Free Radical Biology & Medicine, Vol. 36, No. 7, pp. 897–910, 2004

0891-5849/$-see front matter

ELSEVIER

doi:10.1016/j.freeradbiomed.2003.12.020

Original Contribution

HYDROXYLATION AT C4′ OR C6 IS ESSENTIAL FOR APOPTOSIS-INDUCING ACTIVITY OF FLAVANONE THROUGH ACTIVATION OF THE CASPASE-3 CASCADE AND PRODUCTION OF REACTIVE OXYGEN SPECIES

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(*Received* 29 *July* 2003; *Revised* 17 *December* 2003; *Accepted* 22 *December* 2003)

Abstract—Previous studies demonstrated that hydroxyl groups play important roles in the antioxidative activities of flavonoids; however, the importance of structurally related hydroxylation in their apoptosis-inducing activities is still undefined. In the present study, flavanone with hydroxylation at C4′ and C6 had a significant cytotoxic effect in human leukemia HL-60 cells accompanied by the occurrence of DNA ladders, apoptotic bodies, and hypodiploid cells, characteristics of apoptosis. The replacement of a hydroxyl group (OH) by a methoxyl (OCH_3) group at C4′ or C6 attenuated the apoptotic effect in cells, and there was no significant cytotocity of flavanone or flavanone with OH or OCH_3 in C7-treated HL-60 cells. Induction of enzyme activity of caspase-3 and -9, but not caspase-1 and -8, accompanied by release of cytocrome C from mitochondria to cytosol and the appearance of cleaved of PARP (85 kDa), D4-GDI (23 kDa), and caspase-3 (p17/p15) fragments, was identified in 4′-OH- or 6-OH- flavanone-treated HL-60 cells. Caspase-3 and -9 inhibitors Ac-DEVD-FMK and Ac-LEHD-FMK, but not caspase-1 and -8 inhibitors Ac-YVAD-FMK and Ac-LETD-FMK, attenuated 4′-OH- or 6-OH-flavanone-induced cell death. And, inhibition of capsase-9 activity by Ac-LEHD-FMK suppresses caspase-3 protein procession induced by 4′-OH- and 6-OH-flavanone, indicative of caspase-9 activation locating upstream of caspase-3. A decrease in the antiapoptotic protein Mcl-1 and increases in the pro-apoptotic proteins Bax and Bad were found in 4′-OH- or 6-OH-flavanone-treated HL-60 cells. Induction of endogenous ROS production was detected in 4′-OH- or 6-OH-flavanone-treated HL-60 cells by the DCHF-DA assay. Antioxidants such as *N*-acetylcysteine (NAC), catalase (CAT), superoxide dismutase (SOD), and allopurinol (ALL), but not pyrrolidine dithiocarbamate (PDTC) or diphenylene iodonium (DPI), significantly inhibited 4′-OH- or 6-OH-flavanone-induced ROS production, with blocking of the apoptosis induced by 4′-OH- or 6-OH flavanone. The apoptosis-inducing activity of 4′-OH- or 6-OH-flavanone was also observed in another leukemia cell line (Jurkat), but was not found in mature monocytic cells (THP-1) and normal human polymorphonuclear neutrophils (PMNs). This suggests that hydroxylation at C4′ or C6 is important to the apoptosis-inducing activities of flavanone through ROS production, and that activation of the caspase-3 cascade, downstream of caspase-9 activation, is involved.

Keywords—4′-OH-flavanone, 6-OH-flavanone, Apoptosis, Reactive oxygen species, Caspase 3, Free radicals

INTRODUCTION

Flavonoids are benzo-γ-pyrone derivatives, and are found extensively in fruits, vegetables, seeds, and medicinal herbs. People have been reported to ingest about 1 g of flavonoids from their diet daily. The biological activities of flavonoids have been extensively examined, and several pharmacological activities including anti-inflammatory, antitumor, and antioxidant properties were identified [1–3]. Although flavonoids possess some promising biological effects, several problems have been reported that impede the practical application of flavonoids as drugs in the treatment of diseases. Recent studies indicated that the differential effects of flavonoids

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are based on substituted functional groups [4,5]. Schroeter et al. reported that flavonoids attenuate neuronal cell death following uptake of oxidized low-density lipoprotein (oxLDL). Furthermore, flavonoids exert protective effects, which appear to be related to specific structural characteristics, particularly relevant being those defining their reduction potentials and partition coefficients [6]. Sekher et al. suggested that the structure of the B ring is the primary determinant of the antioxidant activity of flavonoids when studied through fast-reaction kinetics [7]. Therefore, investigation of the structurally related activities of flavonoids is an important issue for their further development.

Apoptosis is a defined type of cell death and differs from traditional cell death, necrosis. Previous studies have demonstrated that induction of apoptosis in tumor cells prevents the occurrence of cellular inflammatory responses, and exhibits better cancer therapeutic effects than with necrosis [8,9]. Several characteristics of apoptosis have been identified such as DNA fragmentation, chromatin condensation, apoptotic bodies, and these are not observed in necrotic cells [10,11]. Apotosis induced by flavonoids has been demonstrated in several previous studies [12–15]; however, the effect of hydroxyl substitution in their apoptosis-inducing activities is still undefined. Reactive oxygen species (ROS) have been shown to serve as initial molecules to turn on the apoptotic machinery in tumor cells, and several apoptosis-related genes have been identified such as caspases, Bcl-2 family proteins, and poly(ADP-ribose) polymerase (PARP) [16]. Human caspases 1 to 10 have been described, and previous studies demonstrated that activation of the caspase cascade is involved in chemical- and agent-induced apoptosis [17]. Activated executioner caspase-3 exists as inactive pro-caspase-3 in the cytoplasm and is proteolytically activated by multiple cleavages of pro-caspase 3 to generate the cleaved fragments in cells undergoing apoptosis [18]. After caspase-3 activation, some specific substrates for caspase-3 such as PARP and D4-GDI proteins are cleaved, and these are important to the occurrence of apoptosis [19]. PARP is required for DNA repair and activated caspase-3 cleaves PARP at Asp 216 to generate the 85- and 31-kDa apoptotic fragments in coordination with DNA fragmentation during apoptosis [20]. D4-GDI is a negative regulator of the ras-related Rho family of GTPases, and activation of Rho GTPases promotes cytoskeletal and membrane changes associated with apoptotic cells. Activated caspase 3 cleaves D4-GDI to 23- and 5-kDa fragments, and activates Rho GTPases to produce apoptotic morphological changes [21]. Other important apoptotic regulatory genes are Bcl-2 family proteins which modulate the occurrence of apoptosis and tumorigenesis. Members of Bcl-2 family proteins can be divided into two subfamilies: one is antiapoptotic and includes Bcl-2, Mcl-1, and Bcl-XL proteins, and the other is pro apoptotic and includes Bax, Bcl-Xs, and Bad [22,23]. Induction of pro-apoptotic Bcl-2 family proteins and inhibition of anti-apoptotic family proteins have been detected in apoptosis induced by chemicals [24].

Our previous studies demonstrated that flavonoids exhibit activities that inhibit TPA-induced tumor promotion in NIH3T3 cells and LPS-induced NO production in vivo and in vitro, and induce apoptosis in tumor cells [25–27]. In the present study, we provide scientific evidence to demonstrate that hydroxylation at C4′ and C6 is critical to the apoptosis-inducing activity of flavonoids, and that activation of ROS production and the caspase-3 cascade is involved in their apoptotic machinery. The structurally related activities of flavonoids in apoptosis induction were investigated.

MATERIALS AND METHODS

Cell culture

HL-60 human promyeloleukemic cells and THP-1 mature monocytic cells were obtained from American Type Culture Collection (ATCC; Rockville, MD). Human polymorphonuclear cells (PMNs) were obtained from healthy male donors after Ficoll–Hypaque density gradient separation. Human PMNs were washed twice in 0.9% NaCl and resuspended in RPMI-1640 medium. HL-60 and THP-1 cells were grown in RPMI-1640 containing 10% heat-inactivated fetal bovine serum (FBS) and maintained at 37°C in a humidified incubator containing 5% CO_2. Exponentially growing cells were exposed to drugs for the indicated periods. All culture reagents were purchased form Gibco-BRL.

Chemicals

The colorigenic synthetic peptide substrates Ac-DEVD-pNA, Ac-YVAD-pNA, Ac-IETD-pNA, and Ac-LEHD-pNA, as well as protease inhibitors for Ac-DEVD-FMK, Ac-YVAD-FMK, Ac-IETD-FMK, and Ac-LEHD-FMK, were purchased from Calbiochem. Propidium iodide, flavanone, 4′-hydroxyflavanone, 4′-methoxyflavanone, 6-hydroxyflavanone, 6-methoxyflavanone, 7-hydroxyflavanone and 7-methoxyflavanone were purchased from Sigma Chemical Company (St. Louis, MO, USA). Antibodies for PARP, caspase-3, and D4-GDI detection in Western blotting were obtained from IMGENEX. Antibodies for detecting Bcl-2 family proteins and α-tubulin were purchased from Santa Cruz. Dichlorodihydrofluorescein diacetate (DCHF-DA) was obtained from Molecular Probes.

