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Apigenin-induced-apoptosis is mediated by the activation of PKCδ and caspases in leukemia cells

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ABSTRACT

Apigenin, a flavone abundantly found in fruits and vegetables, exhibits antiproliferative, anti-inflammatory, and antimetastatic activities through poorly defined mechanisms. In the present study, the treatment of different cell lines with apigenin resulted in selective antiproliferative and apoptotic effect in monocytic and lymphocytic leukemias. Apigenin-induced-apoptosis was mediated by the activation of caspase-9 and caspase-3. Apigenin was found intracellularly and localized to the mitochondria. Treatment of monocytic cells with apigenin was accompanied by an increase in reactive oxygen species (ROS) and phosphorylation of the MAPKs, p38 and ERK. However, the inhibition of ROS, p38 or ERK failed to block apoptosis, suggesting that these cellular responses induced by apigenin are not essential for the induction of apoptosis. In addition, apigenin induced the activation of PKCδ. Pharmacological inhibition of PKCδ, the expression of dominant-negative PKCδ and silencing of PKCδ in leukemia cells showed that apigenin-induced-apoptosis requires PKCδ activity. Together, these results indicate that this flavonoid provides selective activity to promote caspase-dependent-apoptosis of leukemia cells and uncover an essential role of PKCδ during the induction of apoptosis by apigenin.

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1. Introduction

Apoptosis, or programmed cell death, plays a crucial role in normal development, homeostasis, and in the defense response against pathogens [1]. Essential executioners of apoptosis are the caspases, a family of conserved cysteine proteases [2]. The caspases are expressed as inactive precursors that become activated by apoptotic signals. Initiator caspases, such as caspase-9, receive the apoptotic signal and initiate the activation of caspase-3, a central

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caspase, responsible for cleaving specific cellular proteins during apoptosis [3]. Apoptosis is characterized by various biochemical and morphological changes, including nuclear fragmentation, which lead to the formation of apoptotic bodies [4,5]. Defects impairing the apoptotic machinery have been implicated in the pathogenesis of cancer [6].

Cell responses to apoptotic-inducing-drugs have been associated with ROS production, the inactivation of survival kinases, and the activation of apoptotic kinases. The mitogen-activated protein kinase (MAPK) family, which includes p38, JNK (Jun-N-terminal kinase), and ERK (extracellular signal-regulating kinase) govern, among others, cell proliferation, survival, and stress responses [7]. Activation of p38 is associated with the induction of apoptosis in response to UV-radiation and treatment with chemotherapeutic drugs [8–11]. ERK activation can exert either anti-apoptotic [12,13] or pro-apoptotic [14,15] effects, depending upon the stimuli and cell type. In addition to the MAPKs, members of the protein kinase C family (PKC) regulate survival and death. PKCδ activation was observed in several cell types in response to a variety of apoptotic stimuli [16–20]. Recently, we showed that caspase-3 phosphorylation by PKCδ acts as a pro-apoptotic signal during spontaneous and etoposide-induced apoptosis of monocytes [21].

Myeloblastic cells normally undergo spontaneous apoptosis through a mechanism that requires caspase-3 [22]. However, in leukemia, malignant transformation confers prolonged cellular survival mediated in part by the inhibition of the apoptotic program [6]. Current therapies for leukemia include the treatment with chemotherapeutic drugs to induce cell death. Thus, the search for new potential anti-cancer drugs is an area of active research. Flavonoids are ubiquitous phenolic compounds broadly distributed in fruits and vegetables [23]. Depending on the organization of their cyclic benzene rings and their modifications, flavonoids can be classified into various groups that include flavan-3-ols, flavones, isoflavones, flavanones, and flavonols. Flavonoids have long been recognized as having potential anti-inflammatory, antioxidant, antiviral, anti-microbial, and anti-allergic properties, providing important nutraceutical components of our diet [24–28]. The flavone apigenin and the flavonone naringenin have been previously shown to inhibit with different potency the proliferation of several cancer cells, including breast epithelial, colon cancer, and lymphocytic leukemia cells [29–32]. Naringenin was shown to impair glucose uptake [29], while apigenin induced cell cycle arrest at G2/M [31,33]. However, the molecular mechanisms by which apigenin induces apoptosis remain largely unknown.

In the present study, we examine the mechanisms that mediate apigenin-induced-apoptosis. Our results demonstrate that apigenin induced ROS production and the activation of the MAPK p38 and ERK. While these processes are induced by apigenin, we found that they are not essential for the execution of apoptosis. In contrast, we demonstrate that the activation of caspase-3 and PKCδ are required for apigenin-induced-apoptosis. Together, these studies provide evidence of the selective potential of apigenin to induce cell death in leukemia and uncover novel aspects of the mechanisms required for apigenin-induced-apoptosis.

2. Materials and methods

2.1. Cell culture and reagents

All cells were grown at 37 °C in a humidified atmosphere of 95% air and 5% CO_2 in media supplemented with 100 U/ml penicillin, and 100 μg/ml streptomycin (BioWhittaker). THP-1, U937, HL60, Jurkat, K562, and NIH3T3 fibroblast cells were maintained in RPMI 1640 medium with L-glutamine (BioWhittaker, Walkersville, MD) supplemented with 5% fetal bovine serum (FBS) (Hyclone, Logan, UT) while A549 cells were supplemented with 10% FBS. MCF-7 cells were maintained in DMEM (Gibco) with 5% FBS. Apigenin, naringenin, and the diluent dimethyl sulfoxide (DMSO) were obtained from Sigma–Aldrich (St. Louis, MO). The caspase inhibitor DEVD-FMK was obtained from Enzyme System Products (Livermore, CA). The p38 inhibitor SB203580, the MEK inhibitor PD98059, and the PKCδ inhibitor rottlerin were obtained from Calbiochem (San Diego, CA). The ROS inhibitor EUK-134 was obtained from Cayman Chemical (Ann Arbor, MI). The spin trap DMPO was obtained from Axxora (San Diego, CA).

2.2. Cell viability assay

Cells were plated at a density of 2×10^5 cells/ml into 96-well plates, treated with flavonoids at various concentrations for different lengths of time. Cell viability was assayed with CellTiter 96 Aqueous One Solution Cell Proliferation Assay following the conditions suggested by the manufacturer (Promega, Madison, WI). Absorbance at 490 nm (A_{490}) was meassured using an ELISA plate reader (Bio-Tek ELx800, Winooski, VT using the KC Junior software).

2.3. Assessment of cell survival and apoptosis and cellular staining

Cells were plated at a density of 1×10^6 cells/ml 24 h prior to treatment with flavonoids. After treatment, cells were collected, washed once with PBS and incubated in PBS with 1 μg/ml calcein AM for 30 min and 50 pg/ml propidium iodide (PI; Molecular Probes, Eugene, OR) for 5 min (excitation 490 nm, emission 530 nm; excitation 535 nm and emission 617 nm). Cells were rinsed twice with PBS and at least 200 cells were counted under the microscope. Cells stained green (calcein AM positive) in the absence of red (PI) were considered alive, while green and red cells were considered apoptotic. Percentage of apoptotic cells expresses the number of green and red cells over total number of cells. Intracellular apigenin was stained with 0.025% (w/v) of the flavonoid-stained diphenylboric acid 2-aminoethyl ester (DPBA; Sigma, USA; excitation 490 nm, emission 530 nm) for 1 min using as previously described [34,35]. Mitochondria staining was obtained with Mitotracker deep red (Molecular Probes, Eugene, OR; excitation 644, emission 665 nm). Cells were stained with 25 ng/ml for 30 min at 37 °C. Fluorescence was visualized on an epifluorescence microscope (Olympus, Melville, NY) and digital images were captured using Optronics Imaging System (Goleta, CA).

