT phosphorylation, this flavonoid did not affect p85 PI3K subunit levels, although it induced p110 expression at the longest incubation time assayed (18 h), in agreement with our previous results [9]. Since AKT is a downstream target of PI3K, the increased levels of p110 might lead to an increased PI3K activity and to membrane translocation of AKT, which would increase cell survival; however, it should be mentioned that the enzymatic activity of PI-3-kinase can be modified without changing either p85 or p110 subunits levels. Thus, selective blockade of PI3K pathway by the pharmacological inhibitor wortmannin prevented activation of this kinase at short (15 min) and long (1080 min) incubation times and, as previously reported [9], inhibited epicatechin-induced survival, which suggests that HepG2 survival depends on the induction of PI3K/AKT signaling pathway.

ERK1/2 belongs to the MAPKs superfamily and are required for cell survival [15,16] as is the AKT/PI3K signaling pathway and the cross-talk between PI3K and ERKs [15]. As can be observed in controls, a sustained activation of ERK1/2 is essential for HepG2 survival/proliferation [15], whereas ERK1/2 phosphorylation was increased during all incubation times with epicatechin. Thus,

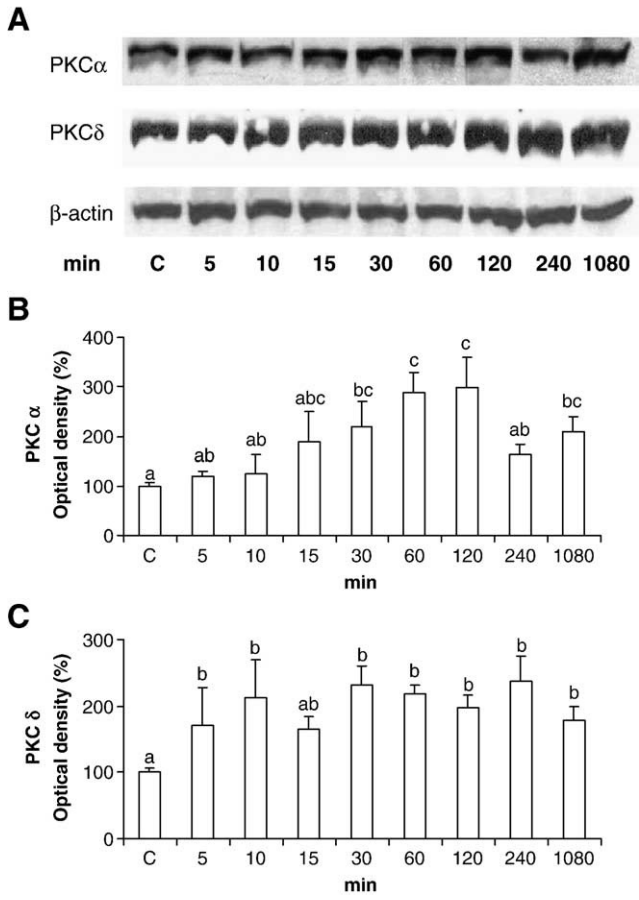

Fig. 6. Time-dependent effects of epicatechin on PKC-α and PKC-δ levels. (A) Bands of representative experiments. Percentage of (B) PKC-α and (C) PKC-δ relative to controls (means ± S.D., n=6). The same blots were reprobed with β-actin. Different letters denote statistically significant differences, P<.05.

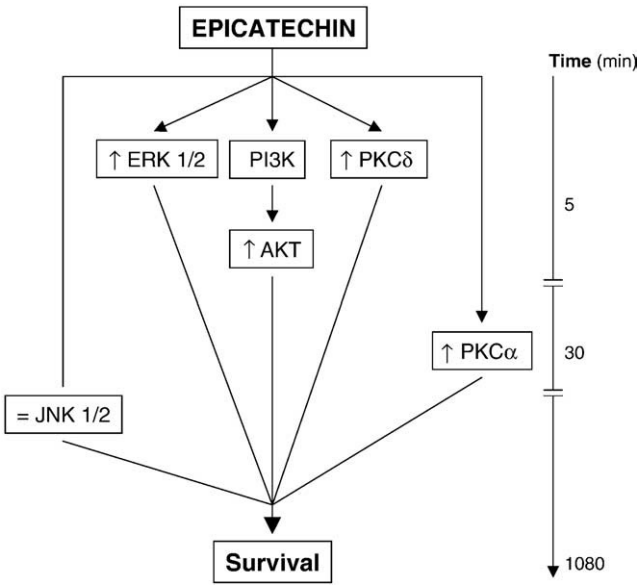

Fig. 7. Schematic overview showing the analyzed targets involved in the epicatechin-induced survival in HepG2 cells. ↑, increased activity; =, unaltered activity. Sharp arrows indicate positive inputs, whereas a line shows no input.