Cell viability

Cell viability was assessed by MTT staining as described by Mosmann [28]. Briefly, HL-60 cells were plated at a density of 10^5 cells/well into 24-well plates. After overnight growth, cells were treated with a different concentrations of the flavonoids described above for 12 h. At the end of treatment, 30 μl of the tetrazolium compound MTT (3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) and 270 μl of fresh RPMI medium were added. The supernatant was removed and formazon crystals were dissolved by DMSO. After incubation for 4 h at 37°C, 200 μl of 0.1 N HCl in 2-propanol was placed in each well to dissolve the tetrazolium crystals. At the end, the absorbance at 600 nm was recorded using an ELISA plate reader.

Determination of ROS production

ROS production was monitored by flow cytometry using DCFH-DA [15]. This dye is a stable compound that readily diffuses into cells and is hydrolyzed by intracellular esterase to yield DCFH, which is trapped within cells. Hydrogen peroxide or low-molecular-weight peroxides produced by cells oxidize DCFH to the highly fluorescent compound, 2′, 7′-dichlorofluorescein (DCF). Thus, the fluorescence intensity is proportional to the amount of peroxide produced by the cells. In the present study, HL-60 cells were treated with each indicated flavanone (40 μM) for 2 or 4 h, respectively. Then, the flavanone-treated cells were washed twice with PBS to remove extracellular compounds, and DCHF-DA (100 μM) with or without H_2O_2 (200 μM) as added for 1 more h. Green fluorescence was excited using an argon

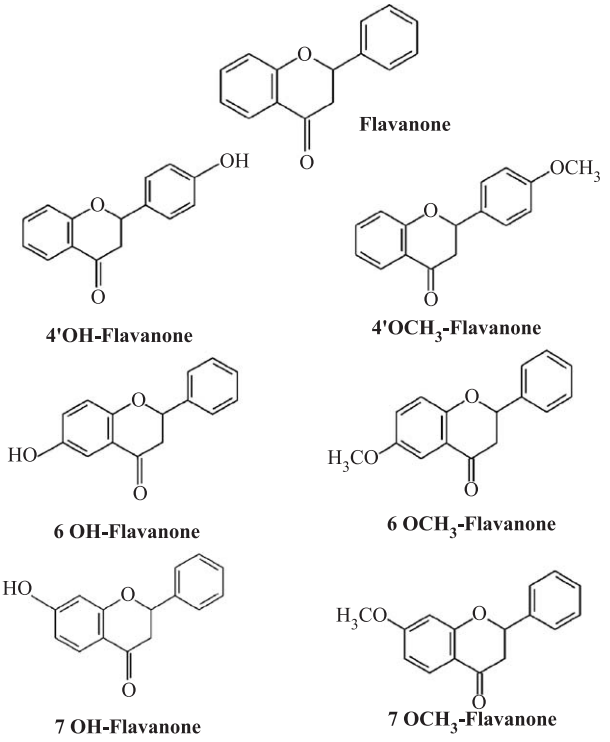

Fig. 1. Chemical structures of flavanone, 4′-hydroxyflavanone (4′-OH-flavanone), 4′-methoxyflavanone (4′-OCH_3-flavanone), 6-hydroxyflavanone (6-OH-flavanone), 6-methoxyflavanone (6-OCH_3-flavanone), 7-hydroxyflavanone (7-OH-flavanone), and 7-methoxyflavanone (7-OCH_3-flavanone).

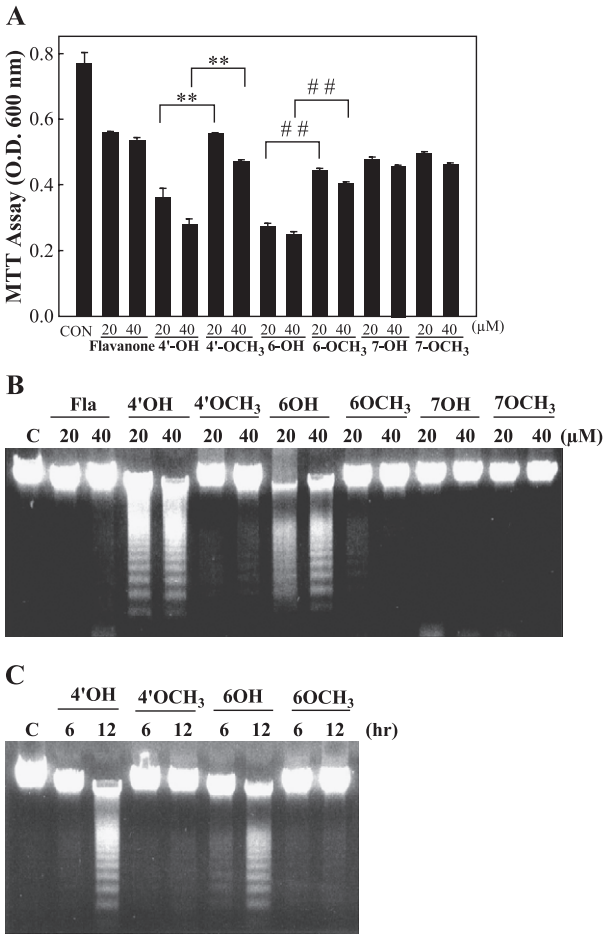

Fig. 2. Analysis of the cell viability and DNA integrity of flavanone-treated HL-60 cells as determined by MTT assay and DNA electrophoresis. (A) HL-60 cells were plated into 24-well plates for 24 h and then treated with different concentrations of the indicated compound (20 and 40 μM) for another 12 h. MTT was added into medium for another 4 h. The viability of cells was detected by measuring the absorbance at 600 nm. Each value is presented as the mean ± SE of three independent experiments. $^{**}p < .01$, significantly different from 4′-OH flavanone and 4′-OCH_3-flavanone. $^{\#\#}p < .01$, significantly different from 6-OH-flavanone and 6-OCH_3-flavanone as analyzed by Student's *t* test. (B) HL-60 cells were treated with different concentrations (20 and 40, μM) of the indicated flavanones for 12 h and DNA from cells was extracted and electrophoresed through a 1.8% agarose gel and visualized by staining with ethidium bromide. (C) The time-dependent appearance of DNA ladders was observed in HL-60 cells treated with 40 μm 4′-OH flavanone and 6-OH flavanone.

laser and was detected using a 525 nm bandpass filter by flow cytometric analysis.

Western blots

Total cellular extracts (20 μg) were prepared and separated on 8% SDS–polyacrylamide minigels for PARP detection and 12% SDS–polyacrylamide minigels for caspase-3, cleaved D4-GDI, the Bcl-2 family, and α-tubulin detection, and transferred to Immobilon polyvinylidene difluoride membranes (Millipore). The membrane was incubated at 4°C with 1% bovine serum albumin at room temperature for 1 h and then incubated with the indicated antibodies for another 3 h at room temperature followed by incubation with alkaline phosphatase-conjugated anti-mouse IgG antibody for 1 h. Protein was visualized by incubation with the colorimetric substrates nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) as described in previous articles [29,30].

Release of cytochrome C from mitochondria in drug-treated cells

Untreated and drug-treated cells were harvested by centrifugation at 1000*g* for 5 min at 4°C. The cells pellets were washed once with ice-cold PBS and resuspended with 5 vol of 20 mM Hepes–KOH (pH 7.5), 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 0.1 mM PMSF, and 250 mM sucrose. The cells were homogenized and centrifuged at 750*g* for 10 min at 4°C. Supernatants were then centrifuged at 10,000*g* for 15 min at 4°C. Pellets were lysed with 0.1 ml of lysis buffer consisting of 10 mM Tris–HCl (pH 7.4), 1 mM EDTA, 1 mM EGTA, 0.15 M NaCl, 5 μg/ml aprotinin, 5 μg/ml leupeptin, 0.5 mM PMSF, 2 mM sodium orthovanadate, and 1% SDS. The lysed solution was used for the identification of mitochondrial cytochrome *C* by immunoblotting. Supernatants were centrifuged at 100,000*g* for 15 min at 4°C, and the supernatants

