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Downregulation of PLK-1 expression in kaempferol-induced apoptosis of MCF-7 cells

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ABSTRACT

The molecular mechanisms underlying the kaempferol-induced cell death have not yet been fully explained. To investigate the role of kaempferol, widely distributed in foods, in tumor progression, human breast cancer cell line, MCF-7, was treated with kaempferol. Apoptosis was indicated by the accumulation of a sub-G1 population, as well as the appearance of 4′-6-diamidino-2-phenylindole (DAPI)-stained apoptotic nuclei in the MCF-7 cells after the administration of kaempferol. Western blot analysis showed cleavage of Poly (ADP-ribose) polymerase (PARP), caspase-7, Bax, and caspase-9 indicating that the intracellular pathway of apoptosis was involved. Kaempferol also downregulated the expression of polo-like kinase 1 (PLK-1), which has been reported to regulate mitotic progression and to be upregulated in several human tumors. Taken together, these findings indicate that kaempferol-induced apoptosis by initiation of intrinsic caspase cascade and downregulation of PLK-1 expression.

1. Introduction

Apoptosis is a selective process for the removal of cells in various biological systems. In contrast to necrosis that simultaneously involves a group of cells, apoptosis may occur in a single cell surrounded by a group of viable cells. Regulation of apoptosis is a complex process and involves a number of cellular proteins, including anti-apoptotic BCL-2, pro-apoptotic Bax, and a series of caspases.

Flavonoids are polyphenolic compounds that are present in high amounts in fruits and vegetables. Several previous studies, including our own results, have indicated that some flavonoids exhibit potent anti-tumor properties and can modulate apoptosis, differentiation, and cell cycle, probably by virtue of their anti-oxidant functions (Fu et al., 2004; Lee et al., 2005, 2007). Among dietary flavonoids, the pharmacological functions of quercetin and kaempferol have been extensively studied (Aligiannis et al., 2001; Lee et al., 1998). It has been suggested that kaempferol inhibits growth of human leukemia cells and v-H-ras NIH3T3 transformed cells. At higher concentrations (>35 μM), kaempferol induces DNA fragmentation (Nguyen et al., 2003),(Sahu and Gray, 1994) and inhibits growth of MCF-7 cells (Sathyamoorthy et al., 1994).

The polo-like kinase-1 (PLK-1), a mammalian Ser/Thr protein kinase, is structurally related to the polo gene product of *Drosophila melanogaster*, Cdc5p of *Saccharomyces cervisiae*, and PLX-1 of *Xenopus laevis*. PLK-1 is a key regulator of the mitotic progression, and it is very closely related to *Drosophila* polo and its enzymatic activity peaks at the onset of mitosis and gradually decreases as the M phase proceeds (Holtrich et al., 1994; Lee et al., 1995). An increasing number of evidence implicates the expression level of PLK-1 in the genesis and progression of tumors. Constitutive expression of PLK-1 in NIH/3T3 cells results in their transformation (Smith et al., 1997) and induces tumor growth in nude mice. More importantly, it has also been shown that PLK-1 mRNA and protein are overexpressed in many tumor cells including cells in breast (Wolf et al., 2000), non-small cells in lungs (Wolf et al., 1997), cells in head/neck (Knecht et al., 1999), cells in colon (Takahashi et al., 2003), and cells in prostate cancers (Weichert et al., 2004). These results suggest that PLK-1 might play an important role in the progression of cell cycle.

Despite the fact that many studies were performed on the function of flavonoids, detailed molecular mechanisms that are responsible for conferring their effects remain largely unknown. The objective of the present work was to assess apoptosis-modulating effects of kaempferol and to clearly elucidate the molecular mechanisms underlying its activity in the apoptosis of human breast cancer MCF-7cells. Therefore, we treated MCF-7 cells with kaempferol and observed that kaempferol exerted distinct pro-apoptotic effects via downregulation of PLK-1. Furthermore, kaempferol-induced apoptosis was dependent

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on the intracellular pathway modulated by caspase-9-mediated caspase cascade.

