Research Article

Quercetin-induced apoptotic cascade in cancer cells: Antioxidant *versus* estrogen receptor α-dependent mechanisms

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The flavonol quercetin, especially abundant in apple, wine, and onions, is reported to have anti-proliferative effects in many cancer cell lines. Antioxidant or pro-oxidant activities and kinase inhibition have been proposed as molecular mechanisms for these effects. In addition, an estrogenic activity has been observed but, at the present, it is poorly understood whether this latter activity plays a role in the quercetin-induced anti-proliferative effects. Here, we studied the molecular mechanisms of quercetin committed to the generation of an apoptotic cascade in cancer cells devoid or containing transfected estrogen receptor α (ERα; *i.e.*, human cervix epitheloid carcinoma HeLa cells). Although none of tested quercetin concentrations increase reactive oxygen species (ROS) generation in HeLa cells, quercetin stimulation prevents the H_2O_2-induced ROS production both in the presence and in the absence of ERα. However, this flavonoid induces the activation of p38/MAPK, leading to the pro-apoptotic caspase-3 activation and to the poly(ADP-ribose) polymerase cleavage only in the presence of ERα. Notably, no down-regulation of survival kinases (*i.e.*, AKT and ERK) was reported. Taken together, these findings suggest that quercetin results in HeLa cell death through an ERα-dependent mechanism involving caspase- and p38 kinase activation. These findings indicate new potential chemopreventive actions of flavonoids on cancer growth.

Keywords: Antioxidant/pro-oxidant / Apoptotic cascade / Cancer cells / Estrogen receptor α / Quercetin

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1 Introduction

Flavonoids are phenolic compounds widely present in fruits, vegetables, cereals, dry legumes, chocolate, and plant-derived beverages, such as tea, coffee, and wine representing, therefore, the most abundant "minor components" in the diet [1].

Flavonoids have multiple biological, pharmacological and therapeutic properties including anti-neoplastic and cytoprotective effects. The close relationship between diet and cancer is suggested by the large variation in rates of specific cancers in different countries and by the spectacular changes observed in the incidence of cancer in migrating populations [2–6]. These observations are strengthened by many epidemiological, animal and cell culture studies [7–10]. Moreover, the ability of plant-based food to block the progression of tumors by directly inducing apoptosis has been reported [6, 11–16].

In general, any of the beneficial effects observed in epidemiological or intervention studies in association with fruit and vegetables intake have been interpreted on the basis that a "high intake of antioxidant-rich foods is inversely related to cancer risk" [17]. As a consequence flavonoids might protect against cancer through inhibition of oxidative damage [18, 19]. The theoretical basis of this protection is well known since the chemical structure of flavonoids is compatible with a one-electron donor activity. They have been demonstrated to function as antioxidants *in vitro* both in cell cultures and cell-free systems by scavenging superoxide anion, singlet oxygen, lipid peroxy-radicals, and/or stabilizing free radicals involved in oxidative proc-

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Abbreviations: DCF, dichlorodihydrofluorescein diacetate; **E2**, 17β-estradiol; **EGF-R**, epidermal growth factor receptor; **ERα**, estrogen receptor α; **ERE**, estrogen responsive element; **ERK**, extracellular regulated kinase; **PARP**, poly(ADP-ribose) polymerase; **PI3K**, phosphatidylinositol 3 kinase; **ROS**, reactive oxygen species

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esses through hydrogenation or complexing with oxidizing species [3, 18, 19]. However, *in vitro* experiments reported that most of dietary compounds with therapeutical properties act as potent pro-oxidants molecules [20, 21] at high concentrations or in presence of transition metals [22]. This pro-oxidant effect increases reactive oxygen species (ROS) production which, acting as second messengers of signaling networks, may induce growth arrest and apoptosis [23].

The different pro-oxidant/antioxidant activities of flavonoids have been usually considered beneficial for the human health and according to this hypothesis, a huge number of preparations are commercially available on the market in the form of plant extracts or mixtures, containing varying amounts of isolated phytochemicals as dietary supplements and as health food products. The commercial success of these supplements is evident, even though several activities and mechanisms, in part or totally independent from phytochemical participation to the intracellular redox balance, have been also described [24–26]. In fact, a spectrum of cellular effects not directly related to the pro-oxidant/antioxidant capacity has been recently reported widening the perspective of research on the relationship between nutrition and health.

It has been proposed that flavonoids have a protective effect on the initiation and/or progression of cancer by inhibiting the epidermal growth factor receptor (EGF-R) tyrosine kinase activity [27]. Many investigators have attributed the growth inhibition effects of flavonoids to the inhibition of the enzymes involved in proliferation and cell survival including protein kinase C, tyrosine kinase, phosphatidyl inositol 3 kinase (PI3K), and extracellular regulated kinase (ERK) [4, 28, 29]. Different flavonoids bind both estrogen receptors (ERα and ERβ) thus modulating the 17β-estradiol (E2)-induced gene transcription. We recently reported that the flavanone naringenin exerts antiproliferative and pro-apoptotic effects by altering selectively ERα signaling, which is important for cyclin D_1 transcription and cell proliferation [14, 15, 30].