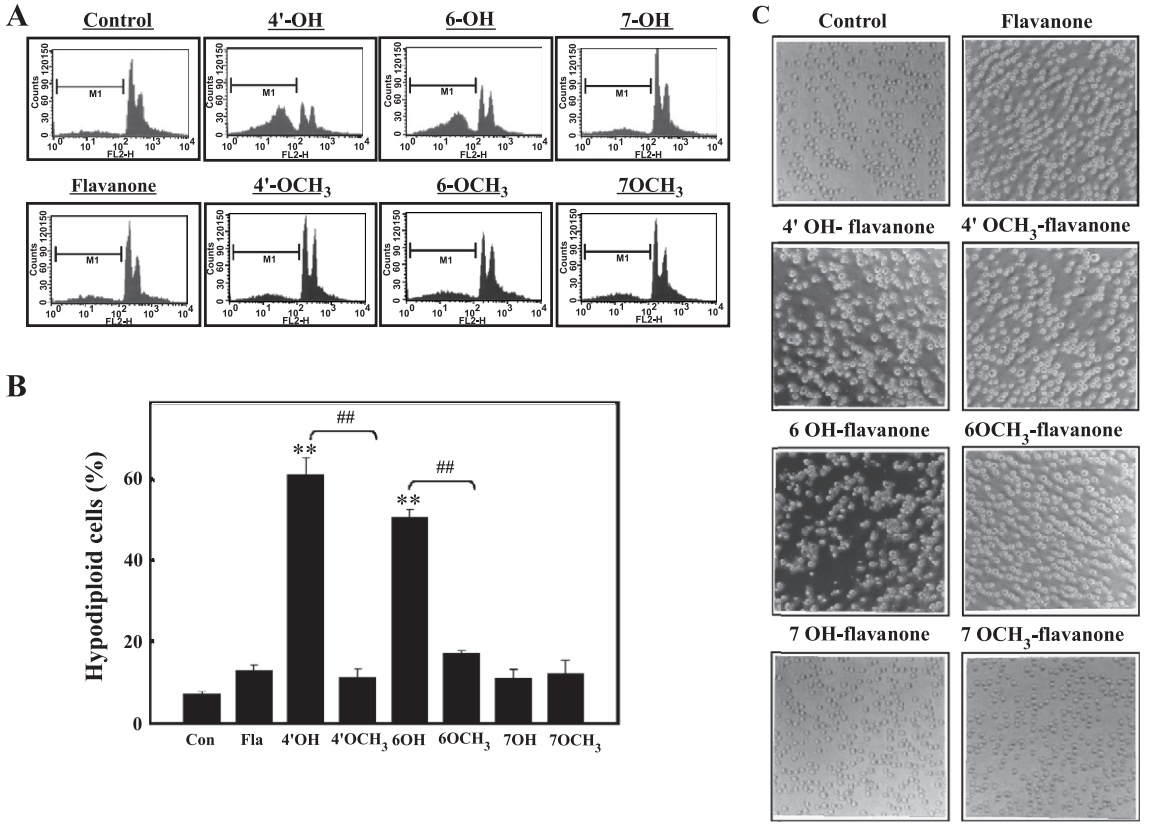

Fig. 3. Appearance of hypodiploid cells, chromosomal condensation, and apoptotic bodies in 4′-OH-flavanone- and 6-OH-flavanone-treated HL-60 cells. (A) HL-60 cells were treated with or without the indicated compounds (40 μM) for 12 h. The sub-G_1 peak was detected by flow cytometry using PI staining. (B) Quantification of the indicated compounds (40 μM) induced the occurrence of hypodiploid cells. Each value is presented as the mean ± SE of three independent experiments. $^{**}p < .01$, significantly different from the control. $^{\#\#}p < .01$, significantly different between the same carbon site with hydroxyl or methoxyl group as analyzed by Student's *t* test. (C) Occurrence of apoptotic bodies of HL-60 cells was detected in 4′-OH-flavanone- and 6-OH-flavanone-treated HL-60 cells for 12 h by light microscopic observation.

obtained were used for identification of cytosolic cytochrome *C* by immunoblotting.

DNA gel electrophoresis

Cells (10^6/ml) given different treatments were collected, washed with PBS twice, and then lysed in 100 ml of lysis buffer (50 mM Tris, pH 8.0, 10 mM EDTA, 0.5% sodium sarkosinate, and 1 mg/ml proteinase K) for 3 h at 56°C and treated with 0.5 mg/ml RNase A for another hour at 56°C DNA was extracted with phenol/chloroform/isoamyl alcohol (25/24/1) before loading. Samples were mixed with loading buffer (50 mM Tris, 10 mM EDTA, 1% (w/w) low-melting-point argarose, 0.025% (w/w) bromophenol blue) and loaded onto a presolidified 2% agarose gel containing 0.1 μg/ml ethidium bromide. The agarose gels were run at 50 V for 90 min in TBE buffer, then observed and photographed under UV light.

Analysis of respective caspase activities

Ac-DEVD-pNA for caspase-3, Ac-YVAD-pNA for caspase-1, Ac-IETD-pNA for caspase-8, and Ac-LEHD-pNA for caspase-9 were used as colorimetric protease substrates. After different treatments, cells were collected and washed three times with PBS and resuspended in 50 mM Tris–HCl (pH 7.4), 1 mM EDTA, and 10 mM EGTA. Cell lysates were clarified by centrifugation at 15,000 rpm for 3 min, and clear lysates containing 50 μg of protein were incubated with 100 μM concentrations of the specific colorimetric substrates at 37°C for 1 h. Alternative activity of caspase-1, -3, -8, or -9 was described as the cleavage

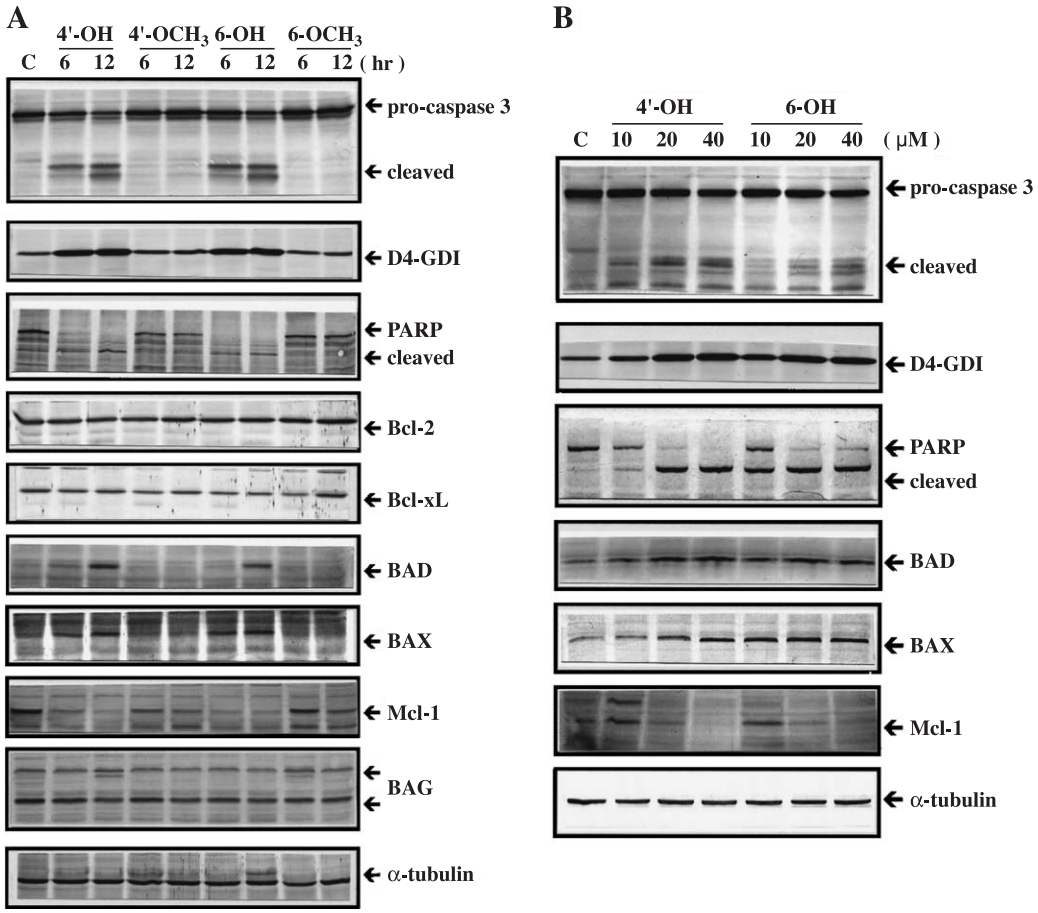

Fig. 4. Involvement of caspase-3 activation, PARP and D4-GDI protein cleavage, Bad and Bax protein expression, and a decrease in Mcl-1 protein in 4′-OH-flavanone- and 6-OH-flavanone-treated HL-60 cells. (A) HL-60 cells were treated with 40 μM concentrations of the indicated flavanones for 12 h. Cleavage of PARP, D4-GDI, and pro-caspase-3 proteins was analyzed by Western blotting as described. Alternative expression of Bcl-2 family proteins in treated HL-60 cells. (B) Dose-dependent effects of 4′-OH-flavanone and 6-OH-flavanone at different concentration (10, 20, and 40 μM) for 12 h on expression of the indicated protein were determined using specific antibodies for Western blot.

of colorimetric substrate by measuring the absorbance at 405 nm.