ERK1/2 activation by the flavonoid, together with the stimulation of AKT/PI3K pathway, could also contribute to cell survival since highly activated ERKs seem to be very important for long-term survival after treatment with catechins [38,39]. However, selective inhibition with PD98059 prevented ERKs phosphorylation without affecting HepG2 viability [9], suggesting that induction of the PI3K/AKT pathway could be sufficient to maintain cell survival. Moreover, it could be suggested that activation of AKT/PI3K and ERKs signaling cascades could be independent, yet required for the effect of epicatechin on cell survival/proliferation pathways as previously reported in hepatic cells [28,40]. The involvement of other signals, such as nuclear factor κB, related to cell cycle and/or cell survival should also be considered [41].

Signaling cascades involving the MAPK-JNK are key mediators of stress signals and seem to be mainly responsible for protective responses, stress-dependent apoptosis and inflammatory reactions [20,21,39,42,43]. The lack of effect of epicatechin on JNK phosphorylated protein levels has been previously reported [30]; neither protein levels nor activity of JNK were altered after a short incubation time with high concentrations of epicatechin or other natural phenolic compounds [30]. ROS generation has been described as a critical upstream activator of JNK [42], and, accordingly, epigallocatechin-induced JNK and p38 activations, which are ROS dependent, were suppressed after treating the cells with *N*-acetylcysteine, but not ERK1/2 activation, indicating that this activation is ROS independent, and that the MAPK signaling pathway plays an important role in cellular long-term survival [38]. Similarly, MAPK signaling pathway was demonstrated to possess a relevant function in EGCG-mediated cytoprotective effects [37]. Furthermore, EGCG induced epidermal growth factor receptor-mediated downstream events [stimulation of ERKs, PI3K, AKT, mammalian target of rapamycin, p70 (S6K)] and suppressed enhanced ROS generation by angiotensin II without affecting cell viability. The cross-talk between JNK and ERK may be essential to prevent a prolonged and therefore lethal JNK activation, and thus, ERKs have been implicated in hepatocyte down-regulation of JNK response [42], although the mechanisms remain to be determined. We have previously reported nonaltered Bax levels after epicatechin treatment [9], which supports the lack of effect of this flavonoid on JNK phosphorylation, as activation of this MAPK could be up-regulated by proapoptotic Bcl-2 family members such as Bim, Bax, Bak and Bad [21,42,43].

The PKC family is constituted by multiple isoenzymes with cell-specific expression, subcellular localization and different roles [17]. PKC-α mediates prosurvival functions in several cells by inhibiting apoptosis, enhancing survival pathways or generating resistance to chemotherapy [17,44,45], whereas PKC-δ has been implicated in the regulation of apoptosis [17,44,46]. In the case of PKC-α, the increase of its levels is rapid and persists over time (up to 18 h), suggesting a role for PKC-α in the epicatechin-induced survival effect on HepG2 cells. PKC-α inhibits apoptosis by regulating the phosphorylation of Bcl-2 and the activation of AKT [44]. Since AKT can be modulated by PKC-α, the observed increased levels of the PKC isoform throughout the treatment might contribute to the activation of AKT phosphorylation and, thus, to the survival effect. These observations are supported by previous studies showing that catechins and other flavonoids could act as PKC activators and exert a cytoprotective effect on cells [24,39]. However, in HepG2 cells, the lack of parallelism between AKT and PKC-α activations suggests that both proteins are regulated by apparently independent transduction pathways with different timing responses.

PKC-δ is associated with inhibition of cell cycle progression and promotion of apoptosis by its translocation from cytosol to nuclei, mitochondria, etc. [17,44], suggesting that PKC-δ may have a negative effect on cell survival [46]. After epicatechin incubation, PKC-δ cytosolic levels increase in a rapid and sustained manner, similarly to PKC-α. It has been described that inhibition of apoptosis rapidly causes a retranslocation of PKC-δ away from the nucleus [47], which could explain the epicatechin-induced accumulation of PKC-δ in the cytosol. Moreover, PKC-δ may also participate in cell survival by interfering with *bcl-2* genes and regulating MAPKs, which would prevent PKC-δ proteolytic activation by caspase-3 [46,48,49].

In conclusion, epicatechin-induced survival in HepG2 cells is a rapid event that requires the integration of different signals and persists over time. These mechanisms involve early activation of key proteins of survival signaling such as PI3K/AKT, ERK1/2 and PKC-α (Fig. 7). Moreover, early lack of PKC-δ translocation away from the cytosol might

also promote cell survival by modulating key survival/proliferative signals. Although further efforts are needed to define the precise role of epicatechin in the regulation of HepG2 cells survival, epicatechin could be promising in the design and development of new treatments for promoting cellular protection and/or survival.

Acknowledgments

A.B. Granado-Serrano is a predoctoral fellow of the Spanish Ministry of Science and Education, and S. Ramos has a Ramón y Cajal contract from the Spanish Ministry of Science and Technology.

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