Therefore, distinct action mechanisms, possibly interacting with one another, for nutritional antioxidant molecules on cell signaling and response can be evoked. As a result, the antioxidant properties could be considered a simplified approach to the function of molecules of nutritional interest due to the fact that their antioxidant capacities are a chemical property that is not necessarily associated to an equivalent biological function [31]. Accordingly, more recently, the impact of flavonoids and, in general, of polyphenols as antioxidants has been reconsidered and questioned, following the evidence that the molecular basis of their activity is much larger than originally considered [32, 33].

The present study was undertaken to examine the molecular mechanism(s) underlying the growth inhibition and cell death effects of quercetin, one of the most frequently studied and ubiquitous bioactive flavonoid, the action mechanisms of which remains poorly understood.

The human cervix epitheloid carcinoma cell line (HeLa) either devoid of any ER isoforms or transiently transfected with the human ERα expression vector were used to reveal the contribution of antioxidant activities, kinases inhibition, and/or ER-dependent mechanism in quercetin-induced anti-proliferative effects.

2 Materials and methods

2.1 Reagents

17β-Estradiol, quercetin, L-glutamine, gentamicin, trypsin, penicillin, DMEM (with and without phenol red), charcoal-stripped fetal calf serum, and GenElute plasmid maxiprep kit were purchased from Sigma-Aldrich (St. Louis, MO, USA). The p38/MAPK inhibitor SB 203580 was purchased from Calbiochem (San Diego, CA, USA) and the ER inhibitor ICI 182,780 was obtained from Tocris (Ballwin, MO, USA). Lipofectamine reagent was obtained from GIBCO-BRL Life-technology (Gaithersburg, MD, USA). The luciferase kit was obtained from Promega (Madison, WI, USA). Bradford protein assay was obtained from Bio-Rad Laboratories (Hercules, CA, USA). The polyclonal anti-phospho-AKT, anti-p38 and anti-phospho-p38 antibodies were obtained from Cell Signaling Technology (Danvers, MA, USA); the polyclonal anti-ERK and the monoclonal anti-phospho-ERK, anti-AKT, anti-caspase-3, anti-poly-(ADP-ribose) polymerase (PARP), and anti-β-actin antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). ECL chemiluminescence reagent for Western blot was obtained from Amersham Bioscience (Uppsala, Sweden).

All other products were obtained from Sigma-Aldrich. Analytical or reagent grade products, without further purification, were used.

2.2 Plasmids, transfection and luciferase assay

The gene reporter plasmids pC3-luciferase (pC3), expression vectors for pCR3.1-β-galactosidase, human pHE0-hERa, and an empty vector, pCMV5, were used. Plasmids were purified for transfection using a plasmid preparation kit according to manufacturer's instructions. A luciferase dose response curve showed that the maximum effect was present when 1 μg plasmid was transfected together with 1 μg pCR3.1-β-galactosidase to normalize for transfection efficiency (~55–65%). HeLa cells were grown to (70% confluence, then transfected with different expression vectors using Lipofectamine Reagent according to the manufacturer's instructions. At 6 h after transfection the medium was changed and 24 h thereafter cells were stimulated as reported.

In some experiments, after transfection, cells were stimulated with different concentrations of E2 or quercetin for 6 h. The cell lysis procedure as well as the subsequent

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measurement of luciferase gene expression was performed using the luciferase kit according to the manufacturer's instructions with an EC & G Berthold luminometer (Bad Wildbad, Germany).

2.3 Cell culture and cell cycle analysis

HeLa cells were routinely grown in air containing 5% CO_2 in modified, phenol red-free, DMEM medium containing 10% charcoal-stripped fetal calf serum, L-glutamine (2 mmol/L), gentamicin (10 mg/mL) and penicillin (100 U/mL). Cells were passaged every 2 days with 0.5 mL trypsin (0.05%) and media changed every 2 days. At 30 h after treatment, cells were harvested with trypsin, centrifuged, stained with trypan blue solution, and counted in a hemocytometer (improved Neubauer chamber) in quadruplicate.

HeLa cells (10^6 cells) were transfected with human pHE0-ERα or pCMV5 expression vectors and, 24 h after, stimulated with quercetin for 30 h in the presence or absence of the ER inhibitor ICI 182,780 (1 μmol/L). After stimulation cells were fixed with 1 mL ice-cold 70% ethanol and subsequently stained with 2 mg/mL DAPI/PBS solution. The fluorescence of DNA was measured with DAKO Galaxyflow cytometer equipped with HBO mercury lamp and the percentage of cells present in sub-G1 phase was calculated using a FloMax© Software.