Flow cytometry analysis

Trypsinized cells were washed with ice-cold PBS and fixed in 70% ethanol at −20°C for at least 1 h. After fixation, cells were washed twice, incubated in 0.5 ml of 0.5% Triton X-100/PBS at 37°C for 30 min with 1 mg/ml RNase A, and stained with 0.5 ml of 50 mg/ml propidium iodide for 10 min. Fluoresence emitted from the propidium–DNA complex was quantitated after excitation of the fluorescent dye by FACScan flow cytometry (Becton Dickinson, San Jose, CA, USA).

Statistics

Values are expressed as means ± S.E. The significance of the difference from the respective controls for each experimental test condition was assayed by using Student's *t* test for each paired experiment. A p value < .01 or < .05 was regarded as indicating a significant difference.

RESULTS

Hydroxylation at C4′ and C6 in flavanone shows the most significant cytotoxic effects in HL-60 cells

Several previous studies have demonstrated the structure–activity relationship (SAR) of flavonoids;

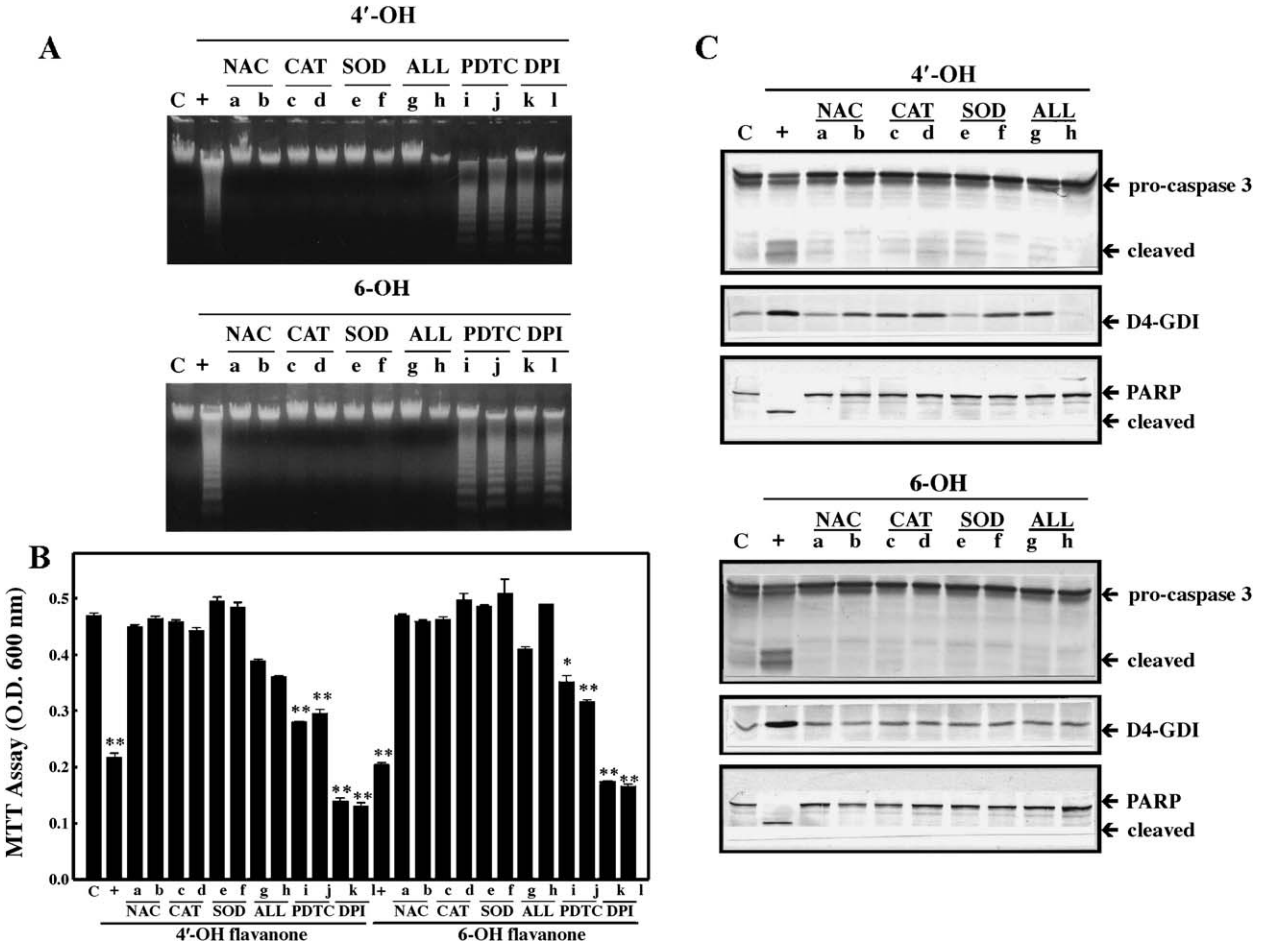

Fig. 5. Effects of NAC, CAT, SOD, ALL, PDTC, and DPI on 4′-OH-flavanone- and 6-OH-flavanone-treated HL-60 cells. (A) HL-60 cells were treated with different concentrations of NAC, CAT, SOD, ALL, PDTC, and DPI for 1 h followed by 4′-OH-flavanone and 6-OH-flavanone (40 μM) treatment for another 12 h. Integrity of DNA was analyzed by electrophoresis as described under Materials and Methods. (B) HL-60 cells were treated with different concentrations of NAC, CAT, SOD, ALL, PDTC, and DPI for 1 h followed by 4′-OH-flavanone and 6-OH-flavanone (40 μM) treatment for a further 12 h. MTT was added to the medium for an additional 4 h. Cell viability was determined as described. Each value is presented as the mean ± SE of three independent experiments. *p < .05 and **p < .01, significantly different from the control as analyzed by Student's *t* test. (C) HL-60 cells were treated with different concentrations of NAC, CAT, SOD, and ALL for 1 h followed by 4′-OH-flavanone and 6-OH-flavanone (40 μM) treatment for a further 12 h. Cleavage of PARP, D4-GDI, and pro-caspase-3 proteins was detected by Western blots as described under Materials and Methods. NAC (a: 2.5 mM, b: 5 mM), CAT (c: 200 U/ml, d: 400 U/ml), SOD (e: 100 μg/ml, f: 200 μg/ml), ALL (g: 50 μM, h: 100 μM), PDTC (i: 20 μM, j: 40 μM), DPI (k: 10 μM, l: 20 μM).

however, the results are still undefined. In the present study we explored seven flavanones with different substitutions at indicated sites including flavanone, 4′-hydroxyflavanone (4′-OH-flavanone), 4′-methoxyflavanone (4′-OCH_3-flavanone), 6-hydroxyflavanone (6-OH-flavanone), 6-methoxyflavanone (6-OCH_3-flavanone), 7-hydroxyflavanone (7-OH-flavanone), and 7-methoxyflavanone (7-OCH_3-flavanone), as well as their apoptosis-inducing activities. The chemical structures of flavanones used in the present study are shown in Fig. 1. Results of the MTT assay showed that 4′-OH-flavanone and 6-OH-flavanone but not others exhibited significant cytotoxicity to human leukemia HL-60 cells. DMSO, even at the highest dose of 0.5%, had no effect on cellular viability of HL-60 cells. To study further the characteristics of cell death induced by 4′-OH-flavanone and 6-OH-flavanone, integrity of DNA, appearance of apoptotic bodies, and ratio of sub-G_1 peaks were analyzed. Results of DNA analysis showed that treatment with 4′-OH-flavanone and 6-OH-flavanone caused the digestion of genomic DNA in a dose- and time-dependent manner, associated with a decrease in intact DNA and the appearance of fragmented DNA (Fig. 2B). Interestingly, neither 4′-CH_3O-flavanone nor 6-CH_3O-flavanone showed apoptotic activity in HL-60 cells (Fig. 2C). Similary, the occurrence of apoptotic bodies detected by light microscopic observation and an increase in the sub-G_1 ratio were detected in 4′-OH- and 6-OH-flavanone- but not 4′-CH_3O-flavanone- nor 6-CH_3O-flavanone-treated HL-60 cells (Fig. 3). No obvi-