2.4. Caspase activity

Lysates from 3×10^6 cells were prepared and incubated in a cytobuffer as previously described [36]. Twenty μM DEVD-AFC was used to determine caspase-3 activity, LEHD-AFC to determine caspase-9, and LETD-AFC for caspase-8 [36]. Released AFC were measured using a Cytofluor 400 fluorimeter (Filters: excitation 400 nm, emission 508 nm; Perspective Co., Framingham, MA).

2.5. HPLC analysis and fractionation

Lysates from 5×10^6 cells were prepared in KPM buffer (50 mM KCl, 50 mM PIPES, 10 mM EGTA, 1.92 mM $MgCl_2$, pH 7, 1 mM DTT, 0.1 mM PMSF, 10 μg/ml cytochalasin B, and 2 μg/ml each of protease inhibitors chymostatin, pepstatin, leupeptin and antipain) by freezing and thawing. Cells were then spun at $100{,}000 \times g$ for 1 h at 4 °C. The supernant named ''supernantant S100'' in the text was transferred to an eppendorf tube while the S100 pellet containing membrane associated fractions was dissolved in DMSO vortexed and kept at −70 °C for further analysis. HPLC was performed using a Waters 2695 ''Alliance'' system equipped with a diode-array-detector (PDA 2996), and a C_{18} column (100 mm × 0.4 mm i.d.). The mobile phase system consisted of the following solvents: (A) H_2O and (B) MeOH–H_2O (90:10). Both solvents (A and B) were adjusted to pH 3.5 with 5% ammonium formate in formic acid. The linear gradient elution was performed employing the EmpowerPro program from (A): 100% (t = 0 min) to (B): 100% in a period 44 min at a flow rate 1 ml min^{-1}. The eluted apigenin was monitored at 280 nm, and identified by comparing the chromatographic retention time as well as absorption spectrum with that of authentic standard (Sigma, USA). All solvents were of HPLC-grade and were filtered and degassed before use.

2.6. Protein analysis by Western blot

Extracts from 3×10^6 cells were prepared by incubating them for 30 min on ice in lysis buffer (50 mM Tris, 10 mM EDTA, 0.5% NP-40, 10 mM Na-glycerophosphate, 5 mM Na-pyrophosphate, 50 mM NaF, 1 mM orthovanadate, 1 mM DTT, 0.1 mM PMSF, 2 μg/ml of protease inhibitors: chymostatin, pepstatin, antipain, and leupeptin). Cell lysates were centrifuged ($14{,}000 \times g$ for 10 min at 4 °C) and the supernatants were stored at −70 °C for future analysis. Equal amounts of protein were loaded and separated by SDS-PAGE, transferred onto nitrocellulose membranes and probed with antibodies of interest followed by horseradish peroxidase conjugated secondary antibody and visualized by enhanced chemiluminescence (Amersham, Arlington Heights, IL). Phospho-p38, phospho-ERK, total ERK, and total p38 antibodies were obtained from Cell Signaling (Boston, MA). PKCδ antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

2.7. Determination of reactive oxygen species (ROS)

1×10^6 cells/ml were treated for 30 min with 20 μM of the ROS scavenger EUK-134 (Cayman, Ann Arbor, MI) prior to the addition of apigenin. Cells were rinsed with PBS, and cultured in RPMI (no phenol red) for 30 min in the presence of 20 mM DMPO (Sigma), a spin trap for $O_2^{\bullet -}$. Cells were centrifuged for 5 min at 1000 rpm 4 °C and the supernatants were used to measure ROS production. Spin-trapping measurements of oxygen radical generation was performed using a Bruker ER 300 spectrometer (Billerica, MA). The samples were loaded into a quartz flat cell and measured at X-band in a TM_{110} cavity. Spectra were obtained using the following parameters: microwave power; 20 mW, modulation amplitude; 0.5 G, modulation frequency; 100 kHz. To quantify rates of $^{\bullet}O_2^{\bullet -}$ generation, adduct signals were corrected for trapping efficiency and decay. Rates of $^{\bullet}O_2^{\bullet -}$ formation were determined from the DMPO-OOH signal over the first 20 min of acquisition as previously described [37].

2.8. Transient transfection and siRNA

THP-1 cells were washed once in PBS and resuspended in the specified electroporation buffer (Amaxa, Cologne, Germany) to a final concentration of 2×10^6 cells/ml. Empty vector (pcDNA3-HA), 0.5 μg or the dominant negative PKCδ mutant pcDNA3-PKCδ-DN-HA [21] were mixed with 0.1 ml of cell suspension, transferred to a 2.0 mm cuvette and nucleofected using the Amaxa Nucleofector™ apparatus according to manufacturer's specifications. Twenty-four after transfection cells were treated with 50 μM apigenin or diluent alone (No treated) for 9 h. For silencing experiments, THP-1 cells were transfected with 150 nM siRNA-PKCδ [the siRNA sequences for targeting PKCδ were sense: 5′-GGCUGAGUUCUGGCUGGACTT-3′ on PKCδ], the same duplex siRNA-PKCδ labeled with rhodamine (siRNA-PKCδ-R) or a random rhodamine-labeled-negative control (sense: 5′-UUCUCCGAACGUGUCACGUTT-3′, Qiagen) were utilized. Forty hours after transfection, cells were treated for 12 h with 50 μM apigenin or diluent to induce apoptosis as described above. Cells were then rinsed with PBS and fixed with 2% paraformaldehyde (Sigma, St. Louis, MO) for 5 min at room temperature and treated with 0.2% Trion-X-100 (BioRad, Hercules, CA) followed by rinse with PBS. Cells were stained with 0.5 μg/ml of DAPI (4′, 6′-diamidino-2-phenylindole, Sigma) for 15 min at 4 °C. Percentage of apoptosis was determined by nuclear fragmentation of cells. Nuclear morphology of 200 cells in each experiment was visualized using Olympus fluorescence microscope and Image ProPlus software (excitation at 300 nm; emission at 461 nm). In silencing experiments only red cells were counted to determine their apoptotic status.

2.9. Immunoprecipitations and in vitro kinase assays

Immunoprecipitations with anti-PKCδ antibodies were carried out as previously described [21]. After immunoprecipitation, kinase assays were performed by incubating protein A-loaded beads for 1 h at 37 °C in the presence of 20 μl kinase assay buffer 25 mM Hepes pH 7.3, 10 mM $MnCl_2$, 1 mM $MgCl_2$, 1 mM DTT) containing 5 μCi of [γ-^{32}P] ATP (Perkin Elmer, Boston, MA), 0.5 mM ATP. To each reaction, 5 μg of histone H2B (Boehringer Mannheim, Roche, Indianapolis, IN) was added as exogenous substrate. Reactions were stopped by the addition of 10 μl of 5X Laemmli buffer. Samples were boiled for 5 min and loaded onto a SDS-PAGE.