2.4 Measurements of ROS

HeLa cells were grown to ~70% confluence, and then transfected with different expression vectors. Cells were harvested, and re-suspended in PBS with 10 μmol/L dichlorodihydrofluorescein diacetate (DCF; Molecular Probes, Eugene, OR, USA) for 30 min at 37°C in the dark. After equilibrating for 30 min, the fluorescence was measured under continuous gentle magnetic stirring at 37°C in a Perkin-Elmer LS-50B spectrofluorimeter. Excitation wavelengths were set at 498 nm and emission at 530 nm. Cells were treated with quercetin (1–50 μmol/L) or DMSO (vehicle) for 15 min and fluorescence was registered as arbitrary units. In some experiments 600 μmol/L H_2O_2 (final concentration) was added after quercetin stimulation and fluorescence was registered after 15 min.

2.5 Electrophoresis and immunoblotting

When indicated, 5 μmol/L p38 inhibitor SB 203580 or 1 μmol/L ER inhibitor ICI 182,780 was added 15 min before treatment with different concentration of quercetin for different times. HeLa (transfected with pHE0-hERα or pCMV5 expression vectors) and HepG2 were lysed and solubilized in 0.125 mol/L Tris-HCl (pH 6.8) containing 10% SDS, 1 mmol/L PMSF and 5 μg/mL leupeptin and boiled for 2 min. Proteins were quantified using the Bradford protein assay. Solubilized proteins (20 μg) were resolved using 10% SDS-PAGE at 100 V for 1 h. The proteins were then electrophoretically transferred to nitrocellulose overnight at 30 V at 4°C. The nitrocellulose was treated with 3% BSA in 138 mmol/L NaCl, 26.8 mmol/L KCl, 25 mmol/L Tris-HCl (pH 8.0), 0.05% Tween-20, 0.1% BSA, and then probed at 4°C overnight with either one of anti-phospho-ERK, anti-phospho-AKT, anti-phospho-p38, anti-caspase-3, or anti-PARP antibodies (1 μg/mL). The nitrocellulose was stripped by Restore Western Blot Stripping Buffer (Pierce Chemical Company, Rockford, IL, USA) for 10 min at room temperature and then probed with either one of anti-ERK, anti-AKT, anti-p38 (1 μg/mL) antibodies. Anti-β-actin antibody (1 μg/mL) was used to normalize the sample loading. Antibody reaction was visualized with chemiluminescence reagent for Western blot. The densitometric analyses were performed by ImageJ software for Windows.

2.6 Statistical analysis

A statistical analysis was performed using Student's *t*-test with the GraphPad INSTAT3 software system for Windows. In all cases p values below 0.05 were considered significant.

3 Results

3.1 Quercetin effect on HeLa cell growth

The growth of HeLa cells transfected with either the empty plasmid or ERα expression vectors was examined at 30 h after stimulation of different concentrations of quercetin. Empty plasmid-transfected HeLa cells growth was not affected by any concentration of quercetin, suggesting that the presence of ERα is necessary for the flavonoid effects (Fig. 1a). On the other hand, quercetin stimulation decreased the number of ERα-transfected HeLa cells with respect to un-stimulated cells (Fig. 1a) in a dose-dependent manner within the range utilized (0.001–100 μmol/L). The quercetin effect on the cell number after 30 h of stimulation was compared with the effect of E2. As expected [34], 0.001–0.1 μmol/L E2 increased cell number only in ERα-containing HeLa cells (Fig. 1b). Moreover, quercetin stimulation of ERα transfected HeLa cell still decreased cell number even in the presence of 0.01 μmol/L E2 (Fig. 1c).

3.2 Quercetin as pro-oxidant/antioxidant

To determine whether quercetin induces ROS generation, HeLa cells, transfected with empty or ERα vectors, were exposed to different concentration of quercetin (1–50 μmol/L) or 600 μmol/L H_2O_2 and changes in DCF fluorescence were measured. Neither vehicle (Fig. 2a) nor quercetin (50 μmol/L) (Fig. 2c) caused any increase in ROS generation after 15 min of treatment in both ERα-contain-