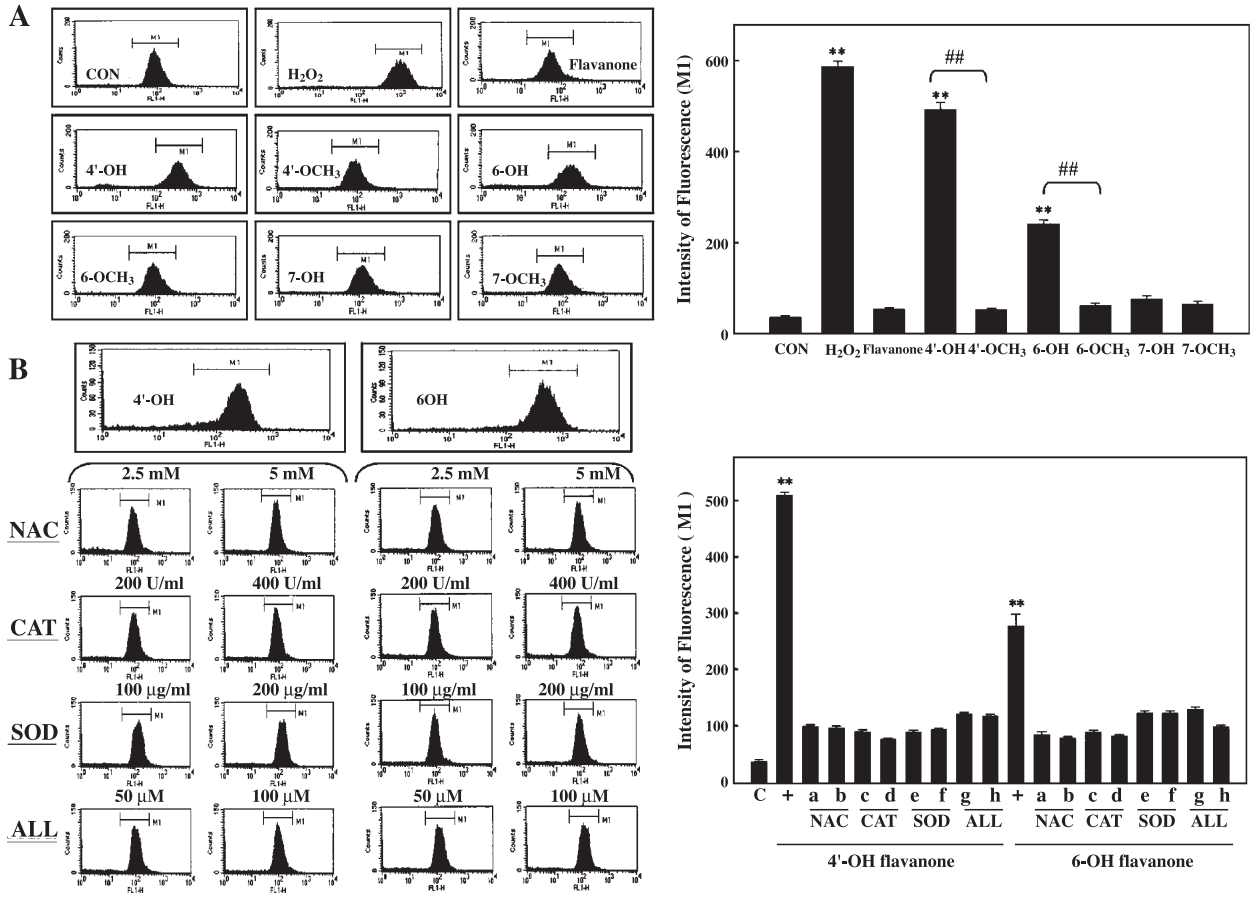

Fig. 6. Involvement of ROS production in 4′-OH-flavanone- and 6-OH-flavanone-treated HL-60 cells. (A) HL-60 cells were treated with 200 μM H_2O_2 or a 40 μM concentration of the indicated flavanone. After treatment, cells were washed twice with PBS to remove extracellular compounds, and addition of DCHF-DA was performed for 1 more h. The fluorescence intensity of cells was detected by flow cytometry analysis. (B) HL-60 cells were treated with different concentration of NAC, CAT, SOD, and ALL for 1 h followed by 4′-OH-flavanone and 6-OH-flavanone (40 μM) treatment for another hour. After treatment, cells were washed twice with PBS to remove extracellular compounds, and addition of DCHF-DA was performed for 1 more h. The fluorescence intensity of cells was detected by flow cytometry analysis. Each value is presented as the mean ± SE of three independent experiments. $^{**}p < .01$, significantly different from the control. $^{\#\#}p < .01$, significant difference between the same carbon sites with hydroxyl group and methoxyl group as analyzed by Student's *t* test. NAC (a: 2.5 mM, b: 5 mM), CAT (c: 200 U/ml, d: 400 U/ml), SOD (e: 100 μg/ml, f: 200 μg/ml), ALL (g: 50 μM, h: 100 μM).

ous DNA fragmentation, apoptotic bodies, or sub-G_1 peak was detected in DMSO-treated HL-60 cells (data not shown). These data suggest that hydroxylation at C4′ and C6 is a requirement for the apoptosis-inducing activity of flavanones.

Involvement of Caspase 3 cascade activation, PARP and D4-GDI cleavage, and Bcl-2 family proteins in 4′-OH flavanone- and 6-OH flavanone-induced apoptosis

Activation of caspase-3 (CPP32/Yama) causes it to recognize the sequence Asp–Glu–Val–Asp (DEVD) and to cleave a number of proteins, such as PARP and D4-GDI, another hallmark of apoptosis. Fig. 4 shows that exposure of HL-60 cells to 4′-OH-flavanone and 6-OH-flavanone causes degradation of 116-kDa PARP to 85-kDa fragments and the production of cleaved D4-GDI protein (23 kDa) in a dose- and time-dependent manner. The protein cleavages were associated with activation of caspase-3 brought about by its cleavage, represented here as a decline in the pro-caspase-3 on the Western blot and production of cleaved fragments. Bcl-2 family proteins are important apoptotic regulators and are located upstream of caspase activation. In 4′-OH-flavanone- and 6-OH-flavanone-treated HL-60 cells, dose-dependent a decreases in Mcl-1 protein and increases in Bad and Bax protein were detected; however, Bcl-2 and Bag protein remained unchanged. In the respective 4′-OCH_3-flavanone- and 6-OCH_3-flavanone-treated HL-60 cells, no significant apoptosis-related gene expression was observed. These results indicate that hydroxylation at the C4′ and C6 positions induces apoptosis through activation of the caspase-3 cascade.

ROS production involves in 4′-OH-flavanone- and 6-OH-flavanone-induced apoptosis

It is important to identify why hydroxylation at C4′ or C6 of flavanone induces apoptosis in HL-60 cells. ROS have been shown to play an important role in the

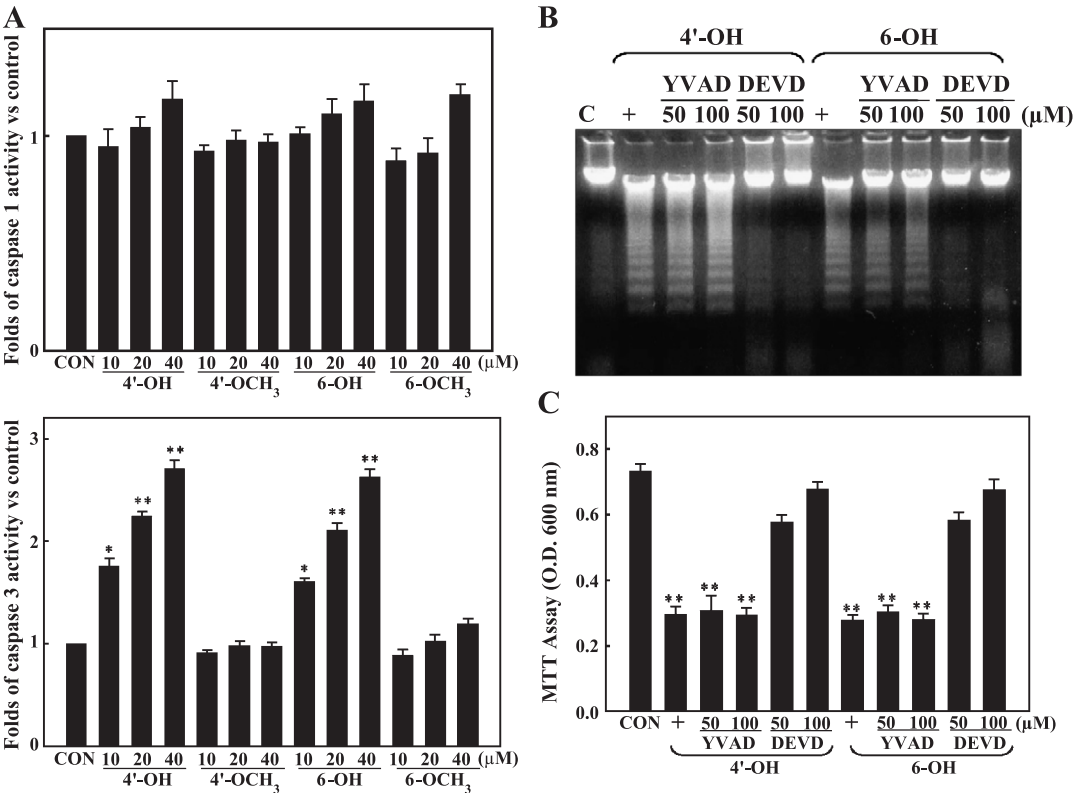

Fig. 7. Specific induction of caspase-3 activities in 4′-OH-flavanone- and 6-OH-flavanone-treated HL-60 cells. (A) HL-60 cells were treated with different concentrations of compounds (10, 20, and 40 μM) for 12 h. Cells were harvested and lysised in lysis buffer. Enzyme activity of caspase-1 and caspase-3 like proteases was analyzed as described under Materials and Methods. (B) HL-60 cells were pretreated with either inhibitor, Ac-DEVD-FMK or Ac-YVAD-FMK (50 or 100 μM), for 3 h followed by 4′-OH flavanone or 6-OH flavanone (40 μM) for a another 12 h. DNA fragmentation was analyzed by 1.8% agarose electrophoresis. (C) Cell viability was examined by MTT assay. Each value is presented as the mean ± SE of three independent experiments. *$p < .05$ and **$p < .01$, significantly different from the control as analyzed by Student's t test.

induction of apoptosis, and both pro-oxidant and anti-oxidant activities of flavonoids have been identified. Results of flow cytometry analysis using DCHF-DA as a fluorescent ROS indicator showed that an increase in intracellular peroxide levels was found in 4′-OH-flavanone- and 6-OH-flavanone-treated but in not other flavanone-treated HL-60 cells. The addition of H_2O_2 also induced intracellular peroxide as an internal control. Additionally, antioxidants such as *N*-acetylcysteine (NAC), catalase (CAT), superoxide dismutase (SOD), allopurinol (ALL), pyrrolidine dithiocarbamate (PDTC), and diphenylene iodonium (DPI) were used in the present study to investigate if ROS production is an essential event for the apoptosis-inducing activities of 4′-OH-flavanone and 6-OH-flavanone. Results in Figs. 5A and B show that pretreatment with NAC, CAT, SOD, and ALL, but not DPI or PDTC, protects HL-60 cells from 4′-OH-flavanone- and 6-OH-flavanone-induced cytotoxicity and DNA fragmentation in a dose-dependent manner. The preventive mechanism of NAC, CAT, SOD, and ALL in 4′-OH-flavanone- and 6-OH−flavanone-induced apoptosis was blocking of caspase-3 activation and PARP and D4-GDI protein cleavage. Neither PDTC (an NF-κB inhibitor) nor DPI (an NADPH oxidase inhibitor) suppressed expression of the indicated genes induced by 4′-OH-flavanone and 6-OH-flavanone in HL-60 cells. In addition, NAC, CAT, SOD, and ALL, but not PDTC or DPI, efficiently suppressed the intracellular peroxide level induced by 4′-OH-flavanone and 6-OH-flavanone. These results suggest that the differential apoptosis-inducing activity of flavanones is mediated by alternative ROS production, and ROS production

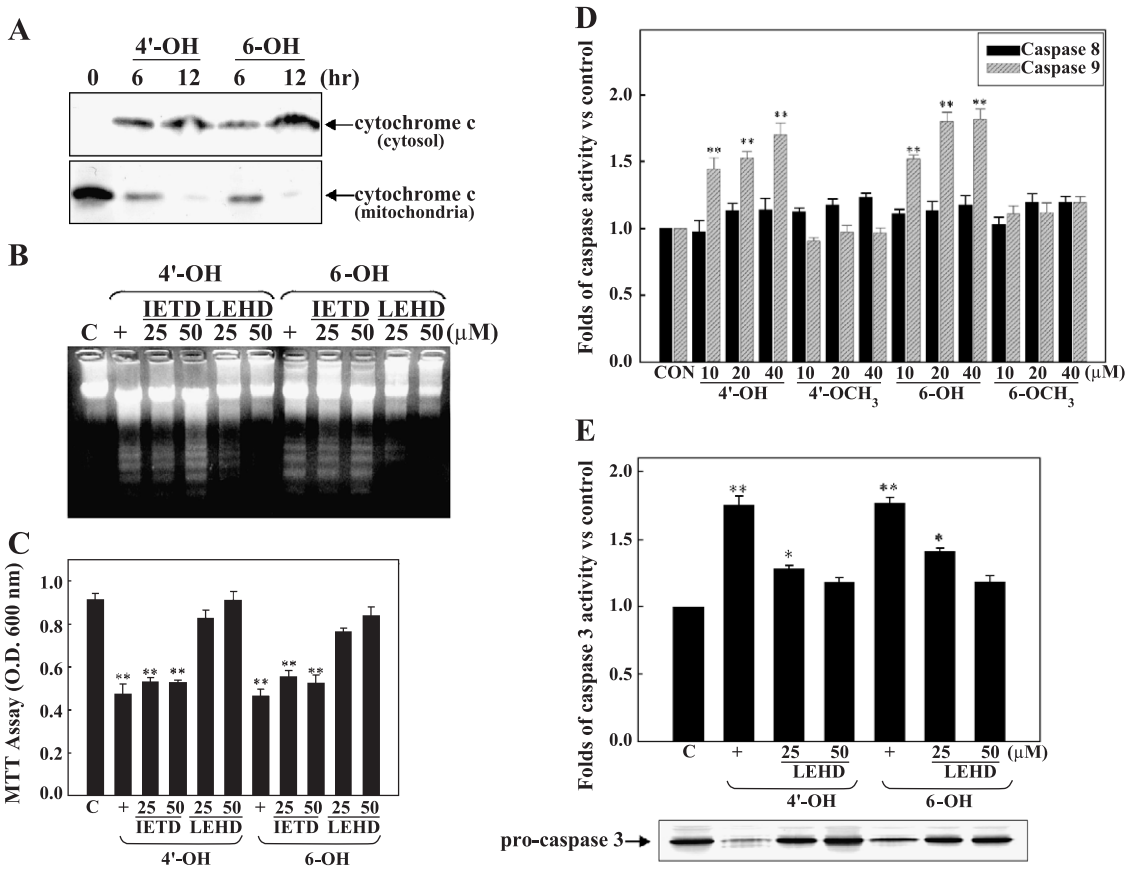

Fig. 8. Activation of caspase-9 enzyme activity and release of cytochrome C from mitochondria to cytosol participated in 4′-OH- and 6-OH-flavanone-induced apoptosis in HL60 cells. (A) HL-60 cells were treated with 4′-OH- or 6-OH-flavanone for 12 h. Release of cytochrome C from mitochondria to cytosol was detected by Western blot. (B) HL-60 cells were pretreated with the caspase-8 inhibitor Ac-IETD-FMK or caspase-9 inhibitor Ac-LEHD-FMK (25 or 50 μM), for 3 h followed by 4′-OH- or 6-OH-flavanone (40 μM) treatment for another 12 h. Integrity of DNA was analyzed by electrophoresis using 1.8% agarose. (C) Viability of cells given different treatments as described in (B) was analyzed by MTT assay. MTT was added into the medium at the end of reaction for another 4 h. (D) HL-60 cells were treated with different concentrations of the indicated compounds (10, 20, and 40 μM) for 12 h. Activity of caspase-8- and caspase-9-like proteases was analyzed as described under Materials and Methods. (E) HL-60 cells were pretreated with Ac-LEHD-FMK (25 or 50 μM) for 3 h followed by 4′-OH- or 6-OH-flavanone (40 μM) for another 12 h. Enzyme activity of caspase-3-like proteases was analyzed (top) and caspase-3 protein procession was detected by Western blot (bottom).

participates in 4′-OH-flavanone- and 6-OH-flavanone-induced apoptosis in HL-60 cells (Fig. 6).

Activation of caspase-3, not caspase-1, is involved in 4′-OH-flavanone- and 6-OH-flavanone-induced apoptosis

Cleavage of caspase-3 and its downstream molecules, PARP and D4-GDI, was examined; however, no evidence supported the importance of caspase-3 activation in 4′-OH-flavanone- and 6-OH-flavanone-treated HL-60 cells. Two colorimetric substrates, Ac-DEVA-pNA for caspase 3-related activities and Ac-YVAD-pNA for caspase-1-related activities, were used in this study. As illustrated in Fig. 7, 4′-OH-flavanone and 6-OH-flavanone induced remarkable increases in DEVD-specific, but not YVAD-specific, caspase activity in HL-60 cells. Either of the two enzyme activities was found in 4′-OCH_3-flavanone- or 6-OCH_3-flavanone-treated cells. The data indicated that activation of caspase-3, but not caspase-1, activity was exhibited in 4′-OH-flavanone- and 6-OH-flavanone- but not 4′-OCH_3-flavanone- or 6-OCH_3-flavanone-treated cells. To further determine if the activation of caspase 3-like protease is necessary for apoptosis induced by 4′-OH-flavanone and 6-OH flavanone, caspase inhibitors including the caspase-3-like protease inhibitor Ac-DEVD-FMK and the caspase-1-like protease inhibitor Ac-YVAD-FMK were used to block intracellular protease, and 4′-OH-flavanone- and 6-OH-flavanone-induced DNA ladders were analyzed by agarose electrophoresis. Results in Fig. 7 show that the caspase-3-like inhibitor Ac-DEVD-FMK (50 and 100 μM) inhibited the occurrence of DNA ladders induced by 4′-OH-flavanone and 6-OH-flavanone. However, Ac-YVAD-FMK, an inhibitor of caspase-1-like activity, at the same concentrations, had no obvious preventive effect. These data demonstrate that activation of caspase-3-like activity is a downstream event in 4′-OH-flavanone- and 6-OH-flavanone-induced apoptosis.