2. Materials and methods

2.1. Antibodies and materials

Antibodies against PLK-1, pro-caspase-7, pro-caspase-9, actin, Bax, and cleaved poly(ADP-ribose) polymerase (PARP) were obtained from Santa Cruz (Santa Cruz, CA), and phospho PLK-1 (Thr210) antibody was acquired from BioLegend (San Diego, USA).

2.2. Cell culture and kaempferol treatment

The spontaneously immortalized human adenocarcinoma MCF-7 cells were grown in Dulbecco's modified Eagle's medium (DMEM) (WelGENE Inc., Daegu, Korea) supplemented with 10% (v/v) fetal bovine serum (FBS) (WelGENE Inc., Daegu, Korea) and 100 U/ml of penicillin/streptomycin. Cultures were maintained at 37 °C in a water-saturated atmosphere of 5% CO_2 in air. After 24 h, the cells were washed once with Dulbecco's Phosphate Buffered Saline (DPBS) (WelGENE Inc., Daegu, Korea) and treated with 50 μM of kaempferol in a serum-free medium. The cells were incubated for additional times (from 0 to 48 h).

2.3. Measurement of apoptosis

2.3.1. Determination of cell viability

MCF-7 cells were plated at a density of 5×10^4 cells in 96-well plates, and cell viability was evaluated via conventional 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assay. After incubation, cells were treated with MTT solution (final concentration, 0.25 mg/ml) for 2 h at 37 °C. Dark blue formazan crystals formed in intact cells were then dissolved using dimethyl sulfoxide (DMSO), and absorbance at 570 nm was measured with an ELISA reader. The results were then expressed as percentages of MTT reduction, with absorbance by control cells being arbitrarily set as 100%.

2.3.2. 4′,6-diamidino-2-phenylindole (DAPI) staining

MCF-7 cells were grown in DMEM medium containing 10% FBS and then treated with 50 μM kaempferol in serum-free medium. The cells were then washed twice with cold PBS, fixed in ice-cold methanol/acetic acid (1:1, v/v) for 5 min, and then stained with 0.8 mg/ml of 4′,6-diamidino-2-phenylindole (DAPI) for 30 min. Stained nuclei were visualized and photographed using a Zeiss Axiovert 200 microscope at the fluorescence DAPI region (excitation, 351 nm; emission, 380 nm). Apoptotic cells were morphologically defined by cytoplasmic and nuclear shrinkage and by chromatin condensation or fragmentation.

2.3.3. Fluorescence-activated cell sorter (FACS) analysis

The cells were trypsinized and collected by centrifugation at 2000 ×*g* for 2 min. The collected cells were washed with ice-cold PBS and resuspended in 1×10^6 cells/ml. For fixation, cell suspension was kept overnight at 4 °C after the addition of 70% ethanol. The cells were then stained with propidium iodide solution (50 μg/ml propidium iodide, 0.1% Triton X-100, 0.1 mM EDTA, and 50 μg/ml RNase) for 20 min at 4 °C. The stained DNA was analyzed by using a flow cytometer (FACS Calibur; Becton Dickinson, BD Biosciences, NJ, USA). The cells were excited at 488 nm and their emission was determined between 515 and 545 nm. The appearance of sub-diploid DNA peak is a specific marker of apoptosis; necrosis induced by metabolic poisons or lysis produced by complement did not induce any sub-G_1 peak in the DNA fluorescence histogram (Darzynkiewicz et al., 1992).

2.4. Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNAs were isolated using RNase mini kit (QIAGEN GmbH, Germany) according to manufacturer's instructions. First-strand cDNA was synthesized by reverse transcriptase using random hexamers.

