Research Article

Time-course regulation of quercetin on cell survival/ proliferation pathways in human hepatoma cells

Ana Belén Granado-Serrano, María Angeles Martín, Laura Bravo, Luis Goya and Sonia Ramos

Department of Metabolism and Nutrition, Instituto del Frío, Consejo Superior de Investigaciones Científicas (CSIC), José Antonio Novais 10, Ciudad Universitaria, Madrid, Spain

Quercetin, a dietary flavonoid, has been shown to possess anticarcinogenic properties, but the precise molecular mechanisms of action are not thoroughly elucidated. This study was aimed at investigating the time-course regulation effect of quercetin on survival/proliferation pathways in a human hepatoma cell line (HepG2). Quercetin induced a significant time-dependent inactivation of the major survival signaling proteins, *i.e.*, phosphatidylinositol 3-kinase (PI 3-kinase)/protein kinase B (AKT), extracellular regulated kinase (ERK), protein kinase C-α (PKC-α), in concert with a time-dependent activation of key death-related signals: *c-jun* amino-terminal kinase (JNK) and PKC-δ. These data suggest that quercetin exerts a tight regulation of survival/proliferation pathways that requires the integration of different signals and persists over time, being the balance of these regulatory signals what determines the fate of HepG2 cells.

Keywords: AKT/PI 3-kinase pathway / HepG2 cells / MAPK / PKC / Quercetin

Received: June 7, 2007; revised: September 10, 2007; accepted: September 14, 2007

1 Introduction

Many natural dietary polyphenols found in fruits, vegetables, spices, and tea have been shown to exert potential anticarcinogenic activities [1, 2]. Quercetin is one of the most common flavonoids found in the diet [2] and it is extensively metabolized during absorption in the small intestine and in the liver [3]. Previous studies have shown that quercetin is a potent inhibitor of tumor initiation *in vivo* and possesses antiproliferative activities against tumor cells *in vitro* [4]. Quercetin is also a potent chemopreventive agent inducing apoptosis in different cancer cell lines [5–9]. Recent evidence indicates that quercetin suppresses a number of key elements in cellular signal transduction pathways linked to the apoptotic cell death [2, 8, 10, 11], although the detailed molecular mechanisms of action remain to be elucidated.

Correspondence: Dr. Sonia Ramos, Department of Metabolism and Nutrition, Instituto del Frío, Consejo Superior de Investigaciones Científicas (CSIC), José Antonio Novais 10, Ciudad Universitaria, 28040, Madrid, Spain
E-mail: s.ramos@if.csic.es
Fax: +34-91-549-36-27

Abbreviations: ERK, extracellular regulated kinase; **FBS,** fetal bovine serum; **HepG2,** human hepatoma cell line; **JNK,** *c-jun* **amino-terminal kinase; MAPK,** mitogen-activated protein kinase; **PI 3-kinase,** phosphatidylinositol 3-kinase; **PKC,** protein kinase C

Apoptosis is a complex process and involves regulation of a number of cellular genes, including *bcl-2*, and a family of zymogen cytosolic proteases, caspases. In this regard, we have previously shown that quercetin induces apoptotic cellular death by activating caspase-3 and caspase-9 in human hepatoma (HepG2) cells in a dose-dependent manner [11]. Moreover, quercetin activates cleavage of PARP (poly ADP-ribose polymerase) [6, 12], release of cytochrome *c* [12] and produces DNA fragmentation [6, 12, 13]. Downregulation of antiapoptotic Bcl-2 proteins, Bcl-x_L and Bcl-2 [11, 14], and upregulation of proapoptotic Bcl-2 proteins such as Bax and Bad in diverse cancer cell lines [6, 14] by quercetin have also been demonstrated.

In addition to proteins that have a direct effect on apoptosis, several other signaling pathways are indirectly involved in the regulation of apoptotic cell death. Signaling pathways through phosphatidylinositol 3-kinase (PI 3-kinase)/AKT, mitogen-activated protein kinase (MAPKs) such as extracellular regulated kinase (ERK) or *c-jun* amino-terminal kinase (JNK), and protein kinase C (PKC) indirectly regulate programed cell death [15, 16]. Prominent among the signaling events inhibited by quercetin are phosphorylations catalyzed by protein kinases. Thus, quercetin-induced inactivation of PI 3-kinase [5, 9, 17] and AKT [6, 11, 18] leads to the induction of apoptosis and to the blockade of cell survival/proliferation [16]. Moreover, both activation and inhibition of ERK and/or JNK have

© 2008 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

been shown to contribute to the induction of apoptosis, depending on the experimental conditions [6, 18, 19]. Besides, quercetin dose dependently decreased the expression of PKC-α, but not PKC-δ in murine melanoma cells resulting in apoptosis induction [20], although in other cell models inhibition of PKC-α and activation of PKC-δ by flavonoids have also been related to apoptosis [17, 21, 22]. On the other hand, it could be highlighted that most of these studies have been carried out after long-term treatments with quercetin. However, since potential variations of the observed effects could take place in a time-course manner during the activation of these pathways [23, 24], it is essential to characterize the role played by quercetin in the regulation of signaling routes indirectly related to apoptosis of HepG2 cells.

In the present work, the time-course mechanisms of the quercetin on key prosurvival pathways, namely AKT/PI 3-kinase, MAPKs (ERK and JNK), and PKC (isoforms α and δ) on HepG2 cells have been investigated. Results show that quercetin induces the inhibition and activation of crucial proteins in survival/proliferation pathways. This feature is an early event which requires the integration of different signals and becomes apparent through the time.