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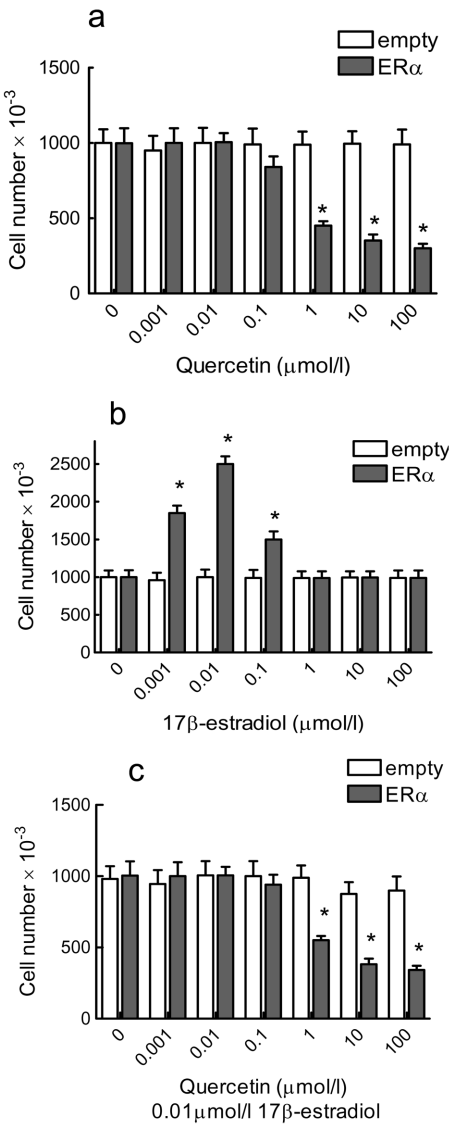

Figure 1. Quercetin effect on HeLa cell growth. HeLa cells, transfected with empty (open bars) or human ERα (filled bars) expression vectors, were grown for 30 h in the presence of different concentration of quercetin (a) or 17β-estradiol (E2, b). In (c), HeLa cells were stimulated with different quercetin concentrations in the presence of 0.01 μmol/L E2. Data are the mean ± SD of four independent experiments carried out in duplicate. * $p < 0.001$, calculated with Student's *t*-test, was compared with non-stimulated control values (0, vehicle).

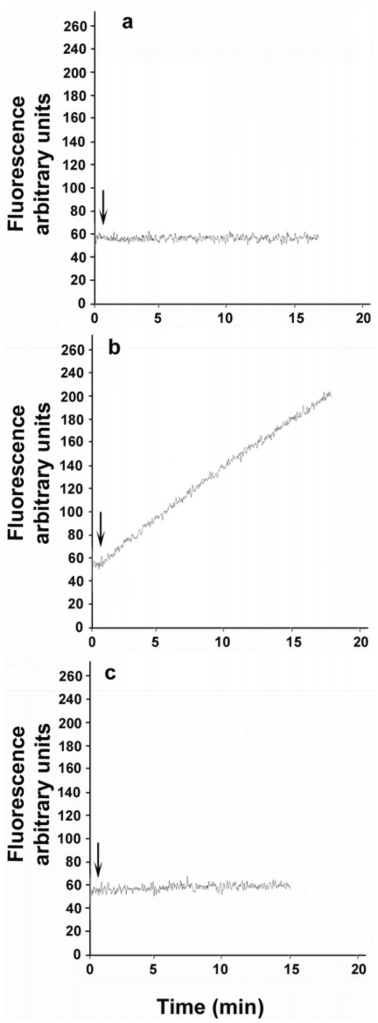

Figure 2. Quercetin effect on ROS generation. HeLa cells, transfected with ERα expression vector, were exposed to vehicle (a) or H_2O_2 (600 μmol/L, b) or quercetin (50 μmol/L, c) and changes in DCF fluorescence were measured. Panels represent original outputs (arbitrary units) of the registrations captured by the spectrofluorimeter during 15-min substance administration.

ing and ERα-devoid (data not shown) HeLa cells, whereas H_2O_2 (600 μmol/L, Fig. 2b) caused a rapid and marked increase in ROS generation (284 ± 18% over the control). Similar results were obtained at all tested quercetin concentrations (1–50 μmol/L, data not shown).

On the other hand, quercetin prevented, in a dose-response manner, H_2O_2-induced ROS production both ERα-devoid (Fig. 3a) and ERα-containing (Fig. 3b) HeLa cells. In fact, 1 μmol/L quercetin impaired ROS production of about 50%, while 50 μmol/L completely blocked H_2O_2-induced ROS production, confirming the potent antioxidant effect of this flavonoid.

3.3 Effect of quercetin as kinase inhibitor

ERK and AKT play a pivotal role in cell proliferation, differentiation, and survival. It has been suggested that quercetin-induced cell death could be caused by down-regulating these kinases [4]. To test this possibility, HeLa cells containing ERα or devoid of ERα were exposed to 1–50 μmol/L quercetin for various times and activation of ERK and AKT was evaluated by detecting their phosphorylation forms. In ERα-containing or ERα-devoid HeLa cells quer-

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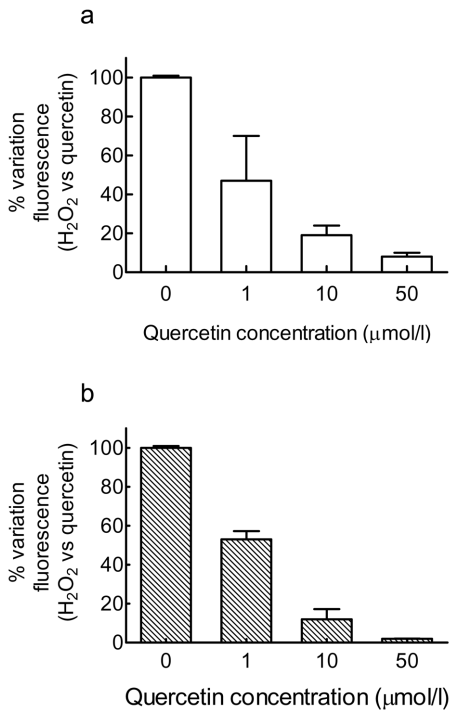

Figure 3. Quercetin effect on H_2O_2-induced ROS production. HeLa cells, transfected with empty (a) or human ERα (b) expression vectors, were pre-treated with different quercetin concentrations before exposure to vehicle (0) or H_2O_2 (600 μmol/L). Data, expressed as % of variation between H_2O_2-stimulated fluorescence *versus* quercetin-stimulated fluorescence, are the mean ± SD of three independent experiments carried out in duplicate.

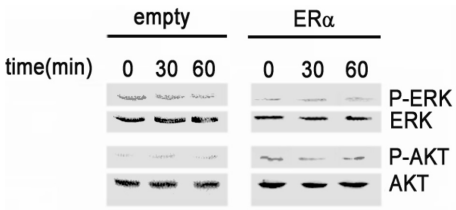

Figure 4. Quercetin effect on ERK and AKT activity. Western blot analysis of phosphorylated and un-phosphorylated ERK, and AKT, were performed, as described in Section 2, on HeLa cells transfected with empty or human ERα expression vectors, stimulated with vehicle (0) or for 30 or 60 min with quercetin (1 μmol/L). Typical blot chosen among three independent experiments. For details see the main text.

cetin failed to induce ERK and AKT phosphorylation (Fig. 4) at any of tested concentrations (data not shown). Notably, quercetin did not decrease the basal, constitutive, phosphorylation status of either kinases or the expression level of total ERK and AKT (*i.e.*, phosphorylated and non-phosphorylated) (Fig. 4).

Previously, we reported that the flavanone naringenin, in the presence of ERα, drives the cells out of cell cycle by the pro-apoptotic p38 pathway activation [14]. Thus, we evaluated the ability of quercetin in modulating this ERα-dependent kinase activation. Quercetin induced a rapid (15 min) and sustained (120 min) increase of p38/MAPK phosphorylation only in ERα-transfected HeLa cells (Fig. 5), suggesting that quercetin induces the ERα-dependent activation of a pro-apoptotic cascade.

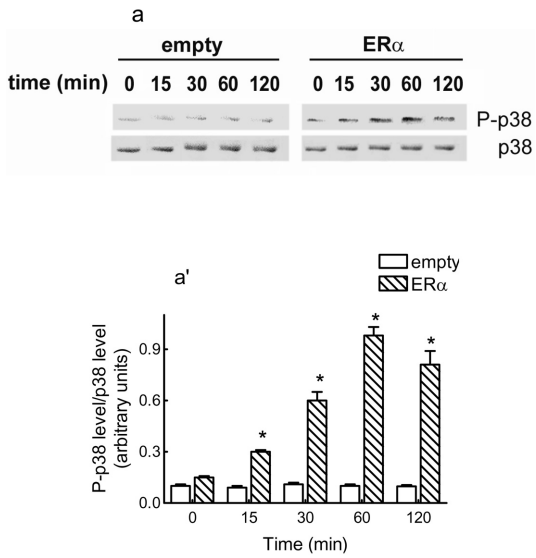

Figure 5. Quercetin effect on p38 activity. Western blot analysis of phosphorylated and un-phosphorylated p38 were performed, as described in Section 2, on HeLa cells transfected with empty or human ERα expression vectors, stimulated with vehicle (0) or quercetin (1 μmol/L) at different times. (a) A typical blot of three independent experiments; (a′) the densitometric analysis. Data are the mean ± SD. * $p < 0.001$, calculated with Student's *t*-test, was compared with non-stimulated control values (0, vehicle).

3.4 Quercetin as pro-apoptotic agent

To verify that the decreased cell number reported in Fig. 1 is associated to the quercetin-induced apoptosis, flow cytometry of ERα-containing or ERα-devoid HeLa cells was performed 30 h after flavonoid treatment. The typical plot of transfected-HeLa cell population is illustrated in Fig. 6 (Control). The first peak indicates the cell number present in G1 phase of the cell cycle (51.0 ± 7%) followed by S phase (15.4 ± 3.5%), and by the peak of G2/M phase (19.6 ± 2.9%). This trend was unchanged after quercetin stimulation of empty vector-transfected HeLa cells (Fig. 6, lane empty). On the other hand, in the presence of ERα the cell number present in sub-G1 region increased 30 h after quercetin stimulation (Fig. 6, line ERα), strongly suggesting the presence of DNA fragmentation. This quercetin effect was completely blocked pre-treating cells with the ER inhibitor ICI 182,780 (Fig. 6, lane quercetin+ICI).

To determine whether the quercetin-induced increase of cell population present in the sub-G1 phase was related to the induction of an apoptotic cascade, we analyzed the acti-

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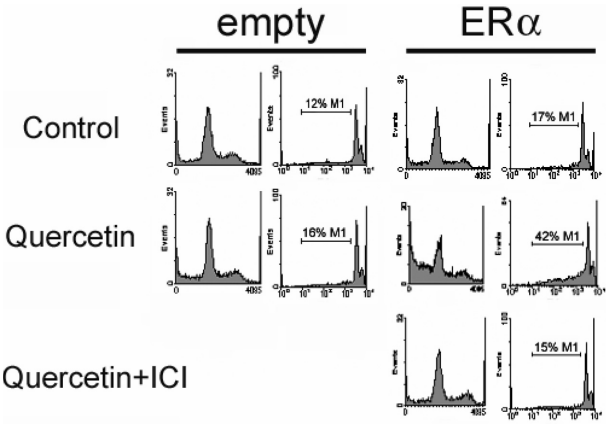

Figure 6. Cell cycle distribution (left panels) or the cell number present in sub-G1 phase (right panels) was performed by cyto-fluorimetric analysis of HeLa cells transfected with empty or human ERα expression vectors after 30 h of cell treatment with quercetin (1 μmol/L) in the presence or absence of the ER inhibitor ICI 182,780 (ICI, 1 μmol/L) and compared with cells treated with vehicle (Control).

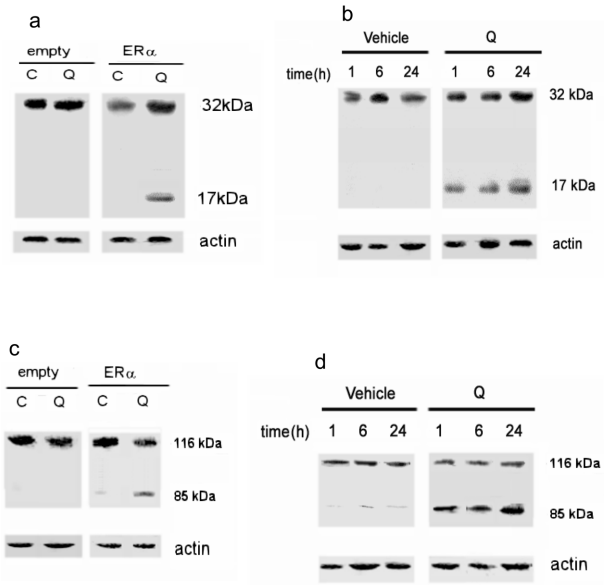

Figure 7. Western blot analysis of caspase-3 (a and b) and PARP (c and d) activation was performed on HeLa cells transfected with empty or human ERα expression vectors treated for 24 h with vehicle (C) or quercetin (Q, 1 μmol/L) or at different times. Typical blot of threre independent experiments. For details see the main text.

vation of the caspase-3 proform and the cleavage of its substrate PARP, well-known markers of apoptosis in several cell types. The 32-kDa proform of caspase-3 was expressed in HeLa cells transfected with either empty or ERα expression vectors (Fig. 7a). The 17-kDa band corresponding to the active subunit of caspase-3 was present only in ERα-containing HeLa cells stimulated for 1–24 h with quercetin (Figs. 7a and b).