The primers for PLK1 amplification were 5′-GACGTGTTCTC-CTCCTTT-3′ and 5′-AGATGCTTCAGACAGATCCC-3′. The primers for GAPDH amplification were 5′-GGGCATGAACCATGAGAAGT-3′ and 5′-AAGCAGGGATGATGTTCTGG-3′.

The PCR-amplified products were separated by electrophoresis on a 1% agarose gel and were visualized by ethidium bromide staining.

2.5. Western blot analysis

Cells in 100 mm dishes were washed thrice in ice-cold PBS, scraped from the dish, and then collected in lysis buffer [50 mM Tris–HCl (pH 8.0), 150 mM NaCl, 0.1% SDS, 1% NP-40, 0.5% sodium deoxycholate, protease inhibitor, 1 mM Na_3VO_4]. After the cells were incubated on ice for 30 min, the lysates were centrifuged (12,000 ×*g*, 15 min), and the proteins in the lysates were quantified with micro BCA™ protein assay kit (PIERCE, Rockford, IL). An equal amount of protein (50 μg) was then separated on NuPAGE® 4–12% Bis–Tris Gel (Invitrogen, Carlsbad, CA) and then transferred onto a nitrocellulose transfer membrane (Schleicher & Schuell Bio Science, Dassel, Germany). These membranes were blocked with 5% non-fat dry milk (BD Biosciences, NJ, USA) and 0.2% NP-40 in Tris-buffered saline (TBS), and subsequently probed with primary antibody in TBS, which contained 5% non-fat dry milk and 0.2% NP-40. The antibody–antigen complexes were then detected using goat anti-mouse IgG or goat anti-rabbit IgG peroxide conjugates, followed by the use of an enhanced chemiluminescence (ECL) detection kit (Amersham Bioscience, Buckinghamshire, UK).

2.6. Statistical analysis

Data are expressed as means ± S.D. of at least three independent experiments. Statistical comparisons were made using an unpaired two-tailed Student's *t*-test with a confidence level of 95%. The significance level was set at $P<0.05$.

3. Results

3.1. Apoptosis induced by kaempferol in MCF-7 cells

Kaempferol inhibits the proliferation and induces apoptosis in various cancer cell lines (Sathyamoorthy et al., 1994). To elucidate the underlying mechanisms of kaempferol-induced apoptosis, we determined the effect of kaempferol on the viability of human breast cancer MCF-7 cells. Thus, MCF-7 cells were treated with 50 μM kaempferol for up to 48 h, and the cell survival and viability were determined at several time points using the MTT assay. Fig. 1A shows that kaempferol reduced cell viability in a time-dependent manner.

We then evaluated nuclear fragmentation, which is a fairly reliable indicator of apoptotic cell death. To assess the apoptotic effect of kaempferol, MCF-7 cells treated with 50 μM kaempferol for 6, 24, and 48 h were analyzed by DAPI staining. As shown in Fig. 1B, kaempferol treatment of MCF-7 cells induced apoptosis characterized by nuclear condensation and fragmentation in a time-dependent manner.

Flow cytometric analysis of DNA content also indicated that kaempferol-induced apoptosis in MCF-7 cells (Fig. 1C). When exposed to kaempferol for 24 h, the percentage of apoptotic cells (a sub-G1 population) showed a marked increase (98.4%) compared with the control (2.3%). These data clearly demonstrate that kaempferol-induced apoptosis.