2 Materials and methods

2.1 Materials and chemicals

Quercetin (98%), antimouse IgG-Agarose, L-α-phosphatidyl-L-serine, gentamicin, penicillin G, and streptomycin were purchased from Sigma Chemical (Madrid, Spain). L-α-phosphatidylinositol was from Jena Bioscience (Diffractia, Madrid, Spain) Anti-AKT and antiphospho-Ser473-AKT, as well as anti-ERK1/2 and antiphospho-ERK1/2 recognizing phosphorylated Thr202/Thy204 of ERK1/2, anti-JNK1/2 and antiphospho-JNK1/2 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). Anti-p110β (catalytic PI 3-kinase subunit), anti-p-Tyr Py20, anti-PKCα, and anti-PKCδ were purchased from Santa Cruz (sc-7175, sc-508, sc-208, and sc-937, respectively, Santa Cruz Biotechnology, Santa Cruz, CA). Anti-p85α/β (regulatory PI 3-kinase subunit) was from Upsate (Upstate Biotechnology Lake Placid, NY). Materials and chemicals for electrophoresis were from Bio-Rad (BioRad 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.

2.2 Cell culture and quercetin treatment

Human hepatoma HepG2 cells were grown in DMEM F-12 medium from Biowhitaker (Innogenetics), supplemented with 2.5% Biowhitaker fetal bovine serum (FBS) and the following antibiotics: gentamicin, penicillin, and streptomycin (50 mg/L). Cells were maintained at 370C 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 in order to avoid the influence of the growth factors contained in the FBS on the results. To study the time-course effects of the flavonoid, cells were treated with 50 μM quercetin and then harvested at different times (5, 10, 15, 30, 60, 120, 240, and 1080 min). All treatments contained the same concentration of DMSO (0.05%).

2.3 Preparation of total cell lysates

To detect AKT, phospho-AKT, ERK1/2, and phospho-ERK1/2, PI-3-kinase subunits (p85 and p110) and activity, as well as PKC-α and PKC-δ, 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 DTT, 0.1% Triton X-100, 200 mM β-glycerolphosphate, 0.1 mM Na_3VO_4, 2 μg/mL leupeptin, and 1 mM PMSF. The supernatants were collected, assayed for protein concentration by using the BioRad protein assay kit according to the manufacturer's specifications, aliquoted and stored at –80°C until used for Western blot analyses, immunoprecipitation, and later PI 3-kinase activity determination.

2.4 Purification of nuclear and cytosolic extracts

Cells were resuspended at 4°C in 10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 0.2 mM PMSF (buffer A); allowed to swell on ice for 10 min; and then vortexed for 10 s. Samples were centrifuged at 10 000 × *g* for 2 min and the supernatant containing the cytosolic fraction was stored at –80°C. The pellet was resuspended in cold buffer B (20 mM HEPES, pH 7.9, 25% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 0.2 mM PMSF, 2.5 μg/mL leupeptin, 2.5 μg/mL aprotinin) and incubated on ice for 20 min for high salt extraction. Cellular debris was removed by centrifugation at 13 000 × *g* for 10 min at 4°C, and the supernatant fraction containing nuclear protein extract was stored at –80°C.

2.5 Western blot analysis

Equal amounts of proteins (100 μg) were separated by SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) filters (Protein Sequencing Membrane, BioRad). Membranes were probed with the corresponding primary antibody followed by incubation with peroxide-conjugated antirabbit Ig (GE Healthcare, Madrid, Spain). Blots were developed with the ECL system (GE Healthcare). Normalization of Western blot was ensured by β-actin and bands

© 2008 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

were quantified by laser scanning densitometry (Molecular Dynamics, Sunnyvale, CA)

2.6 Immunoprecipitation and PI 3-kinase activity

Cell lysates containing 100–500 μg proteins were immunoprecipitated overnight at 4°C with gentle rotation in presence of 1–2 μg of anti-*p*-Tyr(P) primary antibody, followed by the addition of antimouse IgG-agarose beads. After mixing for 2 h, the pellets were collected by centrifugation and the supernatants were discarded. Then the immune complexes were collected on antimouse IgG-agarose beads and were washed twice with each of the following buffers: (1) PBS with 1% Triton X-100; (2) 0.1 M Tris pH 7.5, 0.5 M LiCl; and (3) 10 mM Tris pH 7.5, 0.1 M NaCl. The pellets were saved for PI 3-kinase activity determination.

PI 3-kinase activity was assayed by phosphorylation of phosphatidylinositol (PI) with [^{32}P]ATP (GE Healthcare). Phosphorylated PI was analyzed by TLC as previously described [25]. The products of the radioactive reaction were visualized by autoradiography and quantified by densitometry.

2.7 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 p110 PI 3-kinase subunit). $p < 0.05$ was considered significant.

An SPSS version 12.0 program was used.

3 Results

To test whether quercetin-induced apoptosis is accompanied by time and/or phosphorylation signaling events, HepG2 cells were treated with a fixed concentration of this flavonoid (50 μM) for different times (0–1080 min) according to results of previous experiments [11], and possible alterations either in total and/or phosphorylated levels of different protein kinases were analyzed.