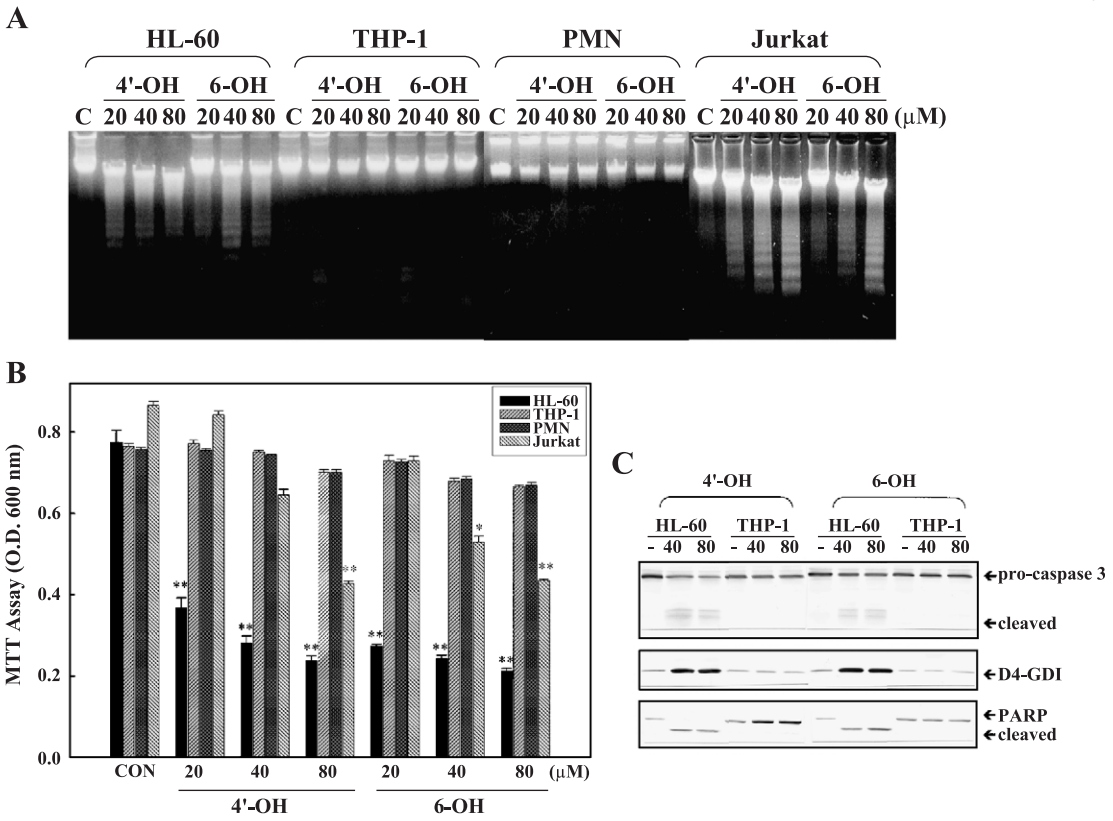

Fig. 9. 4′-OH-flavanone and 6-OH-flavanone showed differential apoptosis-inducing activitiy in leukemia cells (Jurkat and HL-60), preferential mature cells (THP-1) and normal human polymorphonuclear (PMN) cells. (A) Equal numbers of HL-60, THP-1, PMN, and Jurkat cells were treated with different concentrations of 4′-OH- or 6-OH-flavanone (20, 40, and 80 μM) for another 12 h. DNA fragmentation was analyzed by electrophoresis. (B) Cell viability was examined by MTT assay as described previously. Each value is presented as the mean ± SE of three independent experiments. $^{**}p < .01$, significantly different from control as analyzed by Student's *t* test. (C) Equal numbers of HL-60 cells and THP-1 cells were treated with different concentrations of 4′-OH- or 6-OH-flavanone (40 and 80 μM) for another 12 h. Cleavage of PARP, D4-GDI, and caspase-3 proteins was detected by Western blot as described previously.

Involvement of pro-initiator caspase-9 activation and release of cytocrome C from mitochondria to cytosol in 4′-OH-flavanone- and 6-OH-flavanone-induced apoptosis

Previous data showed that an increase in Bax and Bad proteins occurred in 4′-OH- and 6-OH-flavanone-induced apoptosis. It is suggested that the mitochondrial pathway might play an important role in 4′-OH- or 6-OH-flavanone-induced apoptosis. In Fig. 8A, release of cytochrome *C* from mitochondria to cytosol was observed in 4′-OH- and 6-OH flavanone-treated HL-60 cells in a time-dependent manner. A caspase-9 peptidyl inhibitor, Ac-LEHD-FMK, but not the caspase 8 peptidyl inhibitor, Ac-IETD-FMK, significantly inhibited 4′-OH- and 6-OH-flavanone-induced apoptosis, as demonstrated by MTT and DNA fragmentation assays (Figs. 8B, C). Additionally, detection of caspase-8 and caspase-9 activities in 4′-OH- and 6-OH-flavanone-treated cells was performed using specific colorimetric substrates (Ac-IETD-pNA for caspase-8 and Ac-LEHD-pNA for caspase-9). Results in Fig. 8D show that there was a significant increase in LEHD-specific, but not IETD-specific, activity in HL-60 cells after 4′-OH- or 6-OH-flavanone treatment. And the caspase-9 inhibitor Ac-LEHD-FMK significantly reduced caspase-3 enzyme activity and protein procession induced by 4′-OH- or 6-OH-flavanone (Fig. 8E). These data indicate that activation of caspase-9 participated in 4′-OH- and 6-OH-flavanone-induced apoptosis, and was located upstream of caspase-3 activation.

The apoptosis inducing activity of 4′-OH-flavanone and 6-OH-flavanone was identified in another leukemia cell line (Jurkat), but not in mature monocytic cells line (THP-1) and normal human PMNs

THP-1 is a mature, well-differentiated monocytic cell line, and previous studies had indicated that THP-1 exhibits physiological characteristics of normal cells [31,32]. To determine if 4′-OH-flavanone and 6-OH-flavanone exhibit differential apoptosis-inducing activities between leukemia and mature blood cells, leukemia cells (Jurkat and HL-60), mature monocytic cells (THP-1), and primary human PMNs were used in the present study. The apoptosis-inducing activities of 4′-OH-flavanone and 6-OH-flavanone were observed in both leukemia cells lines (Jurkat and HL-60), but not in THP-1 cells and primary PMNs, as analyzed by DNA fragmentation and MTT assays (Figs. 9A, B). Results of Western blotting showed that 4′-OH-flavanone and 6-OH-flavanone induced cleavage of caspase-3, PARP, and D4-GDI protein in HL-60 cells, but not in THP-1 cells (Fig. 9C). These data suggest the specific apoptosis-inducing activities of 4′-OH-flavanone and 6-OH-flavanone in leukemia cells.

DISCUSSION

The present study demonstrates that hydroxylation status is important to the apoptosis-inducing activity of flavanone. Flavanone with a hydroxyl group at C4′ or C6 showed significant apoptosis-inducing activity in HL-60 cells, compared with other structurally related flavanones. The apoptosis induced by 4′-OH flavanone and 6-OH flavanone was in accord with induction of ROS production and activation of the caspase-3 cascade. Antioxidants such as NAC, catalase, and SOD inhibited 4′-OH-flavanone- or 6-OH flavanone-induced apoptosis associated with the suppression of caspase-3 activation. Interestingly, neither DNA fragmentation nor caspase-3 activation was previously examined in 4′-OH-flavanone- or 6-OH-flavanone-treated mature monocytic THP-1 cells and human PMNs. These data showed that (1) a hydroxyl group at C4′ and C6 is an apoptotic determinant of flavanone; (2) ROS production and caspase-3 activation are involved in the apoptotic machinery induced by 4′-OH-flavanone and 6-OH-flavanone; and (3) differential cytotoxic effects of 4′-OH-flavanone and 6-OH-flavanone in poor leukemia (HL60) cells and mature monocytic (THP-1) cells and human PMNs. ROS are a family of active molecules including superoxide (O_2^{-}), peroxyl ($ROO^{\bullet}$), hydroxyl ($OH^{\bullet}$), and nitric oxide ($NO^{\bullet}$) that are involved in the modulation of biological functions of cells. Several previous studies have indicated that ROS play different roles in vivo; some are beneficial and are related to energy production, phagocytosis, regulation of cell growth, and synthesis of biologically important compounds [33,34]. Growth factors such as epidermal growth factor (EGF) and platelet-derived growth factor (PDGF) induce proliferation accompanied by ROS production [35,36]. However, a large amount of ROS may cause damage because they can attack lipids in cell membranes, proteins in tissue or enzymes, and DNA. This oxidative damage is thought to play a causative role in aging and several human diseases such as heart disease, cataracts, and cancer [37,38]. These data indicate that ROS are a family of multifunctional molecules, and are involved in the regulation of physiological activities and the occurrence of human diseases. Flavonoids have been shown to possess both ROS-producing (pro-oxidant) and ROS-scavenging (antioxidant) activities [39–41]. Previous studies demonstrated that flavonoids prevent ROS-induced DNA damage, lipid peroxidation, and cell death through antioxidant activities [42,43]. The structure-related antioxidant activities of flavonoids have been extensively studied. Wang and Burda reported that the aromatic