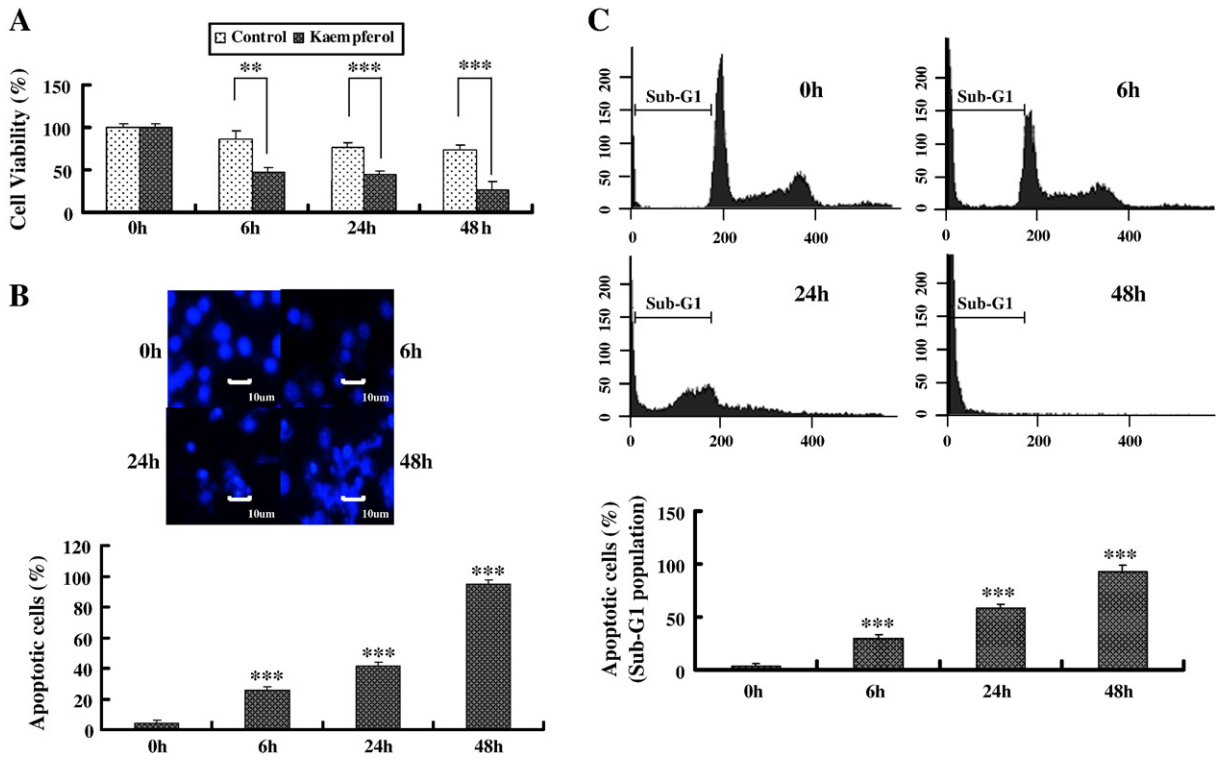

Fig. 1. Kaempferol induces apoptosis in MCF-7 cells. (A) Cell survival assay of MCF-7 cells treated with kaempferol. MCF-7 cells were exposed to kaempferol at a final concentration of 50 μM. At indicated time points (0, 6, 24 or 48 h), cell viability was determined by MTT assay. Data are presented as mean ± S.D. of three independent experiments. (B) MCF-7 cells on cover slip were treated with kaempferol as described. At 6, 24, or 48 h, the cells were fixed and DNA was stained with 4′,6-diamino-2-phenylindole. (C) FACS profiles, kaempferol treatment induces apoptosis. After treatment of the MCF-7 cells with kaempferol, the cells were harvested at 6, 24, or 48 h and analyzed. The positions of cell cycles were labeled. The intensity of fluorescence, which is proportional to the DNA content, is given on *x*-axis, and the number of fluorescent cells is given on *y*-axis. The results shown are typical results obtained in three independent experiments that gave similar results. The graphs are representations of cell viability (A) or the apoptotic cells (B and C) after treatment with 50 μM kaempferol. Each bar represents mean ± S.D., $n = 3$, $^{**}P<0.01$, $^{***}P<0.001$ vs. control.