3.1 AKT phosphorylation

AKT phosphorylation is associated with activation of this kinase, which has been demonstrated to protect cells from apoptosis [16]. As shown in Fig. 1, total AKT protein levels did not change during the incubation with quercetin, whereas AKT phosphorylation protein levels varied in a time-dependent manner. This inhibition of AKT phosphorylation was evident at an early time-point of treatment (5 min) and maintained up to 4 h, recovering then to controls after 18 h of incubation. Thus, quercetin treatment

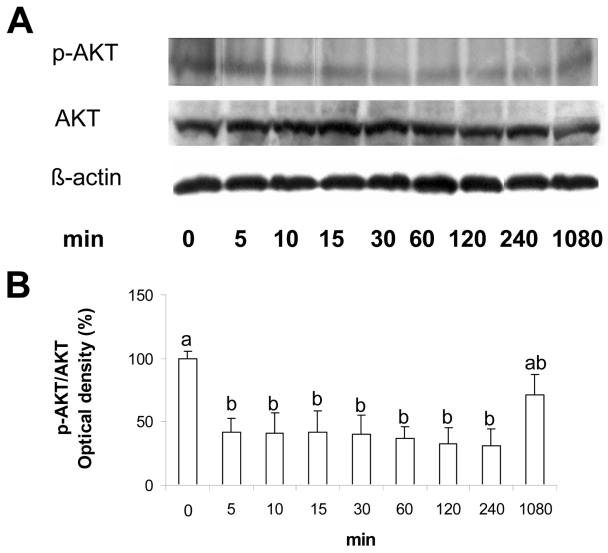

Figure 1. Time-dependent effect of 50 μM quercetin on basal levels of phosphorylated AKT (Ser473) and total AKT. (A) Bands of a representative experiment. (B) Percentage values of the p-AKT/AKT ratio relative to the control condition (means ± SD, $n = 6$). Normalization of Western blots was ensured by β-actin. Means without a common letter differ, $p < 0.05$.

seemed to be more effective in inhibiting phosphorylation of AKT rather than its expression. Moreover, dephosphorylation of AKT was transient and returned to basal levels after 18 h.

3.2 PI 3-kinase protein levels and activity

PI 3-kinase pathway is regulated by several growth factors and the PI 3-kinase/AKT signaling transduction pathway plays a protective role in induced apoptosis [15]. Thus, the effect of quercetin on the levels of the p85 regulatory subunit and the p110 catalytic subunit of PI 3-kinase was examined. Figure 2 shows that the expression of both subunits was not altered by quercetin treatment. However, PI 3-kinase activity was inhibited as early as after 5 min of incubation, and this reduced enzymatic activity was sustained in time, showing the lowest values at the longest incubation time (18 h) (Fig. 3).

3.3 ERK phosphorylation

ERK signaling pathway is activated in response to certain situations of cellular stress and it is implicated in cellular survival signaling [15]. To further elucidate the involvement of other possible kinases in quercetin-induced signaling modulation, the two bands corresponding to ERK1 (44 kDa) and ERK2 (42 kDa) protein levels (total and phosphorylated or activated form, respectively) were investigated. Total ERK1/2 protein levels did not change during the treatment with quercetin, similarly to what was

© 2008 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

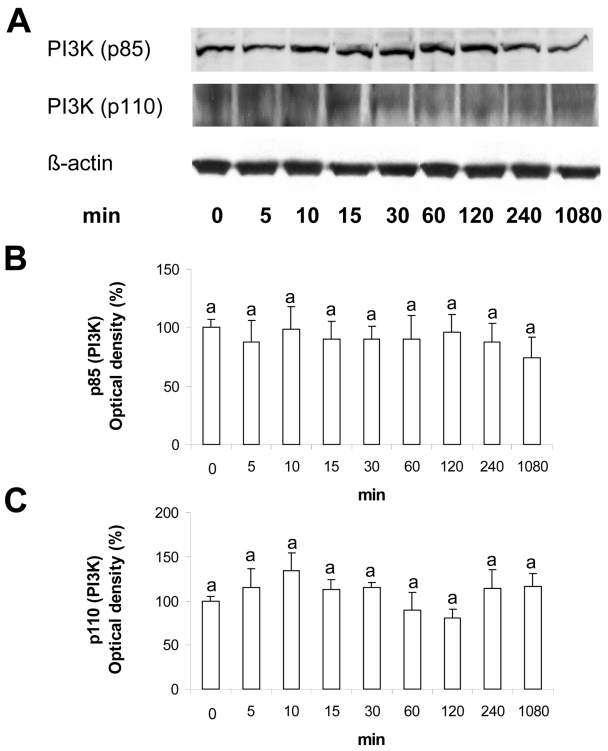

Figure 2. Time-dependent effects of quercetin (50 μM) on basal levels of p85 and p110 subunits of PI 3-kinase. (A) Representative blots of both PI 3-kinase subunits. Values of (B) p85 and (C) p110 PI 3-kinase subunits are shown as a percent relative to the control condition (means ± SD, $n = 5$). Normalization of Western blots was ensured by β-actin. Means without a common letter differ, $p < 0.05$.