OH group is very important to the antioxidative effects of flavonoids, and flavonoids with 3′, 4′-OH groups in the B ring and a 2,3-double bond in conjugation with a 4-oxo group in the C ring such as quercetin exhibited potent inhibitory activities on H_2O_2-induced oxidative stress and calcium dysregulation [5,41]. Cao et al. indicated that flavonoids contain multiple hydroxyl (OH) substitutions which show significant antiperoxyl radical activities, and di-OH substitutions at 3′ and 4′, but not a single OH at position 5, are particularly important to the peroxyl radical scavenging activity of flavonoids [44]. These data suggest that the more OH substitutions there are in the backbone structure of flavonoids, the stronger the antioxidant activity will be. Furthermore, the ability to penetrate the cell membrane of flavonoids was reported as an important issue in antioxidant activation, and the more lipophilic a compound, the better it can penetrate lipid membranes. Structural analysis showed that reducing OH groups increased the lipophilicity of flavonoids. However, better lipophilicity did not parallel the flavonoids' ability to antagonize H_2O_2-induced oxidative stress [5], indicating that the structural compositions of flavonoids govern the antioxidant potency of flavonoids. In the present study, flavanones contained a respective OH or OCH_3 group at 4′, 6′, or 7 in a structure without a 2,3-double bond in the C ring, which was a crucial group for the antioxidant activity of flavonoids as suggested by Wang et al. This indicated that these flavanones might be lipophilic enough to penetrate lipid membrane and have low antioxidant activity in cells.

In contrast to antioxidant activity, some flavonoids with multiple OH substitutions such as quercetin have been shown to be mutagenic in vitro through pro-oxidant rather than antioxidant action [45]. Although considerable physiological and pharmacological functions have been reported for various flavonoids, it is extremely important to understand the antioxidant and pro-oxidant behavior of flavonoids and their related SARs. Our previous studies demonstrated that wogonin, fisetin, emodin, and quercetin, which contain four, five, six, and seven OH groups, respectively, in the backbone of their structures, induced apoptosis in tumor cells independent of ROS production [12,15,19]. Results of the present study show that a single OH substitution at C4′ or C6 is critical to apoptosis induction by flavanone. Induction of intracellular ROS levels by 4′-OH- or 6-OH-flavanone was identified, and antioxidants such as NAC, catalase, and allopurine prevent the apoptosis induced by both of them. These data suggest that the apoptosis induced by 4′-OH-flavanone and 6-OH-flavanone is mediated by their pro-oxidant activities. Based on the results of our serial studies, there are two possible structure-related apoptotic mechanisms induced by flavonoids: one is ROS-independent apoptosis, which occurs when flavonoids contain more than one OH substitution; and the other is ROS-dependent apoptosis when flavonoids possess no or only a single OH substitution. More evidence needs to be provided.

Apoptosis induced by flavonoids has been investigated in many studies; however, the apoptotic mechanism and SARs of 4′-OH-flavanone and 6-OH-flavanone have not previously been clarified. Caspase-3 is an executioner caspase, and exists in the cytoplasm as inactive procaspase-3 that becomes proteolytically activated by multiple cleavages of its 32kDa precursor to generate the 17- or 15-kDa form active in the apoptotic process. Activated caspase-3 cleaves PARP to generate the 85- and 31-kDa fragments during apoptosis [12,19]. In addition, Bcl-2 family proteins have been suggested to be involved in the process of apoptosis. These members are divided into proapoptotic and antiapoptotic proteins depending on their actions. Bax, Bad, Bak, and Bcl-Xs were demonstrated to be proapoptotic and Bcl-2, Bcl-XL, Bag, and Mcl-1 to be antiapoptotic proteins [15]. With respect to the results, the programmed death induced by 4′-OH-flavanone and 6-OH-flavanone was demonstrated to involve a decrease in Mcl-1 protein and increases in Bad and Bax proteins. 4′-OH-flavanone- and 6-OH-flavanone-induced apoptosis was significantly attenuated by the specific caspase-3 peptidyl inhibitor Ac-DEVE-CHO, but not by the broad caspase inhibitor Ac-YVAD-FMK. Thus, specific activation of the caspase-3 cascade was also involved during the apoptosis process.

Pharmacological studies using antioxidants or inhibitors of ROS-producing enzymes have shown that NAC, CAT, SOD, and ALL, but not PDTC and DPI, inhibit 4′-OH-flavanone- and 6-OH flavanone-induced apoptosis. NAC is a thio-containing antioxidant capable of directly

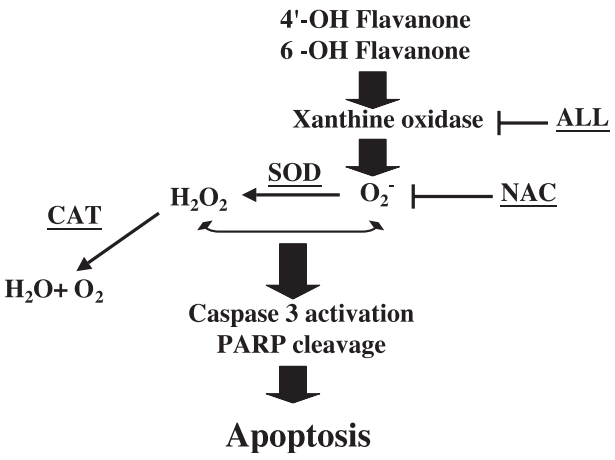

Fig. 10. Tentative model for 4′-OH-flavanone- and 6-OH-flavanone-induced apoptosis in HL-60 cells proposed in the present study. NAC, *N*-acetylcysteine; CAT, catalase; SOD, superoxide dismutase; ALL, allopurinol; PARP, poly(ADP-ribose) polymerase.

inactivating ROS as well as inducing glutathione production. ALL, an inhibitor of xanthine oxidase, exhibited a preventive effect on the apoptosis induced by 4′-OH-flavanone and 6-OH-flavanone, whereas DPI, an inhibitor of NADPH oxidase, had no effect. SOD and CAT, the enzymes involved in the conversions $O_2^{\bullet}$ to H_2O_2 and H_2O_2 to H_2O, respectively, inhibited 4′-OHflavanone- and 6-OH-flavanone-induced cell death.

On the basis of these data, we propose a possible apoptotic mechanism induced by 4′-OH- or 6-OH-flavanone (Fig. 10). The initial event induced by 4′-OH-flavanone and 6-OH-flavanone may be to induce $O_2^{\bullet}$ production by activating xanthine oxidase, because ALL prevented apoptosis. Conversion of $O_2^{\bullet}$ to H_2O_2 is essential for the apoptotic mechanism because addition of SOD had an inhibitory effect. Results of CAT prevention indicated that H_2O_2 is an important apoptotic mediator in 4′-OH-flavanone- and 6-OH-flavanone-induced cell death. Furthermore, decreasing ROS with an antioxidant also blocked activation of the caspase-3 cascade induced by 4′-OH- or 6-OH-flavanone; this indicates that ROS production is located upstream of the caspase-3 cascade and DNA fragmentation. The tentative apoptotic mechanism induced by 4′-OH-flavanone and 6-OH-flavanone is demonstrated in the present study.

Acknowledgment—This study was supported by grants from the National Science Council NSC 91-2320-B-038–040.

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ABBREVIATIONS

Ac-DEVD-FMK — acetyl-Asp–Glu–Val–Asp-fluoromethylketone
Ac-IETD-FMK — acetyl-Ile–Glu–Thr–Asp-fluoromethylketone
Ac-LEHD-FMK — acetyl-Leu–Glu–His–Asp-fluoromethylketone
Ac-YVAD-FMK — acetyl-Tyr–Val–Ala–Asp-fluoromethylketone
ALL — allopurinol
CAT — catalase
DCHF-DA — dichlorodihydrofluorescein diacetate
DCF — 2′,7′-dichlorofluorescein
DPI — pyrrolidine dithiocarbamate
MTT — 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide
NAC — *N*-acetylcysteine
PARP — poly(ADP-ribose) polymerase
PBS — phosphate-bufftered saline
PDTC — diphenylene iodonium
PMN — human polymorphonuclear cell
PMSF — polymethanesulfonyl fluoride
ROS — reactive oxygen species
SAR — structure–activity relationship
SOD — superoxide dismutase