3.2. Effect of kaempferol on PLK-1 expression

It has been shown that the depletion of PLK-1 induces apoptosis in various cancer cell lines by affecting cell-cycle profile and by damaging DNA (Ando et al., 2004; Liu and Erikson, 2003; Spankuch-Schmitt et al., 2002a). Since kaempferol-treated cells showed similar apoptotic characteristics in the present study, we examined whether the kaempferol-induced apoptosis is related to the PLK-1 depletion, by studying the ability of kaempferol to reduce endogenous level of PLK-1 mRNA in MCF-7 cells. As shown in Fig. 2A, Northern blot analysis of total RNA preparation of MCF-7 cells at 6 h of kaempferol treatment clearly showed that the level of PLK-1 mRNA showed a decrease. Furthermore, as seen in Fig. 2B, the expression level of PLK-1 protein also showed a significant decrease following kaempferol treatment: Treatment of MCF-7 cells with 50 μM kaempferol resulted in >70% decrease in the total PLK-1 protein. These results indicate that kaempferol effectively reduced the expression of PLK-1. Blotting with anti-α-tubulin confirmed equal amounts of protein loaded. Therefore, the reduction of both PLK-1 mRNA and protein expressions after treatment of kaempferol suggested that kaempferol-induced apoptosis was most likely mediated by downregulation of PLK-1.

3.3. Effect of kaempferol on activating a family of caspases

To further analyze the apoptosis-associated molecular events induced by kaempferol, Western blot analysis for poly-ADP-ribose polymerase (PARP) was performed (Fig. 3A). PARP synthesizes poly [ADP-ribose] from β-nicotinamide adenine dinucleotide (NDA) in response to DNA breaks. Since it has been demonstrated that the cleavage of PARP by caspase activation is an early event marker for apoptosis (Germain et al., 1999), the induction of apoptosis could be demonstrated by PARP cleavage using anti-PARP antibody. Western blot analysis of MCF-7 cell lysates treated with kaempferol showed proteolytic cleavage of PARP, which began 6 h and reached the maximum 24 h after kaempferol treatment.

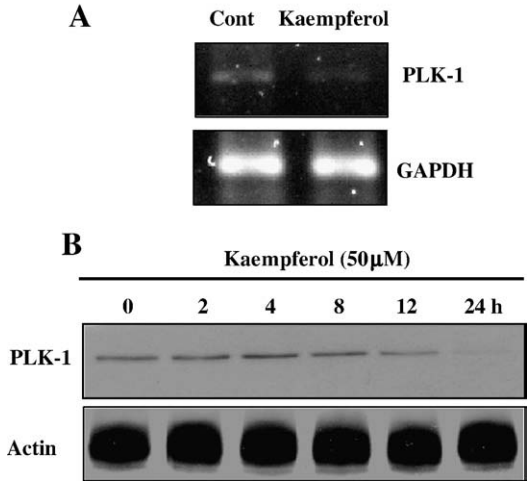

Fig. 2. Downregulation of PLK-1 during kaempferol-induced apoptosis. (A) Effect of kaempferol on the level of plk1 mRNA in MCF-7 cells, quantified by RT-PCR. RNA from an equal number of kaempferol-untreated and -treated MCF-7 cells was extracted. Amplified GAPDH served as an internal control. (B) Western blot analysis: At indicated time points (0, 2, 4, 8, 12, 24 h) after treatment with a final concentration of kaempferol of 50 μM, whole cell lysates were prepared and immunoblotted against anti-PLK-1. Immunoblotting for actin is shown as the control for equal protein loading. Similar results were obtained in three independent experiments.