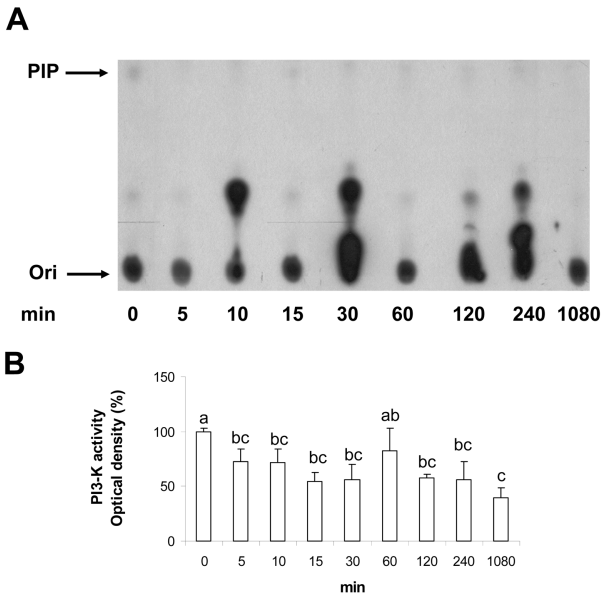

Figure 3. Time-dependent effect of quercetin (50 μM) on PI 3-kinase activity. (A) Bands of a representative experiment. (B) Values (means ± SD, $n = 4$) show the percentage of PI 3-kinase activity relative to the control condition. Different letters denote statistically significant differences, $p < 0.05$.

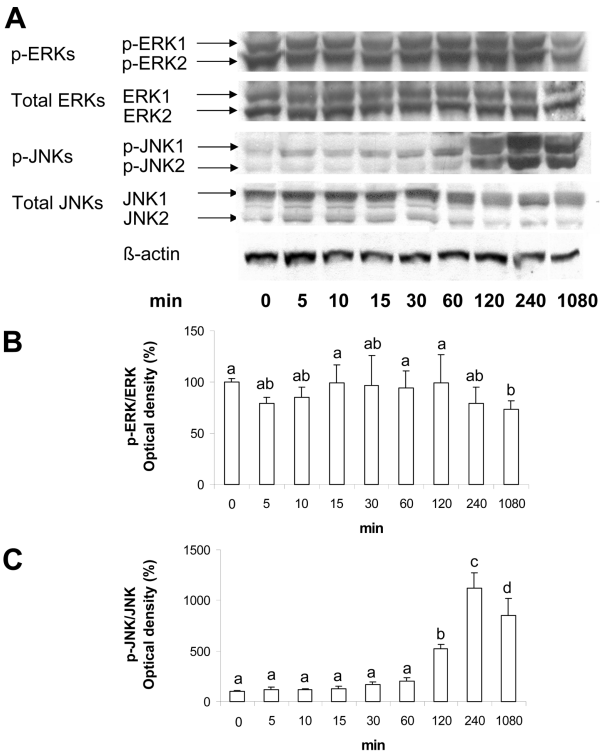

Figure 4. Time-dependent effects of 50 μM quercetin on the basal levels of phosphorylated ERK1/2 (Thr202/Tyr204), total ERK1/2, phosphorylated JNK1/2 (Thr183/Tyr185), and total JNK. (A) Representative blots of both MAPKs. Percentage data of (B) the p-ERK/ERK and (C) p-JNK/JNK ratios relative to controls (means ± SD, $n = 6$). Normalization of Western blots was ensured by β-actin. Means without a common letter differ, $p < 0.05$.

observed for AKT. However, quercetin evoked an inhibitory effect on active ERKs after 18 h of incubation (Fig. 4). Contrary to AKT regulation, quercetin treatment of HepG2 cells resulted in unaffected phosphorylation levels of ERK1/2 at short times, and a significant dephosphorylation of ERK1/2 at 18 h.

3.4 JNK phosphorylation

JNK activation is connected to cell death, although in certain circumstances inhibition of the protein has also been reported in an apoptotic situation [26]. Therefore it was important to study the effect induced by quercetin on HepG2 cells. To this end, total and phosphorylated (active form) protein expressions of JNK1/2 were analyzed. Treatment of HepG2 cells with the flavonoid resulted in an enhanced phosphorylation of JNK1/2 at 2–18 h, whereas quercetin did not affect the levels of the two bands corresponding to JNK1 (54 kDa) and JNK2 (46 kDa) at any of the shorter times tested (0–1 h) (Fig. 4). Total JNK1/2 protein levels did not change during the quercetin exposure, similarly to what was observed for total AKT and ERKs protein expressions.

© 2008 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

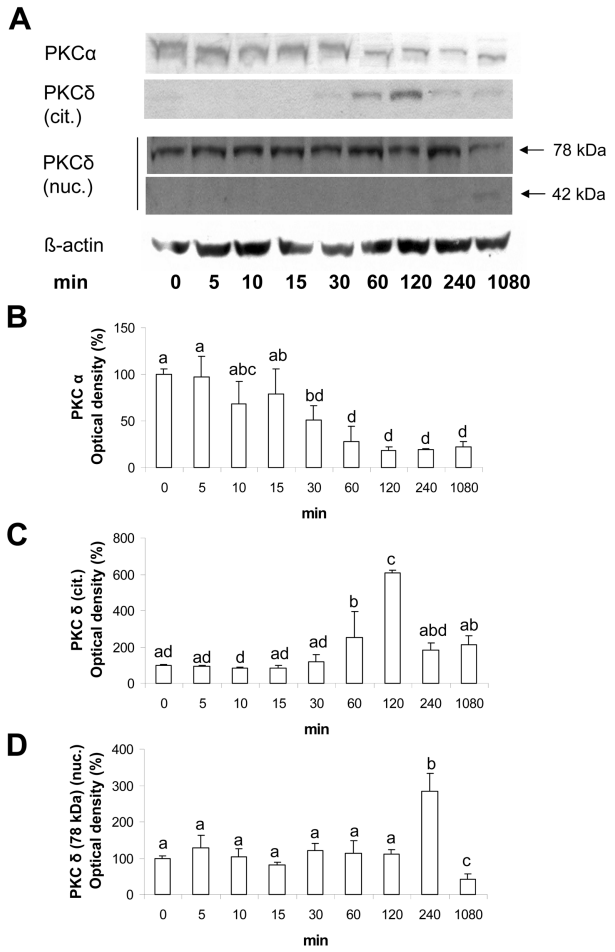

Figure 5. Time-dependent effects of quercetin (50 μM) on PKC-α and PKC-δ. (A) Bands of representative experiments. Percentage of (B) PKC-α, (C) cytosolic PKC-δ, and (D) nuclear PKC-δ (78 kDa) relative to controls (means ± SD, $n = 6$). The same blots were reprobed with β-actin. Different letters denote statistically significant differences, $p < 0.05$.