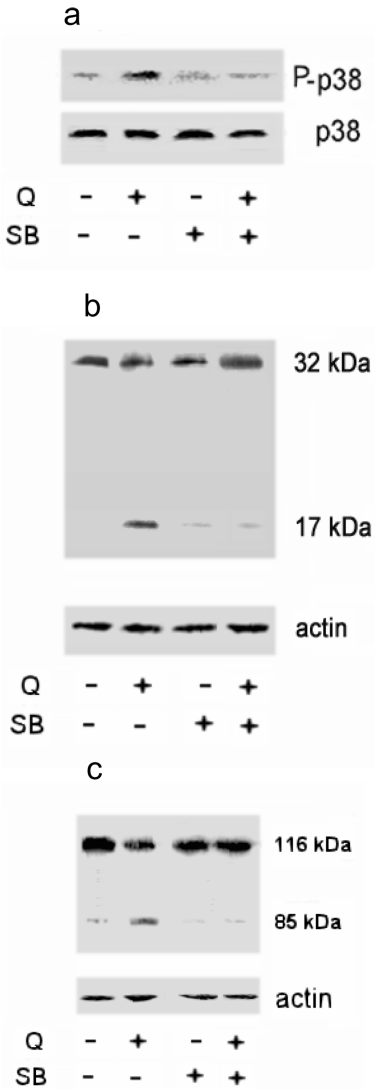

Figure 8. Effects of quercetin (1 μmol/L) on p38 (a), caspase-3 (b), and PARP (c) activation in ERα-transfected HeLa cells. When indicated 5 μmol/L of p38 inhibitor, SB 203580 (SB), was added 15 min before quercetin administration. Typical blot of three independent experiments. For details see the main text.

To confirm that the quercetin-induced appearance of the 17-kDa band was associated with an increase in caspase-3 activity, we analyzed the cleavage of the caspase-3 substrate, the DNA repair enzyme PARP. By Western blot analysis, the treatment of ERα-transfected HeLa cells with quercetin resulted in the conversion of 116-kDa PARP into its inactive 85-kDa fragment (Figs. 7c and d). This result is consistent with the idea that quercetin specifically activated an apoptotic cascade involving the caspase-3 activation and its downstream substrate PARP only in the presence of ERα.

The p38/MAPK pathway involvement in quercetin-induced apoptotic cascade was confirmed by the pre-treatment of transfected cells with the specific p38 inhibitor, SB

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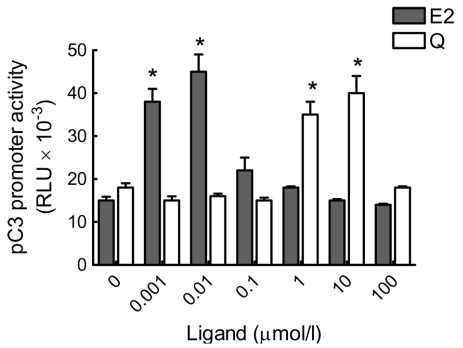

Figure 9. Effect of 17β -estradiol (E2) and quercetin (Q) on pC3 promoter activity. Dose-response curves (0.001–100 μmol/L) of luciferase assay detection on HeLa cells co-transfected with ERα expression vector and pC3-luciferase construct and then treated for 6 h with vehicle (0) or ligands. Data are the means ± SD of four independent experiments. * $p < 0.001$, compared with respective control values (0), was determined using Student's *t*-test.

203580. This inhibitor completely prevented the quercetin-induced p38 phosphorylation (Fig. 8a), caspase-3 activation (Fig. 8b) and PARP cleavage (Fig. 8c), thus linking the rapid quercetin-induced p38/MAPK phosphorylation to the activation of apoptotic cascade.

3.5 Quercetin as modulator of ERα transcriptional activity

Our final target was to assess the effects of quercetin, in comparison with E2, on a well-known E2:ERα complex modulated cell function: estrogen responsive element (ERE)-containing gene transcription. Figure 9 shows the dose-dependent effect of E2 and quercetin on pC3 promoter activity. Although at higher concentration, quercetin induced ERE-containing promoter to a level comparable to that of E2 in HeLa cells transfected with ERα expression vector. No transcriptional activity was present when HeLa cells, transiently transfected with the empty plasmid, were stimulated with the different ERα ligands (data not shown). These results confirm that quercetin can trigger the ERα-mediated genomic mechanism mimicking E2 effects.

4 Discussion

The antioxidant hypothesis at the root of protective effects of dietary components against degenerative diseases states that these compounds (*e.g.*, urate, ascorbate and tocopherols) act as antioxidants *in vivo* and consequently lower the incidence of some diseases [35–39]. The effects of antioxidant supplementation have been studied by many intervention trials, but the results are still controversial [40]. Consequently, the value of supplementation with exogenous antioxidants is increasingly questioned [33, 35, 38, 40]. Flavonoids have since been widely assumed to be included in the class of *in vivo* antioxidant compounds, a concept further inferred from their *in vitro* antioxidant properties. Within the flavonoid family, quercetin seems to be the most potent scavenger of ROS [18, 41] and reactive nitrogen species [42, 43]. Quercetin is suggested to substantially empower the endogenous antioxidant shield due to its contribution to the total plasma antioxidant capacity, which is 6.24 times higher than the reference antioxidant trolox [44]. Our findings confirm that quercetin is an excellent antioxidant *in vitro*, in fact, 50 μmol/L quercetin prevented the H_2O_2-induced ROS production by 95%. However, smallest amount of quercetin (*i.e.*, 1 μmol/L) activates a pro-apoptotic cascade only in ERα-transfected HeLa cells.

Normally, human quercetin plasma concentrations are in the low nanomolar range, but upon quercetin supplementation they may increase to the high nanomolar or low micromolar range [39], whereas the concentration of circulating endogenous antioxidant, ascorbate or urate, has been estimated to be in the range of 159–380 μmol/L for a normal individual [38]. Thus, flavonoids do not appear to be present in the circulation at high enough concentrations to contribute significantly to total antioxidant capacity. The increase in plasma total antioxidant capacity from apple consumption (rich in quercetin) has been explained by a ~37% increase in urate concentration, as a consequence of fructose metabolism, with no detectable effect associated with the apple flavonoids [45].

Moreover, it has been suggested that quercetin and other flavonoids might exert protective effects against cancer cell growth associated with generation of ROS [46, 47]. Indeed, in the last few years it has been emerging that ROS are not only the downstream damaging species produced by radical chain reactions, but also the second messengers of signaling networks [23]. Our results indicated that quercetin did not increase ROS generation in HeLa cells. These results, consistent with findings in human promyeloleukemic HL-60 cells [48] and glioma cells [4], render unlikely that quercetin-induced HeLa cell death is linked to the activation of ROS-dependent signal generation.

Flavonoid effects on cancer progression in malignant cells could be related to the modulation of signal transduction pathways involved in cell proliferation and apoptosis. Several lines of evidence described an important role for ERK and AKT in cell survival signaling and tumorigenesis [49–52]. To explore the underlying mechanism of quercetin-induced cell death, the effect on activation of ERK and AKT was examined. HeLa cells transfected with the empty and ERα expression vectors contain a detectable amount of activated kinases, but quercetin stimulation did not modify the phosphorylation levels of ERK and AKT in both cell lines. As a whole, it seems to be very improbable that pro-oxidant/antioxidant effects and the inhibition of protein kinases may underlie the anti-proliferative effects of flavonoids reported in Fig. 1.

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The present data indicate, for the first time, that the flavonol quercetin inhibits cancer cell proliferation by ERα-dependent p38 pathway activation. This occurs at quercetin concentration physiologically achievable in the plasma after the consumption of meals rich in this flavonol. Ample evidence indicates that the p38 pathway serves an important role in stress and immune response [53, 54]. Furthermore, p38 pathway has been associated with a significant slowing in cell proliferation and with the regulation of the apoptosis [55]. In particular, p38 can sensitize cells to apoptosis through the positive regulation of Fas/CD-95 and Bax expression, which, in turn, activate caspase cascades [55]. We previously showed that E2 induced different effects on the pro-apoptotic p38 pathway depending on the ER isoforms present [34]. In fact, the E2:ERα complex rapidly (15 min) activated the proliferative ERK, PI3K/AKT, and the pro-apoptotic p38 signal transduction pathways. After 30 min of E2 stimulation the expression of Bcl-2, an anti-apoptotic protein, was enhanced and the activation of the p38 was blocked in ERK-dependent manner, thus hampering the apoptosis cascade activation [34].

Present data indicate that quercetin decouples the ERα action mechanisms impairing the ERα ability to activate ERK and PI3K/AKT signal transduction pathways and allowing the sustained ERα-dependent p38 phosphorylation and the down stream caspase-3 activation and PARP cleavage. Recently, we reported a similar effect by stimulating cancer cells with the flavanone naringenin [30]. In particular, naringenin induces the rapid ERα dissociation from plasma membrane and caveolin-1, thus impeding ERα to bind adaptor (MNAR) and signaling (c-Src) proteins involved in the activation of the E2-induced mitogenic/survival signaling cascades (*i.e.*, ERK and AKT) [30]. On the other hand, naringenin induced the ER-dependent p38 kinase activation and apoptosis cascade [14, 30]. Notably, both naringenin [14] and quercetin (present data) still stimulated the activity of ERE-luciferase reporter gene construct in the presence of ERα, strongly suggesting a role of partial agonists for these flavonoids. Thus, both flavonoids influence cancer cell proliferation by acting as selective antagonist of ERα-mediated non-genomic activities. This implies that flavonoids could work as ERα antagonist on specific pathways in that they can selectively activate specific E2-signaling pathways in different organs, resulting in the beneficial effects of E2 in non-reproductive tissues without adverse effects in reproductive organs.

In conclusion, molecules having a chemical structure compatible with a strong putative antioxidant capacity can actually perform activities and roles totally independent from such capacity, and interact with cellular functions at different levels. Diet-health relationship is obviously very complex and food items most probably act through multiple pathways, and the isolation of the specific role of a single component is very difficult. Understanding the cross-talk within these different pathways and the elaborate feedback mechanisms will provide an opportunity to employ these chemicals as novel therapeutics to direct specific responses in target cells or to modulate selectively ER activities in specific target tissues and organs.

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