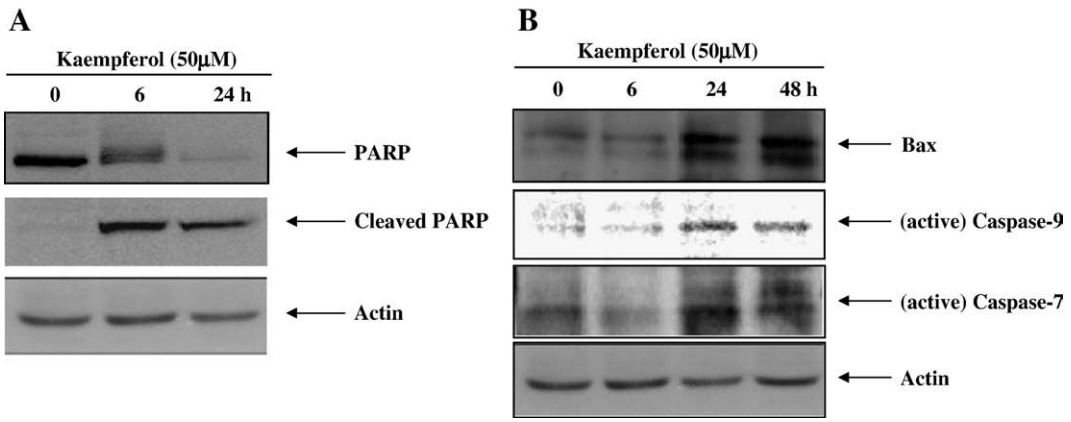

Fig. 3. Western blot analysis. At indicated time points (0, 6, 24, 48 h) after treatment of MCF-7 cells with a final concentration of kaempferol of 50 μM, whole cell lysates were prepared and immunoblotted against PARP (A), Bax, caspase-9, and caspase-7 (B). Immunoblotting for actin is shown as control for equal protein loading. Similar results were obtained in three independent experiments.

The cleavage of PARP implies activation of caspase cascade in kaempferol-induced apoptosis. Apoptosis is known to depend on the death receptor pathway and the intracellular pathway, and activation of p53 has been detected in both the pathways (Igney and Krammer, 2002). Furthermore, activation of p53 upregulates the expression level of Bax, a key pro-apoptotic regulator in the intracellular pathway in PLK-1-depleted cell lines (Ando et al., 2004; Liu and Erikson, 2003). Therefore, in order to assess the possible effect of kaempferol treatment on the expression of Bax, Western blot analysis of Bax was performed in kaempferol-treated cells. As shown in Fig. 3B, kaempferol treatment, which induced PLK1-depletion (Fig. 2A and B), resulted in the induction of Bax expression. Bax, which damages the outer mitochondrial membrane, causes the activation of caspase-9 (Brunelle and Chandel, 2002). The activation of caspase-9 subsequently activates effector caspases-3 and -7 (Twiddy et al., 2006). To determine whether kaempferol-induced apoptosis in MCF-7 cells is also associated with the activation of such caspase cascade, we measured the activation of caspases-9 and -7 by the detection of cleavage of caspases-9 and -7.

Thus, MCF-7 cells were treated with 50 μM kaempferol for 6, 24, and 48 h, and then the cell lysates were examined by Western blot analysis. Because MCF-7 cells do not express caspase-3, Western blot analysis was performed only with anti-caspase-7 and caspase-9 antibodies. As shown in Fig. 3B, the activation of caspase-9, which is caused by Bax, was clearly detected after kaempferol treatment, suggesting the initiation of apoptotic caspase cascade.

4. Discussion

Flavonoids constitute a large series of compounds found in many dietary plants and, therefore, they make up an important part of the diet. Several studies have reported that some flavonoids, including kaempferol and quercetin, exert anti-oxidant effects, and can also inhibit carcinogenesis (Hertog et al., 1993). Kaempferol is a flavonoid present in various natural sources including red wines and leaves of *Gingko biloba* (Watson and Oliveira, 1999), and lower concentrations (10–30 μM) of the flavonoid attenuate apoptosis induced in vascular smooth muscle cells by oxidized low-density lipoproteins (Niering et al., 2005). However, on the contrary, higher concentrations of it have been shown to induce apoptosis in A549 lung cancer cells, accompanied by PARP cleavage and proteolytic activation of caspase-7 (Nguyen et al., 2003): 35 μM of kaempferol reduced the cell viability by half. However, the precise mechanisms of its pro-apoptotic activities remain largely unknown. In the present study, we showed that kaempferol at higher concentrations (50 μM) induced apoptosis in human breast cancer MCF-7 cells through mitochondria-dependent pathway by depletion of PLK-1.