3.5 PKCα and PKCδ expression levels

PKC activation plays a role against induced apoptosis [27], however the individual PKC isoforms play different roles in apoptosis [27]. In this regard, PKC-α has been implicated in apoptosis suppression in different cell lines, whereas PKC-δ has usually been involved in the promotion of apoptosis [28]. To address the role of PKC-α and -δ in quercetin-induced survival/proliferation signaling regulation, their expression levels were examined in a time-course study. Treatment of HepG2 cells with quercetin markedly reduced PKC-α levels after 30 min, and protein levels continued decreasing at 60 min up to 1080 min, showing at these incubation times maintained low values (Fig. 5). Contrary to what was observed for PKC-α, quercetin treatment significantly increased cytosolic PKC-δ levels after 60 min and declined at 240 and 1080 min, showing levels comparable to those of controls, whereas nuclear PKC-δ levels (78 kDa) increased after 240 min, exhibiting later a decrease at 18 h (Fig. 5). In addition to the translocation, PKC-δ catalytic domain (42 kDa) was detected in the nuclear fraction after 4 and 18 h of quercetin treatment, showing higher nuclear levels at the longest incubation time (18 h).

4 Discussion

Many chemopreventive drugs have been shown to induce apoptosis directly by regulating different proteins (caspases, Bcl-2 proteins), but also indirectly through the involvement of other signaling pathways such as PI 3-kinase/AKT, MAPKs, PKC, *etc.* in malignant cells [2, 29]. Currently, much attention has been focused on polyphenolic compounds as they can facilitate a number of apoptotic mechanisms [1, 2]. Quercetin is one of the major flavonoids in certain species of plants and foods that has been found to be chemopreventive. We have recently reported that after 18 h of treatment with 50 μM quercetin, the induced apoptotic effect might have been enhanced by the inhibition of AKT and ERK phosphorylated levels, whereas at this concentration the flavonoid also provoked characteristic morphological alterations of the apoptosis, decreased cell viability and the highest activation of caspases-3 and -9 and expression levels of proapoptotic Bcl-2 family members (Bcl-x_S and Bax) in comparison with other concentrations used [11, 13].

In the present study, we attempted to determine the molecular mechanisms that run together with the apoptotic effects of quercetin and its metabolites potentially generated by HepG2 cells during the treatment by focusing on the time-course regulation of cell survival/proliferation pathways. We provide evidence that quercetin is an effective suppressor of survival/proliferation pathways and induces PI 3-kinase/AKT, ERK and PKC downregulation and JNK upregulation.

Quercetin has been shown to induce apoptosis in a wide variety of cells, including hepatoma cells [5, 6, 8, 9, 11, 14]. Importantly, this flavonoid exerts the apoptotic effect in a selective manner, since the same concentrations of quercetin induced apoptosis in cancer cultured cells but not in their normal counterparts [10]. In humans, a continuous daily intake of polyphenolic dietary supplements such as quercetin might result in steady-state plasma concentrations of quercetin and its metabolites, in levels that might provoke an apoptotic cell death (10–50 μM) [30]. Moreover, rats fed with quercetin showed quercetin metabolites concentrations in plasma of 50 μM, and when animals were adapted to the flavonoid, a concentration of 100 μM (quercetin and metabolites) was reported [31]. However, at present the potential contribution of the quercetin metabolites to the biological activity is unclear and it should not be underestimated [32]; its evaluation will require further studies.

© 2008 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

Quercetin treatment results in a rapid and sustained inactivation of PI 3-kinase activity in HepG2 cells, which could be related to the time course-dependent cellular damage [11], suggesting that this protein plays a role in the survival of HepG2 cells. Antiapoptotic effects of PI 3-kinase are due to its activation of serine/threonine protein kinase AKT [33], which blocks apoptosis through different mechanisms and its phosphorylation has been considered as a critical factor in the aggressiveness of hepatocellular cancer [33]. In the present report, quercetin inhibits AKT phosphorylation rapidly, occurring within minutes of treatment, and transiently, which agrees with previous studies showing the requirement of an eventual decrease in the AKT phosphorylated levels for the induction of apoptosis and the later AKT activation response as a self-defense mechanism to protect cells from death [23]. Because AKT is a downstream target of PI 3-kinase and despite the unaffected protein levels of PI 3-kinase subunits (p85 and p110), the observed inhibition of PI 3-kinase activity throughout the time of treatment, suggests that quercetin may also inhibit AKT phosphorylation. This observation is supported by previous studies showing that quercetin and other flavonoids are PI 3-kinase inhibitors, and these phenolic compounds reduced the enzymatic PI 3-kinase activity without changing neither p85 nor p110 subunit levels [5, 9, 17]. In addition, by suppressing the activation of AKT, quercetin can promote apoptosis insofar as inhibition of AKT would prevent Bad phosphorylation, increasing its proapoptotic activity [33].