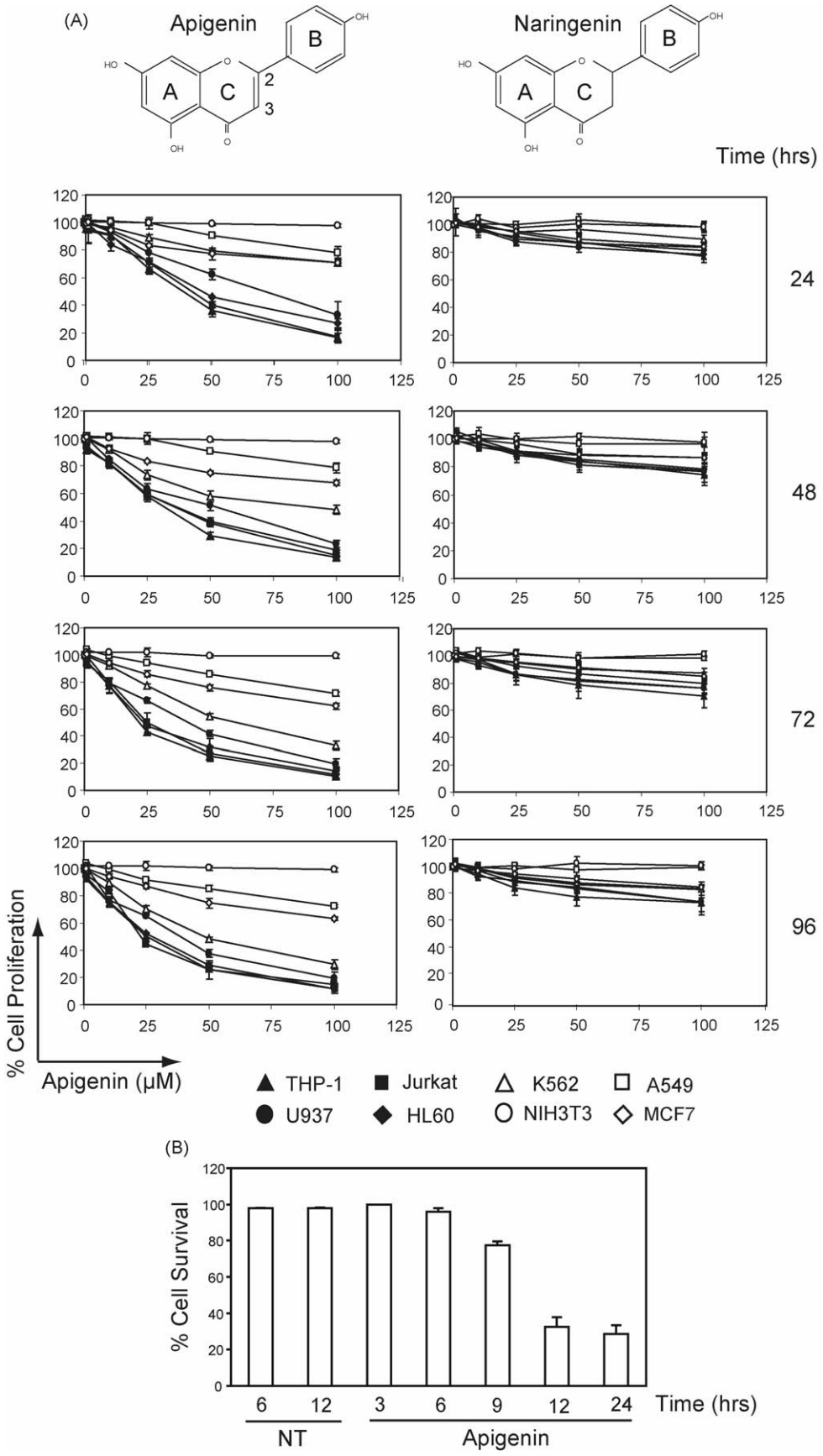

Fig. 1 – Effect of apigenin and naringenin in cell proliferation. (A) THP-1, U937, HL60, Jurkat, K562, A549, MCF-7 and NIH3T3 cells were treated with various doses of apigenin or naringenin for 24, 48, 72 and 96 h. After the treatment, the percentage

2.10. Statistical Analysis

All data are expressed as mean ± S.E.M. and Student's *t*-test comparisons were conducted to analyzed statistical significance. Statistical significance is stated in the text.

3. Results

3.1. Apigenin inhibits the viability of monocytic leukemia cells

We investigated the effect of the structurally-related flavonoids apigenin and naringenin on the viability of different cancer cells, including the human monocytic leukemia THP-1 and U937 lines, the promyelocytic HL60 line, the acute T cell leukemia Jurkat line, the K562 chronic myelogenous leukemia line, the lung epithelial A549 line, the breast epithelial MCF7 line, and the fibroblast NIH-3T3 line (Fig. 1A). Treatment with up to 100 μM apigenin for 96 h did not affect proliferation of the fibroblast NIH3T3 line (Fig. 1A, see NIH3T3). Moreover, apigenin only reduced the viability of epithelial cells by 30% after 96 h (Fig. 1A, see MCF7 and A549). In contrast, apigenin reduced viability by 80% or more on all the leukemia cells tested (Fig. 1A, see THP-1, U937, K562 and HL60). The apigenin IC_{50} values were approximately 30 μM for most of the leukemia and 50 μM for the terminal blast crisis chronic myelogenous leukemia cell line K562 (Table 1). Treatment with the related flavonoid naringenin resulted in only a minor effect on cell viability, with less than a 20% reduction after 96 h on all of the cells tested (Fig. 1A). These results indicate that, despite the chemical similarity between these two flavonoids, apigenin is significantly more potent in inhibiting the viability of cancer cells and its effect is more effective for leukemia cells.

Table 1 – IC_{50} for apigenin-treated cells

Cell type	24 h	48 h	72 h	96 h
THP-1	37.8 ± 4.2	31.9 ± 3.2	31.9 ± 2.7	27.8 ± 3.9
U937	64.7 ± 2.8	50.6 ± 3.2	41.3 ± 2.2	39.7 ± 2.1
HL60	50.1 ± 0.3	38.9 ± 2.2	31.1 ± 3.9	29.2 ± 1.7
Jurkat	39.9 ± 2.8	35.8 ± 2.6	29.0 ± 6.2	29.6 ± 1.6
K562		85.6 ± 3.5	62.0 ± 2.5	54.4 ± 4.0

The effect on proliferation was determined in different cell lines treated for various lengths of time with apigenin. Data represents the mean ± S.E.M. ($N = 9$).

3.2. Apigenin-induced-cell death is mediated by a caspase-dependent pathway

To establish whether apigenin induces apoptosis, we determined the number of apoptotic cells, after the treatment of the THP-1 cells with 50 μM apigenin for different lengths of time, using the calcein AM/PI viability assay (Fig. 1B). We found that the viability of THP-1 cells was reduced by 20% after 9 h and by 70% after 12 h of the apigenin treatment (Fig. 1B).

To determine whether apigenin-induced cell death involved activation of the apoptotic machinery, we first investigated the effect of apigenin on the activation of caspases. For this purpose, THP-1 cells were treated with 50 μM apigenin for various lengths of time and caspase-9, caspase-8, and caspase-3 activities were measured using the fluorogenic substrates LEHD-AFC, LETD-AFC, and DEVD-AFC, respectively. In THP-1 cells, high caspase-9 activity was observed 6 h after apigenin addition, decreasing after 12 h (Fig. 2A). Caspase-3 activity was detected 3 h after the addition of apigenin (Fig. 2B). Similar to caspase-9, the activity was sustained at 12 h, but decreased after longer treatments (Fig. 2B). Similar results were obtained in the other leukemias (data not shown). In contrast, caspase-8 was not activated by apigenin (data not shown) in all the leukemia cells tested.

We next investigated whether caspase-3 activity was required for apigenin-induced cell death. THP-1 cells were incubated with the caspase-3 inhibitor DEVD-FMK at 20 μM for 1 h prior to the addition of 50 μM apigenin. We found that after 12 h of apigenin treatment 70% cells were apoptotic, as shown by calcein AM/PI staining. Whereas pretreatment with the caspase inhibitor DEVD-FMK prior to the addition of apigenin reduced the number of apoptotic cells to less than 10%, a similar percentage of apoptotic cells that was observed in cells untreated or treated with DEVD-FMK alone (Fig. 2C). Consistent with these findings, pretreatment with DEVD-FMK inhibited the activation of caspase-3 to the levels observed in untreated cells (Fig. 2D). These results demonstrate that apigenin induces apoptosis of THP-1 through a caspase-9/caspase-3-mediated pathway.

3.3. Intracellular accumulation of apigenin

To investigate the metabolic fate of apigenin, we determined the levels of this flavone in the growth media, in the soluble (supernatant of S100 centrifugation) and membrane (pellet of S100 centrifugation) fractions of THP-1 cells after treatment with 50 μM apigenin for 3 h. HPLC analyses indicated that apigenin was not converted to any other compound with absorbance at 280 nm (Fig. 3A). Most of the compounds previously reported as resulting from the modification or metabolism of apigenin (e.g., luteolin) would absorb at this wavelength [38]. Interestingly, apigenin was mainly found in the pellet of the S100 centrifugation (Fig. 3A), suggesting its association with a membrane fraction.

To further investigate the presence of apigenin in a membrane fraction, THP-1 cells treated with 50 μM apigenin for different lengths of time were treated with the flavonoid-staining DPBA, as previously described [39]. Immunofluorescence microscopy showed intracellular accumulation of apigenin after 3 h, staining that became more prominent after 12 h of treatment (Fig. 3B). Staining of apigenin-treated cells simultaneously with DPBA and mitotracker showed that apigenin was excluded from the nucleus and plasma

of cell proliferation was calculated as the ratio of treated cells to control cells as determined by the MTT method (A_{490}). Data represents means ± S.E.M. ($N = 9$). (B) THP-1 cells were treated for various lengths of time with 50 μM apigenin or diluent (NT) and stained with calcein AM and PI to evaluate the percentage of cell death. Data represents means ± S.E.M. ($N = 3$).

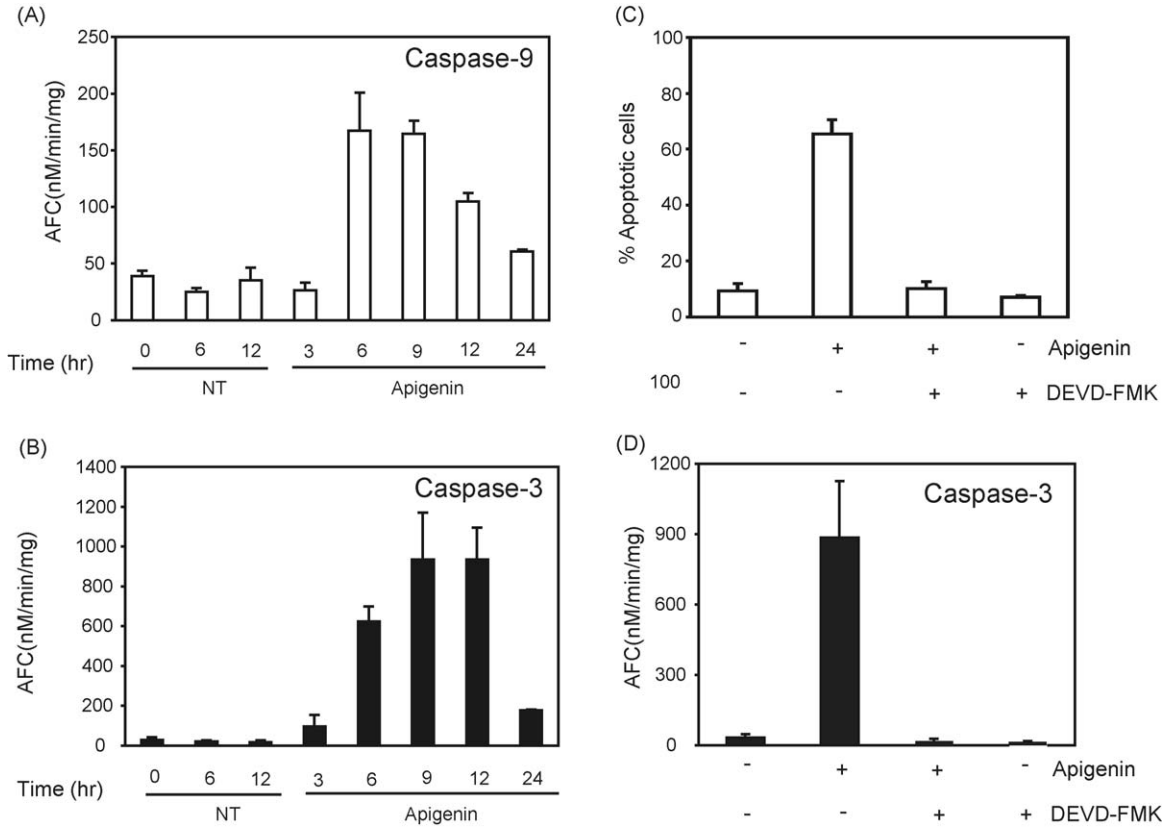

Fig. 2 – Apigenin induces caspase-dependent cell death in monocytic leukemia cells. THP-1 cells were treated for various lengths of time with 50 μM apigenin or DMSO (NT) and caspase activity was established. (A) Caspase-9 activity was determined by the LEHD-AFC assay. (B) Caspase-3 activity was determined by the DEVD-AFC assay. Data represents means ± S.E.M. (N = 3). (C) THP-1 cells were treated for 12 h with 50 μM apigenin alone or pretreated with 20 μM DEVD-FMK for 1 h prior to the addition of apigenin. The percentage of apoptotic cells was determined by calcein AM/PI staining. (D) Lysates from cells treated as described above were used to determined caspase-3 activity by the DEVD-AFC assay. All data in C and D represents means ± S.E.M. (N = 5).

membrane and appeared to colocalize with the mitochondria (Fig. 3C).

3.4. *Apigenin induces the generation ROS and the activation of p38 and ERK*

Since several studies have demonstrated that chemotherapeutic drugs induce cell death via generation of ROS [40,41], we investigated whether apigenin induced the formation of ROS in THP-1 cells. Treatment with 50 μM apigenin for 1 h induced a large and transient ROS production that decreased after 3 h (Fig. 4A, compare with untreated, NT). Because increased ROS production has been associated with activation of MAPK [42], we next examined the effect of apigenin in MAPK. We showed a rapid increase in p38 phosphorylation in THP-1 cells treated with 50 μM apigenin for 1 h (Fig. 4B, P-p38). Moreover, apigenin induced ERK phosphorylation at around 6 h, with delayed kinetics compared to p38 (Fig. 4B). In contrast, no change was observed in JNK phosphorylation in cells treated with apigenin (data not shown).

To determine the role of ROS on apigenin-induced-apoptosis, THP-1 cells were pretreated with 20 μM EUK-134, a synthetic catalytic scavenger of ROS [43], for 1 h prior to the addition of apigenin. Under these conditions, EUK-134 completely blocked the production of apigenin-induced ROS (Fig. 4A, gray bars). Using calcein AM/PI staining we found that cells pretreated for 1 h with 20 μM EUK-134 prior to the addition of 50 μM apigenin for 6, 9, and 12 h showed 80% of apoptotic cells, similar numbers were found in cells treated with apigenin alone (Fig. 4E, no statistical difference $P > 0.05$, Student's *t*-test comparing 12 h apigenin-treated cells with apigenin + EUK-134 treated cells).

We next investigated whether the activation of p38 and ERK was required for apigenin-induced cell death. For this purpose, the number of apoptotic cells was compared in THP-1 cells treated with 50 μM apigenin alone, pretreated for 1 h with either 10 or 25 μM of SB203580, a p38 inhibitor, or with 25 or 50 μM of the ERK inhibitor PD98059 alone or in combination, prior to the addition of apigenin and compared with cells treated with the diluent alone. We found that p38 phosphorylation was reduced in cells pretreated for 1 h with 10 or 25 μM of the p38 inhibitor SB203580 (Fig. 4C). Similarly, ERK phosphorylation was reduced in cells pretreated with PD98059 (Fig. 4D). Pretreatment with SB203580, PD98059 or with both MAPK inhibitors together in apigenin-treated cells failed to reduce the number of apoptotic cells (Fig. 4F, no

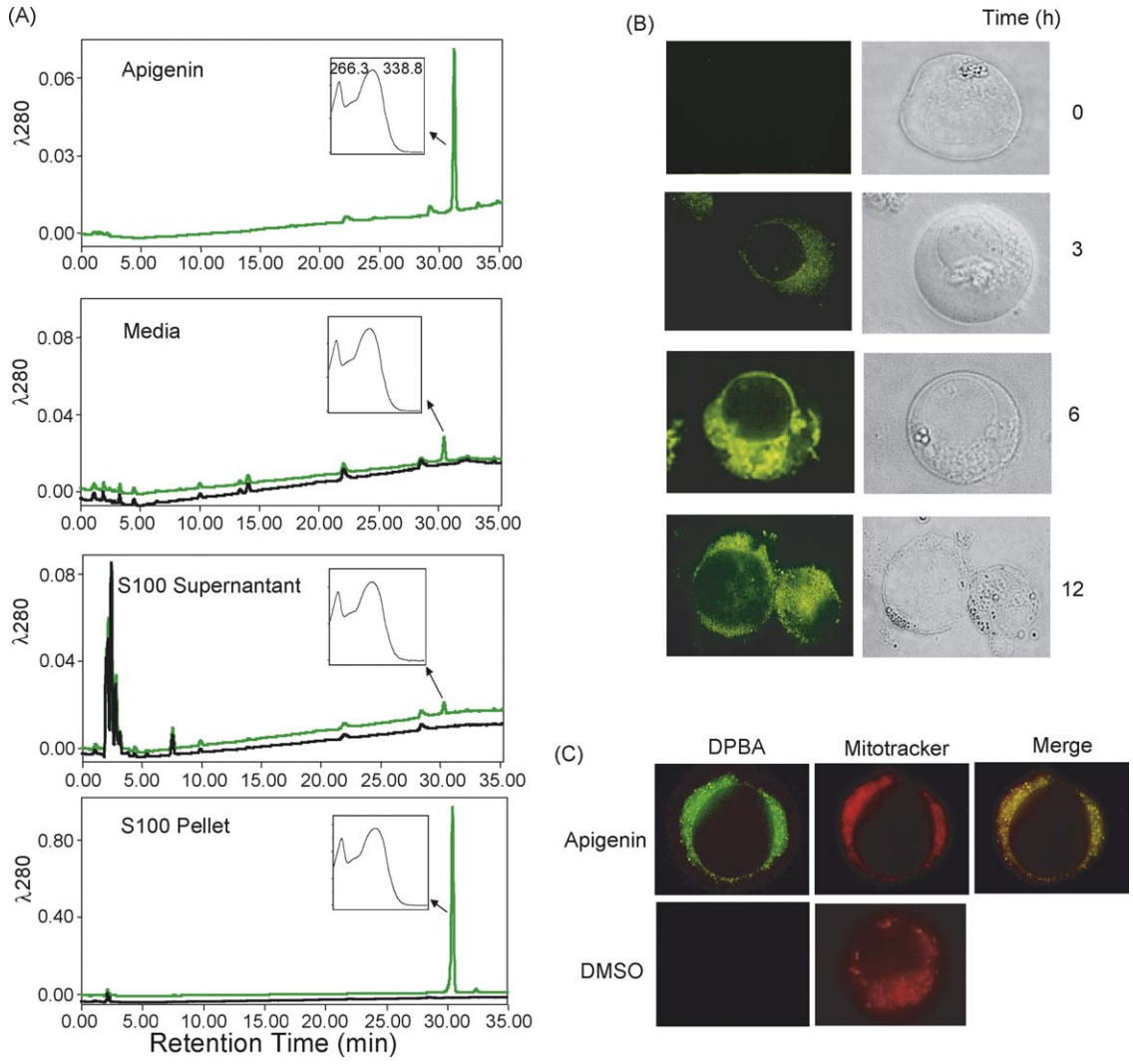

Fig. 3 – Apigenin in THP-1 cells associates. (A) HPLC analysis of media, S100 supernatants, or pellets from THP-1 cells treated for 3 h with 50 μM apigenin. The top panel corresponds to an apigenin standard. (B) THP-1 cells treated for different lengths of time with 50 μM apigenin were stained with DPBA. (C) THP-1 cells treated for 3 h with 50 μM apigenin or with the diluent DMSO were stained with DPBA and mitotracker and visualized by microscopy.

statistical difference $P > 0.5$ compared to apigenin-treated cells, Student's *t*-test). Moreover, MAPK inhibitors failed to inhibit caspase-3 activity in apigenin-treated cells (Fig. 4G, no statistical difference $P > 0.5$ compared to apigenin-treated cells, Student's *t*-test). Taken together, these results demonstrate that apigenin promotes ROS production and the activation of p38 and ERK, but that these events are not necessary for the execution of apoptosis.

3.5. *Activation of PKCδ is essential for apigenin-induced-apoptosis*

We previously showed that PKCδ functions as a pro-apoptotic factor during spontaneous monocyte apoptosis and etoposide-induced apoptosis [21]. To determine whether PKCδ participates in apigenin-induced-apoptosis, lysates from THP-1 cells treated for 3 h with 50 μM apigenin were immunoprecipitated with an anti-PKCδ antibody and subjected to an *in vitro* kinase assay using H2B as a substrate. Immunoprecipitates from cells left untreated (Fig. 5A, see -) or treated for 3 h with DMSO (Fig. 5A, see 0 apigenin) had no detectable PKCδ activity. In contrast, apigenin treatment dramatically increased PKCδ activity, as visualized by the increase in H2B phosphorylation (Fig. 5A).