As shown in Fig. 2, PLK-1 expression was downregulated both transcriptionally and translationally during kaempferol-induced apoptosis. PLK-1 has been shown to play a critical role during G2/M and G1/S cell-cycle transitions and have been found to be constitutively expressed in various human tumors (Smith et al., 1997; Yuan et al., 1997). Several studies have shown that downregulation of PLK-1 by using several anti-sense and small interfering RNA targeted to PLK-1 as well as cytotoxic compounds caused cell-cycle arrest and apoptosis in cancer cell lines (Liu and Erikson, 2003; Spankuch-Schmitt et al., 2002a,b). In accordance with the several reports which suggest that the depletion of PLK-1 induces apoptosis, the kaempferol-mediated apoptosis was associated with a decrease in PLK-1 both in mRNA and protein levels (Fig. 2A and B). However, it is not fully clear whether downregulation of PLK-1 in kaempferol-treated cells is a real cue for apoptosis or is a consequence of the induction of apoptosis.

In the present study, we also showed that kaempferol-induced apoptosis through mitochondria-dependent pathway in MCF-7 cells, and that, when the cells were treated with kaempferol, the cleavages of PARP and procaspases-7 and -9 were clearly detected 24 h after treatment. These time courses of caspase-9 activation after kaempferol treatment showed good correlation with the time courses of the appearance of apoptotic cells, indicating that the caspase-9 activation pathway contributes significantly to the kaempferol-induced apoptosis of MCF-7 cells.

In the present study, DNA fragmentation, accumulation of a sub-G1 population, and the cleavages of caspase-9 and PARP strongly indicate that apoptosis was induced by kaempferol treatment. Two major pathways for the execution of apoptosis in mammalian cells have been described: the initiator caspases-8 and -9. Caspase-8-induced apoptosis is regulated via activation of death receptors of TNF family induced by extracellular ligands (Muzio et al., 1998; Srinivasula et al., 1998; Zou et al., 1999). Upon ligand binding, caspase-8 and downstream caspases-3 and -7 are proteolytically activated, resulting in the cleavage of substrates such as PARP, which leads to cell death.

The activation of caspase-9 is initiated by cellular stresses such as chemical treatment and irradiation, resulting in mitochondria-mediated apoptosis (Hakem et al., 1998). Caspase-7, the key mediator of apoptosis, was clearly activated, as determined by the fragmentation of caspase-7. During the apoptotic process, caspase-7 may be activated by the activation of caspase-9 (Twiddy et al., 2006). Downstream responses to caspase-9 include cleavage of caspase-7 and eventually PARP. In the present study, Western blot analysis of capase-7 and PARP indicated the involvement of caspase-9 in kaempferol-induced apoptosis of MCF-7 cells.

Our present findings represent a significant step forward in our understanding of kaempferol-induced apoptosis and also provide useful information on the molecular mechanisms underlying the effects of kaempferol. However, more studies are needed in order to clearly elucidate the signaling pathways involved in PLK-1 depletion initiated by kaempferol, leading to apoptosis. Furthermore, elucidation of the molecular mechanisms of kaempferol-induced apoptosis is expected to be extremely helpful in its use for chemoprevention or chemotherapy.

In conclusion, this study may allow us to further address the controversial pro-oxidant/anti-oxidant properties, shown by various flavonoids. Our results also deepen our understanding of the molecular mechanisms involved in differential cellular functions associated with flavonoid treatment.

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