Many intracellular proliferative signals are mediated by MAPKs, such as ERKs [15], as well as PI 3-kinase and its downstream target AKT, and also the crosstalk between PI 3-kinase and ERKs, which strengthen the cellular survival/proliferative effect [15, 16]. A sustained activation of ERK1/2 is necessary for cell survival and cell proliferation [15, 34] as can be observed in control cells, whilst ERK1/2 phosphorylation was reduced after a long (18 h) incubation time with quercetin (Fig. 4). Such inhibition of ERK1/2 by the flavonoid could also contribute to the apoptotic process since inactivation of ERKs leads to inhibition of cell proliferation and cell cycle progression [15]. Moreover, the timing of AKT dephosphorylation was not correlated with that of ERK dephosphorylation, suggesting that PI 3-kinase/AKT and ERK pathways are independent and required for the survival of hepatoma cells as previously reported [34]. Furthermore, this lack of parallelism between AKT and ERK inhibition might also be indicative of the involvement of other signals related to cell cycle and/or cell survival such as NFκB (nuclear factor kappa B) [35].

The JNK family belongs to the MAPKs superfamily (same as ERKs) and its activation has been associated to apoptosis [19, 36, 37]. The effect of quercetin on JNK phosphorylation profile evoked a significant activation at 2 h, and the activity remained intensely elevated throughout the experiment (up to 18 h); thus the response was delayed and prolonged. Previous works support this type of JNK activation in a time-dependent manner by quercetin and other polyphenols in vascular smooth muscle and HepG2 cells [19, 26, 37] and it has clearly been demonstrated that activation of the JNK signaling pathway is a common mediator of cell death in hepatic cells [19, 36, 37]. Moreover, as described previously in other studies [36], inhibition of ERK might be sufficient to sensitize hepatic cells to death, since crosstalk between JNK and ERK may be essential to prevent a prolonged and therefore lethal JNK activation. Furthermore, apoptosis can be promoted by the activation of JNK as it could upregulate proapoptotic Bcl-2 family members such as Bim, Bax, Bak, and Bad [19, 36, 37], in agreement with the highest mitochondrial Bax levels detected in our previous study [11]. Similar to ERKs inhibition, the flavonoid-induced JNK activation was delayed, whereas AKT phosphorylated levels decreased fast. Although the mechanisms that modulate AKT and MAPKs (ERK and JNK) in response to the flavonoid treatment are not clear at present, it could be suggested that AKT and MAPKs are regulated by apparently independent transduction pathways with different timing responses, as it has been previously reported in the treatment of cancer cells with other chemopreventive agents [23, 34].

The PKC family consists of multiple isoenzymes with distinct distribution patterns and different functions [27]. PKC-α mediates prosurvival functions in several cell types by reducing apoptosis, enhancing survival or producing resistance to chemotherapy [27], whereas PKC-δ has been related to both cell protection and apoptosis [20, 21, 27]. In the case of PKC-α, the decrease in its levels is rapid, occurring within minutes of treatment, and persists over time (up to 18 h). This suggests that PKC-α inactivation runs together with the apoptotic process of HepG2 cells induced by quercetin. PKC-α inhibits apoptosis by regulating the phosphorylation of Bcl-2 and the activation of AKT [20]. Because AKT can be modulated by PKC-α, the observed decreased levels of the PKC isoform throughout the treatment might contribute to the rapid inhibition of the AKT phosphorylation. This observation is supported by previous studies showing that quercetin and other flavonoids are PKC-α and/or PKC inhibitors [17, 20, 28, 38].

On the other hand, quercetin provokes an accumulation of PKC-δ in the cytosol to diminish later; this cytosolic decrease was followed by a rapid increase of PKC-δ protein levels in the nuclei and by the appearance of its proteolytic domain, indicating that both processes (translocation and proteolysis) were concomitant events in the nuclei. PKC-δ plays an important role in apoptosis by translocating from cytosol to nuclei, mitochondria, *etc.* [20, 27]. It has been described that quercetin did not affect the expression of PKC-δ, but it had a dose-dependent effect on the translocation of PKC-δ from cytosol to nuclei [20], which is in agreement with the results of this study. In addition to the progressive relocalization of PKC-δ, its proteolysis may occur during apoptosis, and both processes (translocation and

© 2008 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

proteolysis) may take place simultaneously as shown before in GH3B6 cells [39]. Moreover, PKC-δ may also participate in the apoptotic effect by regulating MAPKs, being proteolytically activated by caspase-3 and decreasing Bcl-2 and increasing Bax [22, 40, 41].

Data reported in the present study provide new insights into the relative contributions of major survival/proliferation pathways on the effects of quercetin in HepG2. Quercetin inhibition of survival/proliferation routes is a rapid event that is accompanied by early suppression of survival signaling (PI 3-kinase/AKT and PKC-α), intermediate-late activation of JNK and inhibition of ERK1/2, early enhanced translocation and later proteolysis of PKC-δ. Further efforts are needed to define the potential relationship between processes, apoptosis, and survival/proliferation pathways, in HepG2 cells treated with quercetin.

The authors wish to thank Dr. F. Escriva for expert assistance with PI 3-kinase activity assay. 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). A. B. G.–S. is a predoctoral fellow of the Spanish Ministry of Science and Education and S. R. has a Ramón y Cajal contract from the Spanish Ministry of Science and Technology.