To evaluate the role of PKCδ in the apigenin-induced-apoptosis, THP-1 cells were pretreated for 1 h with different doses of the PKCδ inhibitor rottlerin, prior to the addition of 50 μM apigenin. The number of apoptotic cells was determined using calcein AM/PI and the caspase-3 activity was assayed 12 h after the addition of apigenin. The treatment with rottlerin resulted in a significant reduction in the number of apoptotic cells induced by apigenin (Fig. 5B $P < 0.005$ at 10 μM or higher, Student's *t*-test) as well as in the activity of caspase-3 (Fig. 5C $P < 0.01$ 10 μM or higher concentrations, Student's *t*-test). To further delineate the involvement of PKCδ, THP-1 cells were transfected with a construct expressing a

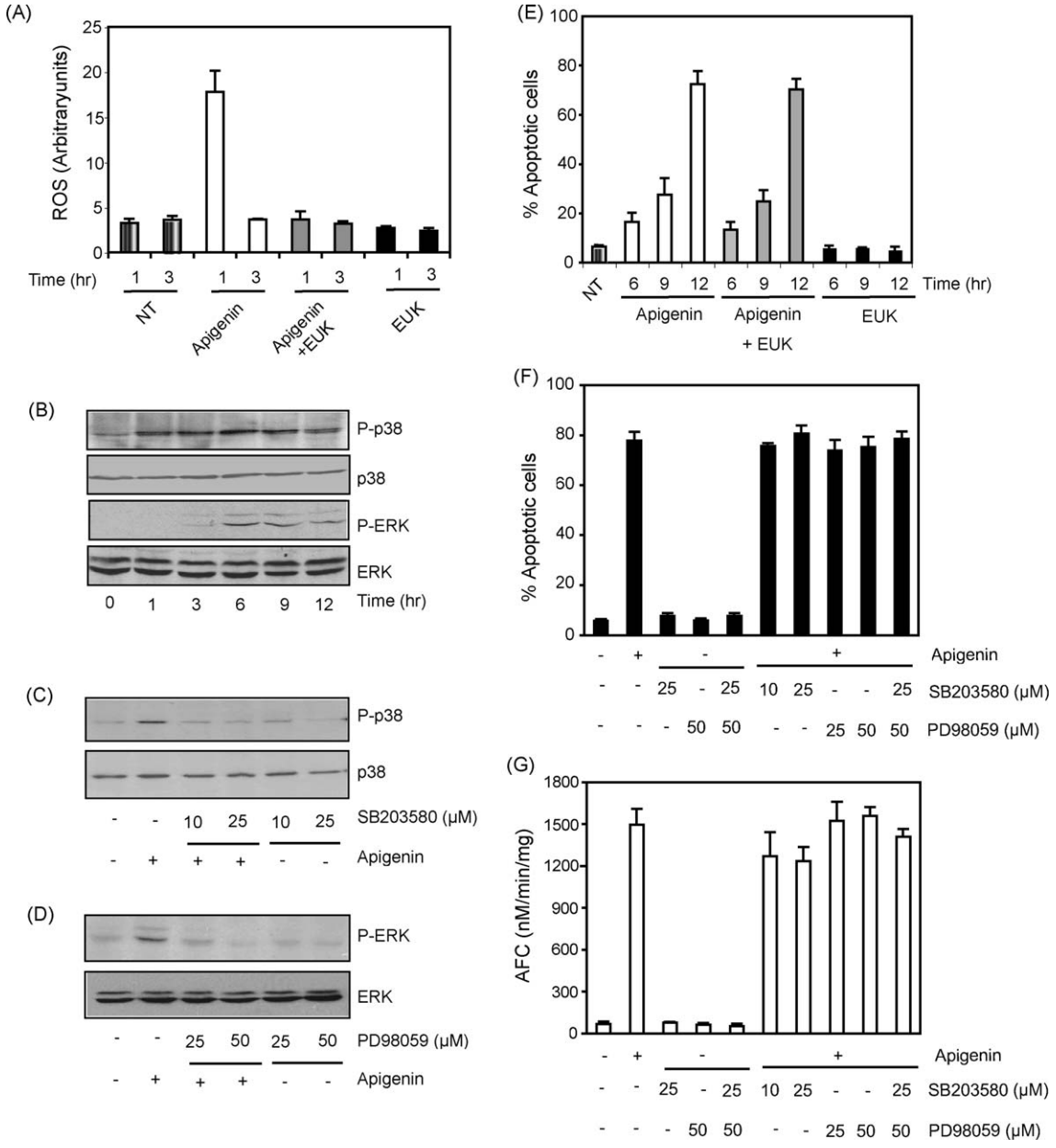

Fig. 4 – Production of ROS and activation of p38 during apigenin-induced-apoptosis. (A) ROS production was analyzed by EPR in THP-1 cells treated for 1 and 3 h with 50 μM apigenin, DMSO (NT), treated with EUK-134 alone or EUK-134 and apigenin. Data represents means ± S.E.M. (N = 3). (B) THP-1 lysates from cells treated with 50 μM apigenin for different lengths of time were analyzed by immunoblots with anti-phospho-p38 (P-p38), anti-phospho-ERK (P-ERK), anti-total-p38 (p38), and anti-ERK (ERK) antibodies. (C) THP-1 cells were treated for 3 h with the apigenin diluent (lane 1) with 50 μM apigenin alone (lane 2), pretreatred with 10 or 25 μM SB203580 for 1 h prior to the addition of apigenin (lanes 3 and 4) or with the SB203580 inhibitor alone (lanes 5 and 6). Lysates were analyzed by immunoblotting. (D) THP-1 cells were treated for 6 h with the apigenin diluent (lane 1) with 50 μM apigenin alone (lane 2), pretreatred with 25 or 50 μM PD98059 for 1 h prior to the addition of apigenin (lanes 3 and 4) or with the PD98059 inhibitor alone (lanes 5 and 6). Lysates were analyzed by immunoblotting. (E) Percentage of apoptotic cells was determined in THP-1 cells treated for different lengths of time with 50 μM apigenin (white bars), 20 μM EUK-134 alone (black bars) or pretreated with EUK134 for 1 h prior to the addition of apigenin (grey bars). Data represents means ± S.E.M. (N = 3). (F) The percentage of apoptotic cells was determined using calcein AM and PI staining on cells treated for 12 h with 50 μM apigenin alone, pretreated with 10 or 25 μM SB203580, 25 or 50 μM PD98059 or both inhibitors together for 1 h prior to the addition of apigenin, with the inhibitors in the absence of apigenin or cells were left untreated. (G) Lysates from the same treatments as described in (F) were used to determine caspase-3 activity by the DEVD-AFC assay. Data represents means ± S.E.M. (N = 5).

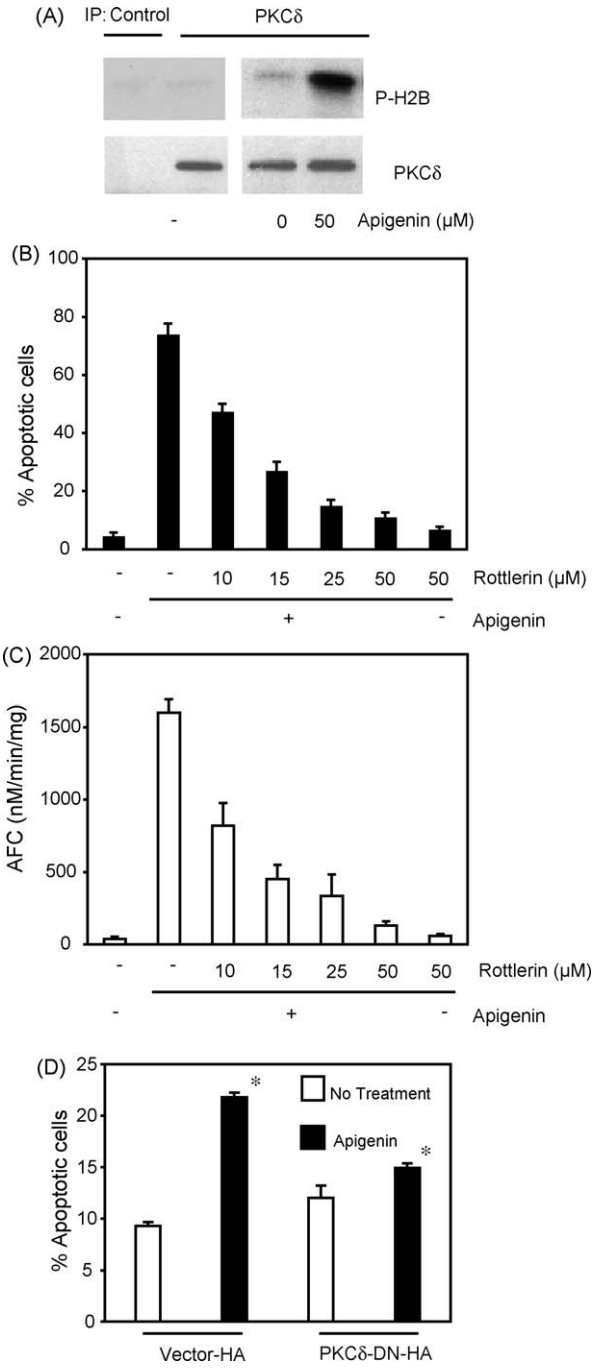

Fig. 5 – PKCδ activity is required for apigenin-induced-apoptosis. (A) THP-1 cells were treated for 3 h with 50 μM apigenin or the diluent. Lysates were immunoprecipitated with anti-PKCδ antibodies or an isotype control (Control) and subjected to *in vitro* kinase assay using H2B as substrate in the presence of [γ-^{32}P] ATP. The kinase reaction products were resolved by SDS-PAGE, transferred to a membrane, and phosphorylated H2B (P-H2B) was visualized by autoradiography. The same membrane was immunobloted with anti-PKCδ antibody to ensure equal loading of the samples (lower panel). (B) The number of apoptotic cells determined by calcein AM/PI staining in THP-1 cells left untreated (−/−), pretreated with indicated doses of rottlerin for 1 h or left untreated (−) prior to the addition of 50 μM apigenin. Results represent means ± S.E.M. (N = 3). (C) Caspase-3 activity determined by DEVD-AFC in lysates from cells treated as in B. Results represent means ± S.E.M. (N = 3). (D) THP-1 cells transfected with a dominant-negative mutant PKCδ-DN-HA or a vector-HA control were left untreated (white) or induce to undergo apoptosis for 9 h with 50 μM apigenin (black). Percentage of apoptotic cells was determined by DNA fragmentation and visualized by DAPI staining. Values are means ± S.E.M. (N = 3; $^{*}P < 0.01$, Student's *t*-test).

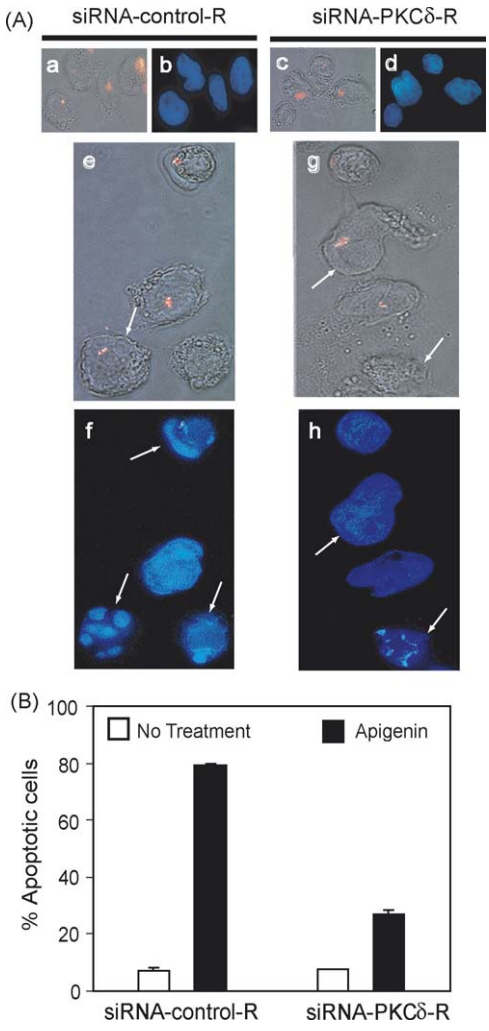

Fig. 6 – Silencing of PKCδ inhibits apigenin-induced-apoptosis. (A) THP-1 cells transfected (red) with siRNA-Control-R or siRNA-PKCδ-R THP-1 cells transfected (red) were left untreated (a, b, c and d) or induced to undergo apoptosis for 12 h with 50 μM apigenin (e, f, g and h) were stained with DAPI. (B) Values represent means ± S.E.M. of DAPI stained cells showing DNA fragmentation over total cells from either cells left untreated (white) or treated with apigenin (black) (N = 3; $^{*}P < 0.01$). DNA morphology of only red transfected cells was taken into consideration.

dominant negative PKCδ mutant (PKCδ-DN-HA) driven by the CMV promoter or vector control. Apoptosis was estimated by DNA fragmentation, twenty-four hours after transfection, in cells treated with 50 μM apigenin for 9 h (Fig. 5D, Apigenin, black), or in cells left untreated (Fig. 5D, No Treatment, white). Expression of PKCδ-DN-HA resulted in a significant reduction of apoptosis in apigenin-treated cells similar to the levels observed in cells transfected with the vector control (Fig. 5D) or in untransfected cells after treatment with apigenin for 9 h (Fig. 2).

To further investigate the role of PKCδ, we determined the effect of silencing PKCδ on apigenin-induced-apoptosis. THP-1 cells were transfected with siRNA-PKCδ duplexes labeled with rhodamine (siRNA-PKCδ-R), and with a rhodamine-labeled random duplex control (siRNA-Control-R). We have previously shown that these siRNA-PKCδ duplexes induce specific down-regulation of PKCδ [21]. We investigated the effect of silencing PKCδ on DNA fragmentation, a process considered a hallmark of apoptosis. For this purpose, THP-1 cells were transfected with siRNA-PKCδ-R or siRNA-Control-R (Fig. 6A, see red cells). Forty hours after transfection, cells were left untreated or induced to undergo apoptosis for 12 h with 50 μM apigenin. After this period, nuclei were stained with DAPI and the number of apoptotic cells was determined as the percentage of cells with fragmented DNA (only nuclei from red fluorescing transfected cells were considered). We found that silencing PKCδ reduced four-fold the number of apoptotic cells as determined by the reduction on the number of cells with DNA fragmentation (Fig. 6B, e and f). Cells transfected with the siRNA-Control underwent DNA fragmentation at higher percentage than the silenced PKCδ cells (Fig. 6B, e and f, see arrows). These results taken together suggest that PKCδ is required for apigenin-induced-apoptosis.

4. Discussion

Plant natural products, such as the flavonoids, are emerging as potent cancer prevention and chemotherapeutic agents. The flavone apigenin, broadly found in many fruits and vegetables [27], has been previously shown to induce cell death with variable efficacy, in human colon carcinoma cell lines, breast epithelial cells, and lymphocytic leukemia cells [30–32]. The mechanisms required for the induction of cell death by apigenin remain elusive. Our results provide novel insights into the mechanisms of apigenin-induced-apoptosis. We show that apigenin is enriched in mitochondria and induces ROS formation in THP-1 cells. However, in sharp contrast from previous models, we demonstrate that production of ROS and the induction of MAPKs are not necessary for caspase-3-dependent-apoptosis. Instead, apigenin induces the activity of PKCδ, which is essential for the apoptotic response, suggesting a novel link between the activation of this kinase and the induction of apoptosis by this flavonoid.

We showed that apigenin is particularly effective in arresting cell proliferation of leukemia cells, but had little or no effect epithelial cells and fibroblasts respectively (Fig. 1A). The arrest of the proliferation of THP-1 cells is associated with the induction of apoptosis, evidenced by the calcein AM/PI dye (Fig. 1B) and by the activation of the caspase-3 and caspase-9 apoptotic cascade (Fig. 2A, B). In contrast to apigenin, the related flavanone naringenin has a very modest effect on the proliferation arrest of leukemia and epithelial cells (Fig. 1A). Previous studies showed that apigenin was more potent in its ability to induce apoptosis of HL-60 lymphoblastic leukemia cells than the flavonols kaempferol and quercetin, leading to the suggestion that the absence of the three-hydroxyl group (C ring) was in part responsible for its potency [32]. Our results suggest that this is probably not the case, since neither apigenin nor naringenin have a 3-hydroxyl group, yet display significant differences in their ability to induce apoptosis of leukemia cells (Fig. 1). It is possible that the planar structure of apigenin conferred by the double bond between carbons 2 and 3 (Fig. 1A) is responsible for the observed difference in potency.

To investigate the fate of apigenin in THP-1 cells, we carried out reverse-phase HPLC experiments. We found no evidence that apigenin is converted to any other metabolite secreted to the growth media or sequestered inside the cells (Fig. 3A). Previous studies have shown that rat liver cells generate, by a cytochrome P450-dependent mechanism, the apigenin mono-hydroxylated compound, luteolin [38]. This does not appear to be a detoxification mechanism employed by THP-1 cells. We found however a very significant fraction (5–15%) of the total apigenin added to the growth media accumulating in the cells. The analysis of S100 supernatant and pellets (Fig. 3A) indicates that much of the cellular apigenin is in an insoluble fraction enriched in cellular membranes. Flavonoids have been shown previously to be associated with cellular membranes, possible due to their hydrophobic nature, however, the specific membranes involved in the process were not defined [44]. Our findings showing the colocalization of the flavonoid-specific-staining DPBA with the mitochondria dye mitotracker suggests the enrichment of apigenin in the mitochondria and are consistent with the accumulation of this flavone observed in an intra-cellular membrane system (Fig. 3B and C). Previously, apigenin was shown to induce mitochondrial depolarization and cytochrome *c* release in HL-60 cells [32]. Our findings suggest that the localization of apigenin in the mitochondria could be responsible for the effects in the alteration of a mitochondrial transmembrane potential often associated with the induction of apoptosis [45]. It is tempting to speculate that the increased accumulation of apigenin in cellular membranes might explain the slow metabolism and elimination reported for apigenin in pharmacokinetic [38], as well as with the high IC_{50} observed (Table 1).

Despite their overall antioxidant activity (Rice-Evans 1997), apigenin and other flavonoids have been shown to induce ROS production [32]. Consistent with these findings, our results show that apigenin induces rapid ROS production in THP-1 cells (Fig. 4A). Interestingly, however, treatment with the ROS scavenger EUK-134 did not inhibit apigenin-induced apoptosis (Fig. 4E). Thus, the induction of ROS and apoptosis can be separated as apoptosis proceeds when ROS production is halted. Induction of ROS has been associated with the activation of the MAPK pathway [46]. In this context, we found that apigenin induces a rapid increase in the phosphorylation of p38 and ERK, although with different kinetics (Fig. 4B and C). However, similar to what we found for ROS, the inhibition of p38, ERK or both MAPK together does not prevent the apoptotic activity of apigenin in THP-1 cells (Fig. 4F and G).

These results suggest that the induction of ROS, p38, and ERK are part of the cellular response to apigenin, but that the activation of MAPK and ROS are not a primary pathway in the induction of apoptosis of THP-1 leukemia cells by apigenin.

Recently, we have identified PKCδ as a positive regulator of spontaneous monocyte apoptosis and etoposide-induced cell death of monocytic leukemia cells [21]. We found here that the activity of PKCδ is significantly increased in THP-1 cells treated with apigenin (Fig. 5A). The inhibition of PKCδ, achieved by either using the PKCδ inhibitor rottlerin or by a dominant negative construct of PKCδ, resulted in a significant inhibition of apigenin-induced apoptosis (Fig. 5B–D). Moreover, silencing of PKCδ reduced the number of apoptotic cells induced by apigenin (Fig. 6). These results suggest that the activation of PKCδ by apigenin is intimately related to the activation of the caspase pathway and the induction of apoptosis.

In addition to their role as anti-oxidants [28], flavonoids are emerging as important signal molecules for microbes, plants, and animals [47]. Indeed, the recently put forward ''xenohormesis'' hypothesis proposes that molecules that participate in the stress-response in plants, such as the flavonoids, have a similar effect on animals, providing an interspecies ''advance warning'' mechanism of upcoming stress [48]. Consistent with such a model, the observed induction of ROS, p38 and ERK by apigenin is likely to lead to the activation of stress-response pathways, recently associated with increased longevity [49]. However, the induction of apoptosis by apigenin in THP-1 cells occurs through a mechanism that is independent of ROS/MAPK and involves the activation of a PKCδ-dependent pathway. The balance between the level of activation of these two pathways could be cell-type specific, consistent with the selective apoptotic effect of apigenin in leukemia (Fig. 1A), and may explain how under certain circumstances flavonoids induce cell death, while in others increase life span. Together, the studies presented here provide novel insights into the molecular mechanism by which the flavone apigenin selectively regulates cell death in leukemia cells and provide the basis to establish how the balance between the players confer the selective effect of flavonoids to regulate cellular life span.

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