The authors have declared no conflict of interest.

5 References

[1] Surh, Y. J., Cancer chemoprevention with dietary phytochemicals. *Nat. Rev. Cancer* 2003, *3*, 768 – 780.

[2] Ramos, S., Effects of dietary flavonoids on apoptotic pathways related to cancer chemoprevention. *J. Nutr. Biochem.* 2007, *18*, 427 – 442.

[3] Spencer, J., Chowrimootoo, G., Choudhury, R., Debnam, E. *et al.*, The small intestine can both absorb and glucuronidate luminal flavonoids. *FEBS Lett.* 1999, *458*, 224 – 230.

[4] Chi, C., Chang, Y., Ou, Y., Hsieh, C. *et al.*, Effect of quercetin on the in vitro and in vivo growth of mouse hepatoma cells. *Oncol. Res.* 1997, *4*, 1021 – 1024.

[5] Singhal, R. L., Yeh, Y. A., Praja, N., Olah, E. *et al.*, Quercetin down-regulates signal transduction in human breast carcinoma cells. *Biochem. Biophys. Res. Commun.* 1995, *208*, 425 – 431.

[6] Nguyen, T., Tran, E., Nguyen, T., Do, P. *et al.*, The role of activated MEK-ERK pathway in quercetin-induced growth inhibition and apoptosis in A549 lung cancer cells. *Carcinogenesis* 2004, *25*, 647 – 659.

[7] Ong, C., Tran, E., Nguyen, T., Ong, C. *et al.*, Quercetin-induced growth inhibition and cell death in nasopharyngeal carcinoma cells are associated with increase in Bad and hypophosphorylated retinoblastoma expressions. *Oncol. Rep.* 2004, *11*, 727 – 733.

[8] Van Erk, M., Roepman, P., van der Lende, T., Stierum, R. *et al.*, Integrated assessment by multiple gene expression analysis of quercetin bioactivity on anticancer-related mechanisms in colon cancer cells in vitro. *Eur. J. Nutr.* 2005, *44*, 143 – 156.

[9] Huynh, H., Nguyen, T., Chan, E., Tran, E., Inhibition of ErbB-2 and ErbB-3 expression by quercetin prevents transforming growth factor alpha (TGF-alpha)- and epidermal growth factor (EGF)-induced human PC-3 prostate cancer cell proliferation. *Int. J. Oncol.* 2003, *23*, 821 – 819.

[10] Chowdhury, S., Kishino, K., Satoh, R., Hashimoto, K. *et al.*, Tumor-specificity and apoptosis-inducing activity of stilbenes and flavonoids. *Anticancer Res.* 2005, *25*, 2055 – 2063.

[11] Granado-Serrano, A., Martín, M., Bravo, L., Goya, L., Ramos, S., Quercetin induces apoptosis via caspase activation, regulation of Bcl-2, and inhibition of PI-3-Kinase/Akt and ERK pathways in a human hepatoma cell line (HepG2). *J. Nutr.* 2006, *136*, 2715 – 2721.

[12] Wang, I., Lin-Shiau, S., Lin, J., Induction of apoptosis by apigenin and related flavonoids through cytochrome c release and activation of caspase-9 and caspase-3 in leukaemia HL-60 cells. *Eur. J. Cancer* 1999, *35*, 1517 – 1525.

[13] Ramos, S., Alia, M., Bravo, L., Goya, L., Comparative effects of food-derived polyphenols on the viability and apoptosis of a human hepatoma cell line (HepG2). *J. Agric. Food Chem.* 2005, *53*, 1271 – 1280.

[14] Vijayababu, M., Kanagaraj, P., Arunkumar, A., Ilangovan, R. *et al.*, Quercetin-induced growth inhibition and cell death in prostatic carcinoma cells (PC-3) are associated with increase in p21 and hypophosphorylated retinoblastoma proteins expression. *J. Cancer Res. Clin. Oncol.* 2005, *131*, 765 – 771.

[15] Ballif, B., Blenis, J., Molecular mechanisms mediating mammalian mitogen-activated protein kinase (MAPK) kinase (MEK)-MAPK cell survival signals. *Cell Growth Differ.* 2001, *12*, 397 – 408.

[16] Nicholson, K., Anderson, N., The protein kinase B/Akt signalling pathway in human malignancy. *Cell. Signal.* 2002, *14*, 381 – 395.

[17] Gamet-Payrastre, L., Manenti, S., Gatracap, M., Tulliez, J. *et al.*, Flavonoids and the inhibition of PKC and PI 3-kinase. *Gen. Pharmacol.* 1999, *32*, 279 – 286.

[18] Spencer, J., Rice-Evans, C., Williams, R., Modulation of pro-survival Akt/protein kinase B and ERK1/2 signaling cascades by quercetin and its in vivo metabolites underlie their action on neuronal viability. *J. Biol. Chem.* 2003, *278*, 34783 – 34793.

[19] Lee, H.-J., Wang, C.-J., Kuo, H.-C., Chou, F.-P. *et al.*, Induction apoptosis of luteolin in human hepatoma HepG2 cells involving mitochondria translocation of Bax/Bak and activation of JNK. *Toxicol. Appl. Pharmacol.* 2005, *203*, 124 – 131.

[20] Zhang, X.-M., Chen, J., Xia, Y.-G., Xu, Q., Apoptosis of murine melanoma B16-BL6 cells induced by quercetin targeting mitochondria, inhibiting expression of PKC-alfa and translocating PKC-delta. *Cancer Chemother. Pharmacol.* 2005, *55*, 251 – 262.

[21] Zhu, B., Yao, Z., Luo, S., Jiang, L. *et al.*, Effects of antisense oligonucleotides of PKC-a on proliferation and apoptosis of HepG2 in vitro. *Hepatolobilliary Pancreat. Dis. Int.* 2005, *4*, 75 – 79.

[22] Yoshida, K., PKCd signaling: Mechanisms of DNA damage response and apoptosis. *Cell. Signal.* 2007, *19*, 782 – 901.

© 2008 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

[23] Zhao, Y., You, H., Yang, Y., Wei, L. *et al.*, Distinctive regulation and function of PI 3K/Akt and MAPKs in doxorubicin-induced apoptosis of human lung adenocarcinoma cells. *J. Cell. Biochem.* 2004, *91*, 621 – 632.

[24] Sharma, S., Gajowniczek, P., Way, I., Lee, D. *et al.*, A common signaling cascade may underlie 'addiction' to the Src, BCR-ABL, and EGF receptor oncogenes. *Cancer Cell* 2006, *10*, 425 – 435.

[25] Valverde, A., Navarro, P., Teruel, T., Conejo, R. *et al.*, Insulin and insulin-like growth factor I up-regulate GLUT-4 gene expression in fetal brown adipocytes in a phosphoinositide 3-kinase-dependent manner. *Biochem. J.* 1999, *337*, 397 – 405.

[26] Pérez-Vizcaíno, F., Bishop-Bailley, D., Lodi, F., Duarte, J. *et al.*, The flavonoid quercetin induces apoptosis and inhibits JNK activation in intimal vascular smooth muscle cells. *Biochem. Biophys. Res. Commun.* 2006, *346*, 919 – 925.

[27] Horbinski, C., Chu, C., Kinase signaling cascades in the mitochondrion: A matter of life or death. *Free Radic. Biol. Med.* 2005, *38*, 2 – 11.

[28] Tammela, P., Ekokoski, E., García-Horsman, A., Talman, V. *et al.*, Screening of natural compounds and their derivatives as potential kinase C inhibitors. *Drug Dev. Res.* 2004, *63*, 76 – 87.

[29] Kaufmann, S., Earnshaw, W., Induction of apoptosis by cancer chemotherapy. *Exp. Cell Res.* 2000, *256*, 42 – 49.

[30] Wätjen, W., Michels, G., Steffan, B., Niering, P. *et al.*, Low concentrations of flavonoids are protective in rat H4IIE cells whereas high concentrations cause DNA damage and apoptosis. *J. Nutr.* 2005, *135*, 525 – 531.

[31] Manach, C., Morand, C., Demigné, C., Texier, O. *et al.*, Bioavailability of rutin and quercetin in rats. *FEBS Lett.* 1997, *409*, 12 – 16.

[32] Lee-Hilz, Y., Boerboom, A.-M. J., Westphal, A., van Berkel, W. *et al.*, Pro-oxidant activity of flavonoids induces EpRE-mediated gene expression. *Chem. Res. Toxicol.* 2006, *19*, 1499 – 1505.

[33] Nakanishi, K., Sakamoto, M., Yamasaki, S., Todo, S., Hirohashi, S., Akt phosphorylation is a risk factor for early disease recurrence and poor prognosis in hepatocellular carcinoma. *Cancer* 2005, *103*, 307 – 312.

[34] Mitsui, H., Takuwa, N., Maruyama, T., Maekawa, H. *et al.*, The MEK1-ERK map kinase pathway and the PI 3-kinase-Akt pathway independently mediate anti-apoptotic signals in HepG2 liver cancer cells. *Int. J. Cancer* 2001, *92*, 55 – 62.

[35] Hsu, S., Bollag, W., Huang, L., Singh, B. *et al.*, Green tea polyphenols induce differentiation and proliferation in epidermal keratinocytes. *J. Pharmacol. Exp. Ther.* 2003, *306*, 29 – 34.

[36] Czaja, M., The future of GI and liver research: Editorial perspectives III. JNK/AP-1 regulation of hepatocyte death. *Am. J. Physiol. Gastrointest. Liver Physiol.* 2003, *284*, G875 – G879.

[37] Yeh, C., Yen, G., Induction of apoptosis by the anthocyanidins through regulation of Bcl-2 gene and activation of c-Jun N-terminal kinase cascade in hepatoma cells. *J. Agric. Food Chem.* 2005, *53*, 1740 – 1749.

[38] Kang, T., Ling, N., Studies on the inhibitory effects of quercetin on the growth of HL-60 leukemia cells. *Biochem. Pharmacol.* 1997, *54*, 1013 – 1018.

[39] Leverrier, S., Vallentin, A., Joubert, D., Positive feedback of protein kinase C proteolytic actvation during apoptosis. *Biochem. J.* 2002, *368*, 905 – 913.

[40] Cerda, S., Mustafi, R., Little, H., Cohen, G. *et al.*, Protein kinase C delta inhibits Caco-2 cell proliferation by selective changes in cell cycle and cell death regulators. *Oncogene* 2006, *25*, 3123 – 3138.

[41] Jarvis, W., Auer, K., Spector, M., Kunos, G. *et al.*, Positive and negative regulation of JNK1 by protein kinase C and p42 MAPkinase in adult rat hepatocytes. *FEBS Lett.* 1997, *412*, 9 – 14.

© 2008 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim