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Wogonin and fisetin induce apoptosis in human promyeloleukemic cells, accompanied by a decrease of reactive oxygen species, and activation of caspase 3 and Ca^{2+}-dependent endonuclease

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Abstract

Seven structurally related flavonoids including luteolin, nobiletin, wogonin, baicalein, apigenin, myricetin and fisetin were used to study their biological activities on the human leukemia cell line, HL-60. On MTT assay, wogonin, baicalein, apigenin, myricetin and fisetin showed obvious cytotoxic effects on HL-60 cells, with wogonin and fisetin being the most-potent apoptotic inducers among them. The cytotoxic effects of wogonin and fisetin were accompanied by the dose- and time-dependent appearance of characteristics of apoptosis including DNA fragmentation, apoptotic bodies and the sub-G1 ratio. Treatment with an apoptosis-inducing concentration of wogonin or fisetin causes rapid and transient induction of caspase 3/CPP32 activity, but not caspase 1 activity. Further, cleavage of poly(ADP-ribose) polymerase (PARP) and decrease of pro-caspase 3 protein were detected in wogonin- and fisetin-treated HL-60 cells. An increase in the pro-apoptotic protein, bax, and a decrease in the anti-apoptotic protein, Mcl-1, were detected in fisetin- and wogonin-treated HL-60 cells. However, Bcl-2, Bcl-XL, and Bad all remained unchanged in wogonin- and fisetin-treated HL-60 cells. *In vitro* chromatin digestion revealed that endonuclease activity was profoundly enhanced in wogonin- and fisetin-treated HL-60 cells, and the addition of ethylenediaminetetraacetic acid (EDTA) or ethyleneglycoltetraacetic acid (EGTA) into the reaction blocked endonuclease activation and at an optimum pH of 7.5. The caspase 3 inhibitor, Ac-DEVD-CHO, but not the caspase 1 inhibitor, Ac-YVAD-CHO, attenuated wogonin- and fisetin-induced DNA ladders, PARP cleavage, and endonuclease activation. Pretreatment of HL-60 cells with *N*-acetyl-cysteine or catalase efficiently inhibited H_2O_2 (200 μM)-induced apoptosis, but showed no inhibitory effect on wogonin- and fisetin-induced DNA ladders, caspase 3 activation, or bax protein induction. Decrease in endogenous ROS production was detected in wogonin- and fisetin-treated HL-60 cells by DCHF-DA assay. In conclusion, our experiments indicate that a decrease in intracellular peroxide level was involved in wogonin- and fisetin-induced apoptosis; activation of caspase 3 and endonuclease, induction of bax protein and suppression of Mcl-1 protein were detected in the process. © 2002 Published by Elsevier Science Inc.

Keywords: Flavonoids; Apoptosis; Caspase 3; Endonuclease; PARP; Bax; Mcl-1

1. Introduction

Polyphenolic compounds are widely distributed in plants and are common components of human diets [1–3]. Flavonoids are one type of polyphenols including flavones, isoflavones, and flavanones, and some of them have been found to possess antilipoperoxidant, antitumoral and antiplatelet and anti-inflammatory activities [4–6]. Several recent studies demonstrated that flavonoids are able to inhibit the activities of several enzymes including lipoxygenase, cyclooxygenase and xanthine monooxygenase [7–9]. In contrast to the beneficial effects, flavonoids have also been found to be mutagenic. These harmful effects were suspected to result from the prooxidant rather than the antioxidant action of the related flavonoids

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Abbreviations: MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; PARP, poly(ADP-ribosyl) polymerase; DCHF-DA, 2′,7′-dichlorofluorescein-diacetate; DCF, 2′,7′-dichlorofluorescein; Bcl-2, B-cell lymphoma 2; ROS, reactive oxygen species; NAC, *N*-acetyl-L-cysteine.

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[10,11]. Therefore, the biological and pharmacological effects of a flavonoid compound may depend upon its behavior as either an antioxidant or a proxidant. However, the differential biological effects of proxidant and antioxidant of flavonoids are still undefined.

Two distinct modes of cell death, termed apoptosis and necrosis, have been recognized. Morphological and biochemical studies have shown the differences between these two forms of cell death [12,13]. Apoptosis is characterized by cell shrinkage, blebbing of the plasma membrane, and chromatin condensation that are associated with DNA cleavage into ladders [14,15]. Previous studies indicated that cells from a variety of human malignancies have a decreased ability to undergo apoptosis in response to some physiological stimuli [16–18]. Therefore, developing various kinds of effective agents that can enhance the extent of apoptosis might be a promising strategy in the treatment of cancer. Apoptosis signal transduction and execution require the action of the cascade of caspases [19,20]. Human caspases-1 to −10 have been described, and several previous study has demonstrated that activation of caspase cascade is involved in chemicals- and agents-induced apoptosis [21]. Recent studies have shown that Apaf-1, a homolog of CED-4, binds to initiator caspase 9 and causes caspase 9 activation through inducing its oligomerization [22,23]. Activated caspase 9 in turn cleaves and activates executioner caspase 3 [24]. Caspase 3 usually exists in the cytoplasm as an inactive pro-caspase 3 that becomes proteolytically activated by multiple cleavages of its precursor 32 kDa to generate the 20/11 or 17/11 kDa active forms in cells undergoing apoptosis [25]. Downstream of caspase 3 activation, some specific substrates are cleaved that are important for the occurrence of apoptosis such as PARP, actin, and Bid protein. PARP is required for the 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 [26].

Digestion of nuclear DNA into a nucleosomal ladder is an important biochemical hallmark of apoptosis. Several previous studies characterized the endonucleases including DNase I, DNase II, Ca^{2+}/Mg^{2+}-dependent endonuclease and Ca^{2+}/Mn^{2+}-dependent endonuclease that are responsible for the DNA fragmentation in apoptosis [27,28]. In our previous study, we demonstrated that activation of Ca^{2+}-dependent endonuclease is involved in curcumin-induced apoptosis [29]. These data indicated that endonuclease activation is important in the process of apoptosis. However, the mechanisms of activation of apoptotic endonuclease and the key role of the caspase cascade played in the endonulcease activation remain unclear.

Human myelogenous leukemia HL-60 cells are very sensitive to apoptosis in the presence various stimuli. The results of the present study show that wogonin and fisetin exhibit significant apoptosis inducing activities in HL-60 cells among seven structurally related flavonoids tested as demonstrated by morphology, DNA fragmentation and flow cytometry assay. Decrease of intracellular ROS, activation of endonuclease, and caspase 3 activity, decrease in the Mcl-1 protein, and increase in the bax protein were associated with the apoptotsis induced by wogonin and fisetin.

2. Materials and methods

2.1. Cell culture and chemicals

HL-60 human myelogenous leukemia cells were obtained from American Type Culture Collection (ATCC). HL-60 cells were grown at 37° in RPMI 1640 containing 10% heat-inactivated fetal bovine serum in an atmosphere containing 5% CO_2. Exponentially growing HL-60 cells were exposed to drugs for the indicated time periods. The colorigenic synthetic peptide substrates for caspase 3-like proteases (Ac-DEVD-pNA) and for caspase 1 (Ac-YVAD-pNA) were purchased from Calbiochem. The inhibitors for caspase 3-like proteases (Ac-DEVD-CHO) and for caspase 1 (Ac-YVAD-CHO) were purchased from Calbiochem. Propidium iodide was obtained from Sigma.

2.2. Cell viability

Cell viability was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) staining as described by Mosmann [30]. Briefly, HL-60 cells were plated at a density of 10^4 cells/well into 24-well plates and treated with different concentrations of flavonoids for 12 hr. At the end of treatment, 20 μL of MTT (10 mg/mL) was added, and cells were incubated for a further 4 hr. Cell viability was obtained by scanning with an ELISA reader (molecular devices) with a 600-nm filter.

2.3. ROS production determination

ROS production was monitored by flow cytometry using DCHF-DA. This dye is a stable compound that readily diffuses into cells and is hydrolyzed by intracellular esterase to yield DCHF, which is trapped within cells. Hydrogen peroxide or low-molecular-weight peroxides produced by cells oxidizes DCHF to the highly fluorescent compound 2′,7′-dichlorofluorescein (DCF). Thus, the fluorescence intensity is proportional to the amount of peroxide produced by the cells. Cells were treated with wogonin or fisetin or H_2O_2 for 1 hr and DCHF-DA (100 μM) was added into the medium for a further hour. The green fluorescence was excited by using an argon laser and was detected using a 525-nm band pass filter by flow cytometry analysis.

2.4. Preparation of cytosolic extract (CE) from wogonin- and fisetin-induced apoptotic HL-60 cells

Wogonin- or fisetin-treated cells were washed with ice-cold PBS and then with cytosolic extraction buffers of

different pH values including 7.5, 6.5, and 5.5 containing 10 mM Hepes-KOH, 2 mM EDTA, 0.1% Chaps, 5 mM DTT, and the protease inhibitors. Cells were suspended in one of the pH value buffers on ice for 20 min, and vortexed vigorously for 5 min. Nucleus-free cytosolic extract was obtained after centrifugation at 12,000 rpm for 15 min. The concentration of protein of each sample was measured by BCA assay [14].

2.5. In vitro chromatin digestion assay

HL-60 cells were washed twice with cold PBS and pelleted by centrifugation in STKM buffer with different pH values including 7.5, 6.5, and 5.5 containing 0.25 M sucrose, 50 mM Tris–HCl, 25 mM KCl, and 5 mM $MgCl_2$ with 0.25% Triton X-100. Nuclei were pelleted by centrifugation at 800 *g* for 5 min. Sedimented nuclei were washed once with STKM buffer. Nuclei (7×10^6) were resuspended in STKM and stored at -70° before use. Reactions for DNA ladder formation and DNase characterization were performed as described in our previous study [29]. In brief, 10 μL of intact HL-60 nuclei (1×10^6) were mixed with 30 μg cytosolic extract and incubated at 37° for 60 min. To characterize DNase in apoptotic cytosolic extracts, the effect of different pH sets (7.5, 6.5, 5.5) and effects of EDTA or EGTA included in the reaction were examined. After reaction, DNA was extracted from the reacted HL-60 nuclei, and the DNA ladder was detected by agarose gel electrophoresis.

2.6. Western blots

Total cellular extracts (30 μg) were prepared according to our previous paper [21], separated on 8% SDS-polyacrylamide minigels for PARP detection and 12% for SDS-polyacrylamide minigels for caspase 3 and α-tubulin detection, and transferred to immobilon polyvinylidenedifluoride membranes (Millipore). The membrane was incubated overnight at 4° with 1% bovine serum albumin at room temperature for 1 hr and then incubated with anti-PARP, anti-caspase 3, or anti-α-tubulin monoclonal antibodies (Transduction Laboratories) for a further 3 hr at RT followed by incubation with alkaline phosphatase-conjugated anti-mouse IgG antibody for another hour. Protein was visualized by incubating with the colorimetric substrates nitro blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) as described in our previous paper [31].

2.7. DNA gel electrophoresis

Cells 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 hr at 56° and treated with 0.5 mg/mL RNase A for another hour at 56°. 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. The gels were observed and photographed under UV light.

2.8. Activities of caspase 3 and caspase 1 (ICE) proteases

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 enzyme-specific colorimetric substrates including Ac-DEVD-pNA for caspase 3/CPP32 and Ac-YVAD-pNA for caspase 1 at 37° for 1 hr. Alternative activity of caspase 3 or caspase 1 was described as the cleavage of colorimetric substrate by measuring the absorbance at 405 nm.

2.9. Flow cytometry analysis

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

2.10. Statistics

Values are expressed as the mean ± SE. 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 <0.01 or 0.05 was regarded as indicating significant difference.

3. Results

3.1. Induction of apoptosis by wogonin and fisetin

Flavonoids are diphenylpropanes that commonly occur in plants, and more than 4000 flavonoids have been found and are frequently components of the human diet. However, several biological activities of flavonoids are still undefined. To obtain information on whether flavonoids exert inhibitory effects on human leukemia cells, seven structurally related compounds including luteolin, nobiletin, wogonin, baicalein, apigenin, myricetin, and fisetin were first used to study their biological activities here. The

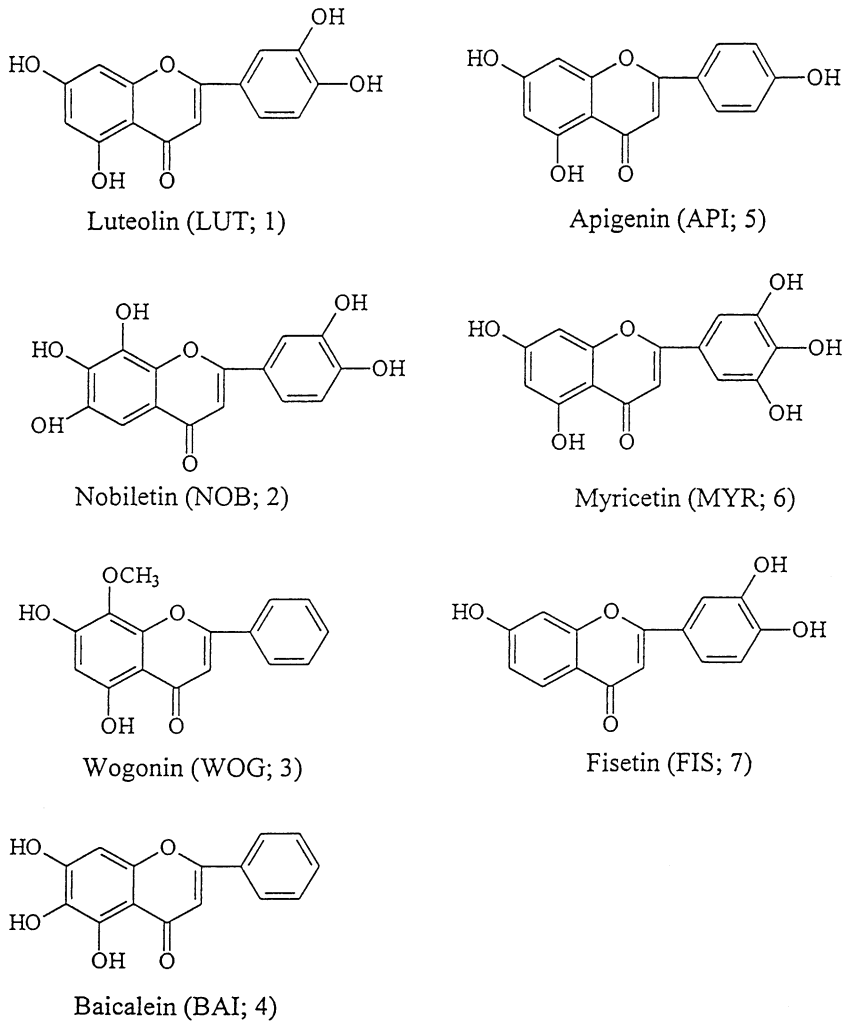

Fig. 1. Structure of the tested flavonoids including luteolin, nobiletin, wogonin, baicalein, apigenin, myricetin and fisetin.

chemical structures of the seven flavonoids used in the present study are shown in Fig. 1, and the effects of these compounds on the viability of cells were investigated by MTT assay (Fig. 2A). When HL-60 cells were treated with various concentrations of each indicated compound (20, 40, and 80 μM) for 12 hr, significant concentration-dependent inhibition on the viability of HL-60 cells was detected in the presence of wogonin, baicalein, apigenin, myricetin, and fisetin, but this was not obvious in luteolin- and nobiletin-treated cells. Among them, wogonin and fisetin showed the most-potent cytotoxicity on HL-60 cells. DMSO, even at the highest dose of 0.5%, showed no effect on cellular viability of HL-60 cells. To characterize cell death induced by flavonoids, the integrity of genomic DNA, occurrence of apoptotic bodies, and the ratio of sub-G1 peak were examined. On analysis of DNA integrity by agarose electrophoresis, the results show that addition of luteolin, nobiletin, wogonin, baicalein, apigenin, myricetin and fisetin caused the digestion of genomic DNA into ladders in dose-dependent manners, associated with a decrease in intact DNA (Fig. 2B). However, luteolin and nobiletin, only at 80 μM, produced few DNA ladders in this assay. In the same part of the experiment, apoptotic bodies by microscopic observation and increase of sub-G1 ratio were detected in wogonin- and fisetin-treated HL-60 cells (Fig. 3). No obvious DNA ladders, apoptotic bodies, or sub-G1 peak were detected in DMSO-treated HL-60 cells (data not shown).

3.2. Stimulation of caspase 3-like activities, not caspase 1-like activities, in wogonin and fisetin-induced apoptosis

Caspases are believed to play a central role in mediating various apoptotic responses and are activated in a sequential cascade of cleavages from their inactive forms. After activation, caspases can cleave their substrates at specific sites such as DXXD↓X for caspase 3 and YXXD↓X for caspase 1. To detect the enzymatic activity of caspases during wogonin- and fisetin-induced apoptosis, 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. 4, wogonin and fisetin induced a dramatic increase in DEVD-specific caspase activity in treated HL-60 cells. Adding the caspase 3 inhibitory peptide, Ac-DEVD-CHO,

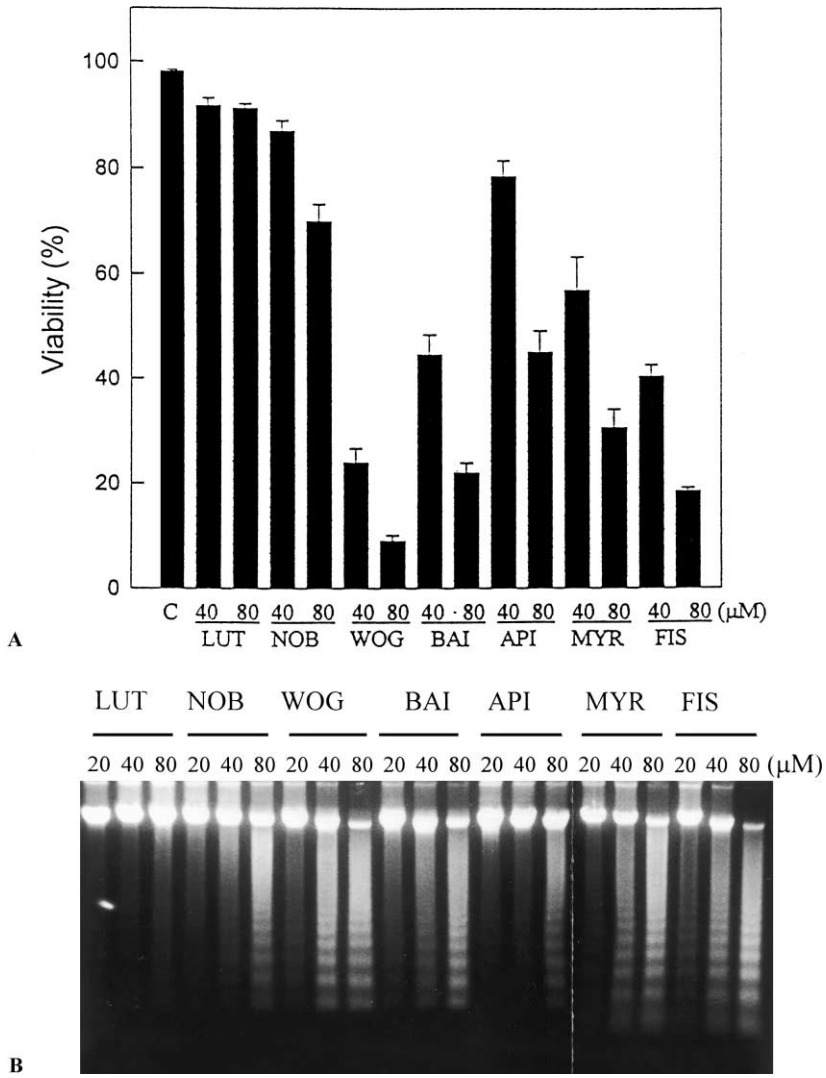

Fig. 2. Analysis of cell viability and DNA integrity in flavonoids-treated HL-60 cells by MTT assay and agarose electrophoresis. (A) HL-60 cells were plated into 24-well plates for 24 hr and then treated with different concentration of indicated compound (40 and 80 μM) for a further 12 hr. MTT was added into medium for an additional 4 hr. The viability of cells was detected by measuring the absorbance at a wavelength of 600 nm. (B) HL-60 cells were treated with different concentrations (20, 40, 80 μM) of the indicated compound for 12 hr and DNA from cells was extracted and electrophoresed through a 1.8% agarose gel and visualized by staining with ethidium bromide.

into the reaction mixture containing cell lysates from 80 μM wogonin-treated HL-60 cells inhibited the increase of caspase 3-like activities. In contrast to the increase in DEVD-specific activity, no significant induction of YVAD-specific activity was observed. Thus, induction of caspase 3-like activity might be involved in wogonin- and fisetin-induced apoptosis.

3.3. Involvement of PARP degradation, caspase 3 activation, and Bcl-2 family proteins in wogonin and fisetin-induced apoptosis

Activation of caspase 3 leads to the cleavage of a number of proteins, one of which is PARP, another hallmark of apoptosis. Fig. 5 shows that exposure of HL-60 cells to wogonin and fisetin caused the degradation of 116 kDa PARP into 85 kDa fragments in a concentration-dependent manner, associated with the activation of caspase 3 brought about by its cleavage, represented here as a decline in its pro-level on the Western blot. Bcl-2 family proteins act as important regulators in apoptosis and are located at the upstream of caspase activation. In wogonin- and fisetin-treated HL-60 cells, decrease in the Mcl-1 protein and increase in the bax protein were detected in dose-dependent manners. In contrast to the expression of bax and Mcl-1 proteins, Bcl-2, Bcl-XL, and Bad proteins remained unchanged in HL-60 cells treated with or without different concentrations of wogonin and fisetin (Fig. 5B).

3.4. Activation of endonuclease activity in wogonin and fisetin-treated HL-60 cells

Internucleosomal DNA fragmentation is thought to be a result of activation of endogenous endonuclease. *In vitro*

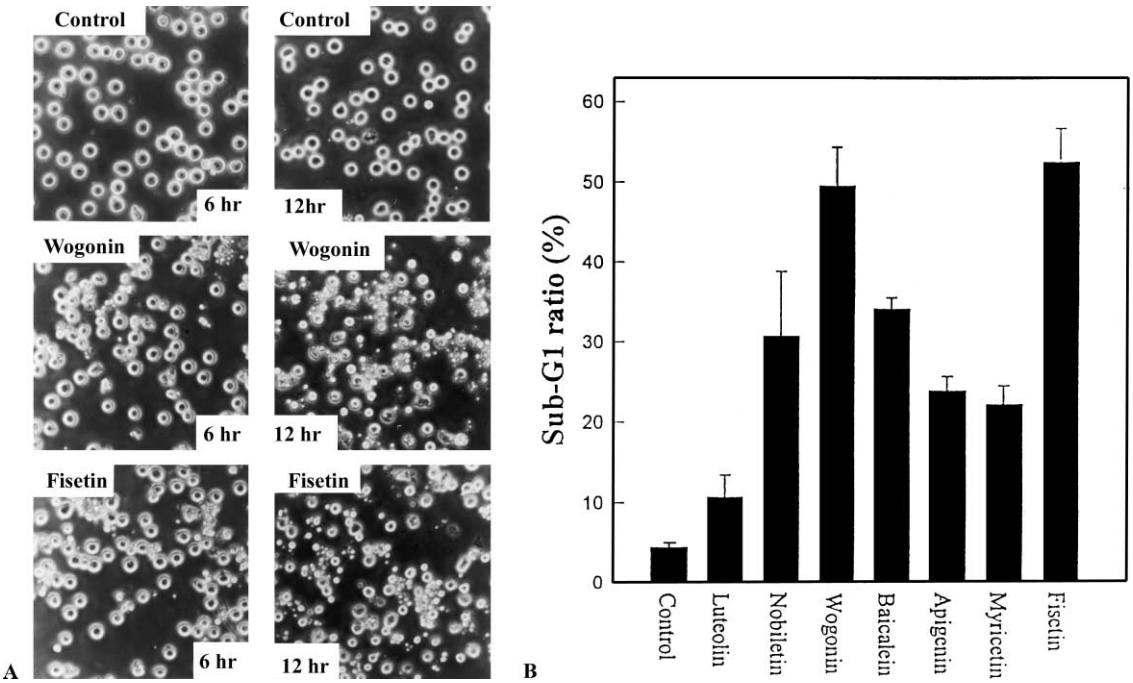

Fig. 3. Appearance of the sub-G1 peak and apoptotic bodies in wogonin- and fisetin-treated HL-60 cells. (A) Occurrence of apoptotic bodies in HL-60 cells was detected in wogonin- and fisetin-treated HL-60 cells for 6 and 12 hr by a light microscopic observation. (B) HL-60 cells were treated with or without the indicated compound (80 μM) for 12 hr. Appearance of a sub-G1 peak was detected by flow cytometry using PI staining. Each value is presented as the mean ± SE of three independent experiments.

chromatin digestion provides a sensitive way to analyze the activity of apoptotic endonuclease. Incubation of the isolated nuclei with cell lysates from untreated HL-60 cells failed to produce DNA fragmentation. When cell lysates extracted from wogonin- and fisetin-treated HL-60 cells were incubated with isolated intact nuclei, the intact DNA was cleaved into fragments (Fig. 6A). Other flavonoids such as luteolin, nobiletin, baicalein, apigenin, and myricetin that induced DNA ladders in Fig. 2 showed only slightly detectable endonucleolytic activity in this assay. Further examination of the properties of endonuclease induced by wogonin and fisetin in HL-60 cells revealed that this endonuclease might be Ca^{2+}-dependent since DNA fragmentation disappeared in calcium free-medium (data not shown) and was attenuated when 5 mM EDTA or EGTA was added to the reaction mixture (Fig. 6B). Furthermore, the optimal

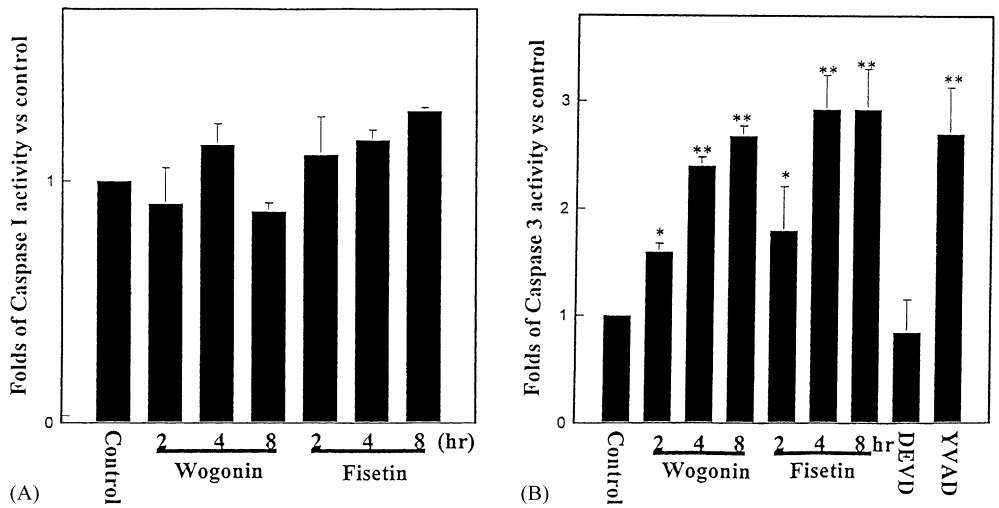

Fig. 4. Activation of caspase 3 but not caspase 1 activity in wogonin- and fisetin-treated HL-60 cells. HL-60 cells were treated with or without wogonin or fisetin (80 μM) for 2, 4, and 8 hr. Cells were harvested and lysed in lysis buffer. Enzymatic activity of caspase 1 (A) and caspase 3 (B)-like proteases were determined by incubation with specific colorigenic substrates, Ac-DEVD-pNA or Ac-YVAD-pNA, as described in Section 2. DEVE and YVAD indicate that the addition of 100 μM of Ac-YVAD-CHO or Ac-DEVD-CHO with the reaction in wogonin (80 μM, 8 hr)-treated HL-60 cell lysates. $^{*}P < 0.05$; $^{**}P < 0.01$.

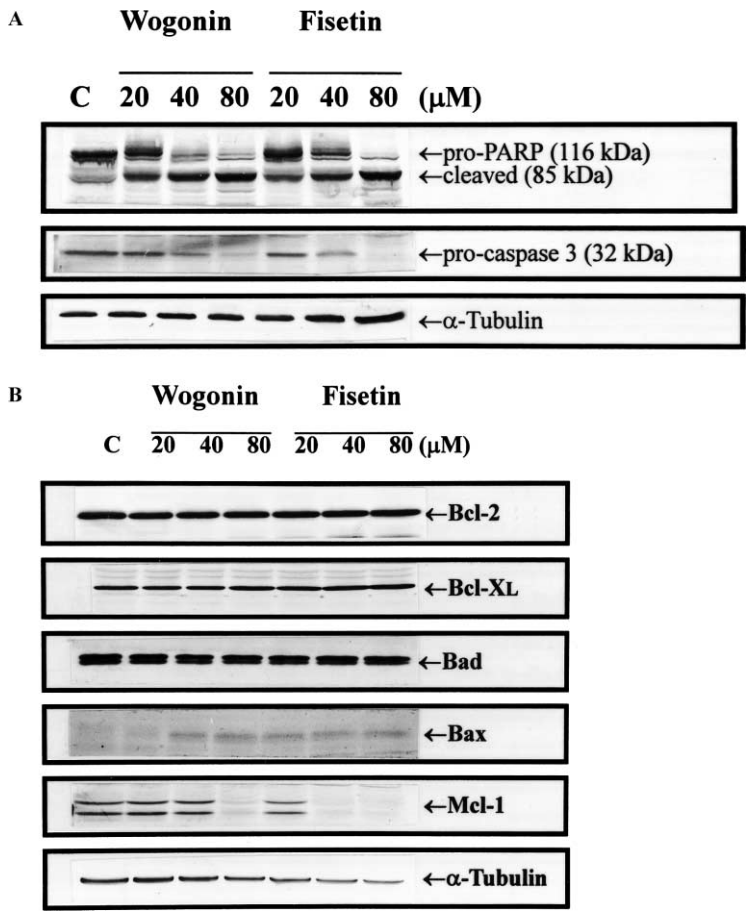

Fig. 5. Involvement of caspase 3 activation, PARP cleavage, induction of bax proteins and decrease in Mcl-1 protein in wogonin- and fisetin-treated HL-60 cells. (A) HL-60 cells were treated with different concentrations (20, 40, and 80 μM) of wogonin or fisetin for 12 hr. PARP cleavage and decrease of pro-caspase 3 were analyzed by Western blotting as described in Section 2. α-Tubulin presented here was used as an internal control. (B) Alternative expression of Bcl-2 family proteins including Bcl-2, Bcl-XL, Bad, Bax and Mcl-1 in wogonin- and fiestin-treated HL-60 cells. Cells were treated with different concentrations (20, 40, and 80 μM) of wogonin and fisetin for 12 hr, and the expression of the indicated protein was evaluated using specific antibodies for Western blot.

pH for activity was around 7.5 because DNA ladders were obviously detected in the reaction mixture with pH 7.5, but was clearly reduced at pH 6.5 or 5.5 (Fig. 6C).

3.5. A caspase 3-like protease inhibitor, Ac-DEVD-CHO, attenuates wogonin and fisetin-induced apoptotic responses associated with decreased endonuclease activity

The above results clearly indicate that caspase 3-like proteases and endonuclease are activated in response to wogonin and fisetin in HL-60 cells. To determine if the activation of caspase 3-like protease is necessary for the induction of apoptosis by wogonin and fisetin, caspase inhibitors including the caspase 3-like protease inhibitor, Ac-DEVD-CHO, and the caspase 1-like protease inhibitor, Ac-YVAD-CHO, were used to block intracellular protease, and wogonin- and fisetin-induced DNA ladders and endonuclease activity were analyzed by agarose electrophoresis and *in vitro* chromatin digestion assay. Results in Fig. 7A and B show that the caspase 3-like inhibitor, Ac-DEVD-CHO (200 μM), was able to inhibit wogonin- and fisetin-induced DNA fragmentation accompanied by suppressing PARP cleavage and decreasing pro-caspase 3 protein. However, Ac-YVAD-CHO, an inhibitor of caspase 1-like activity, had no effect at a similar concentration (200 μM). In order to illustrate if activation of caspase 3-like activity participated in wogonin- and fisetin-induced endonuclease activities, *in vitro* chromatin digestion using cell lysates extracted from HL-60 cells under different treatments was performed. The results in Fig. 7C show that wogonin- and fisetin-induced endonuclease activities were inhibited by Ac-DEVD-CHO (200 μM), but not by Ac-YVAD-CHO (200 μM). These results indicate that activation of caspase 3 and endonuclease activities is involved in wogonin- and fisetin-induced apoptosis, and that endonuclease activation might be located downstream of caspase 3 activation.

3.6. N-acetyl-cysteine (NAC) and catalase have no effect on wogonin and fisetin-induced apoptosis, but Inhibited H_2O_2-induced cell death

ROS producing and scavenging activities play important roles in the biological effects of flavanoids. In order to

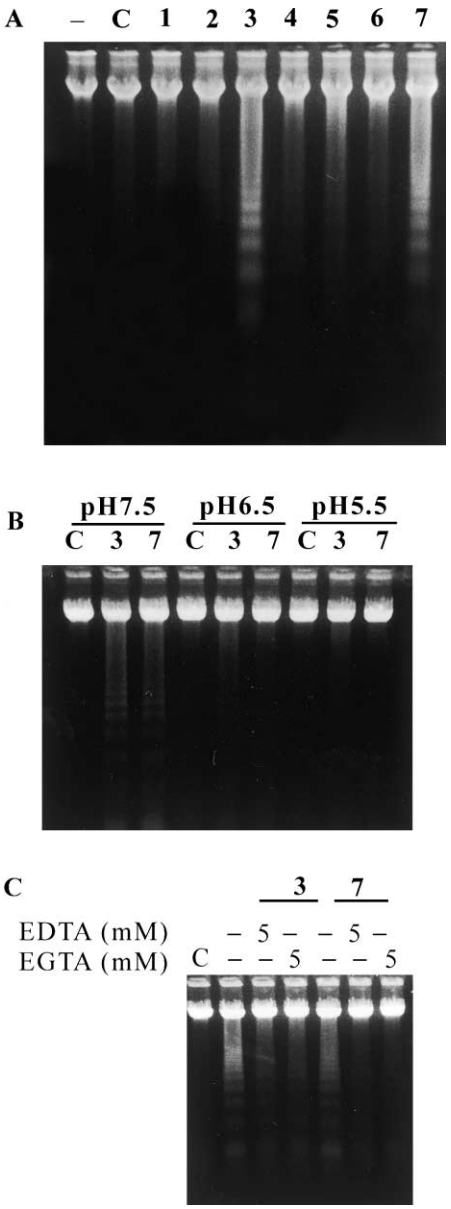

Fig. 6. Analysis of endonuclease activities in wogonin- and fisetin-treated HL-60 cells. Intact HL-60 nuclei and indicated compound-treated lysates (30 μg) were reconstituted for reaction at 37° for 60 min, and the endonuclease activity was assayed by DNA ladder formation. (A) Cell lysates extracted from flavonoid-treated HL-60 cells (30 μg) and analysis of endonuclease activity by *in vitro* chromatin digestion as described in Section 2. (1), Luteolin; (2), nobiletin; (3), wogonin; (4), baicalein; (5), apigenin; (6), myricetin; (7), fisetin. (B) pH-dependent activity of three pH sets (5.5, 6.5, and 7.5) in the presence of 0.1 mM Ca^{2+} and 2 mM Mg^{2+} in wogonin- and fisetin-treated HL-60 cells. Endonuclease activity was analyzed by DNA ladder formation as described in (A). (C) Inhibition of wogonin- and fisetin-induced endonuclease activation by Ca^{2+} chelators, 5 mM EGTA and 5 mM EDTA in a reaction at pH 7.5 containing 0.1 mM Ca^{2+}.

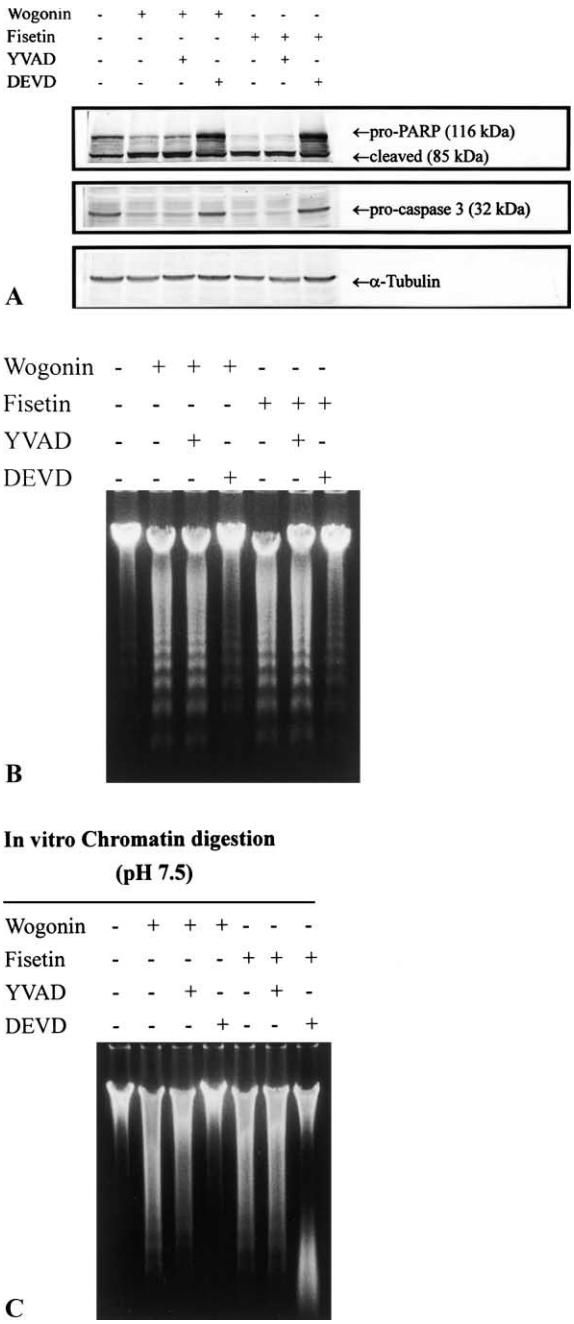

Fig. 7. Effects of caspase 1 and 3 inhibitors on wogonin- and fisetin-induced PARP cleavage, pro-caspase 3 protein, and endonuclease activation in HL-60 cells. HL-60 cells were pretreated with either inhibitor, Ac-DEVD-CHO or Ac-YVAD-CHO (200 μM), for 3 hr followed by 80 μM wogonin and fisetin treatment for a further 12 hr. (A) Expressions of PARP, caspase 3 and α-tubulin proteins were analyzed by Western blotting as described in Fig. 5. (B) The integrity of genomic DNA in each treatment was detected by electrophoresis on a 1.8% agarose. (C) Alternative activity of endonuclease in wogonin- and fisetin-treated HL-60 cell lysate in the presence or absence of a caspase 3 (Ac-DEVD-CHO, 200 μM) or caspase 1 (Ac-YVAD-CHO, 200 μM) inhibitor was measured by *in vitro* chromatin digestion as described in Fig. 6.

demonstrate the role that ROS play in wogonin- and fisetin-induced apoptosis, free radical-scavenging agents, NAC (a: 20 mM; b: 40 mM) and catalase (c: 200 U/mL; d: 400 U/mL), were used in this study. Results in Fig. 8A show that neither NAC nor catalase has any obvious inhibitory effect on wogonin- and fisetin-induced caspase 3 procession, PARP cleavage, decrease in Mcl-1 protein, and increase in bax protein (Fig. 8A). Results of analysis of DNA integrity by agarose electrophoresis show NAC and catalase are unable to block wogonin- and fisetin-induced

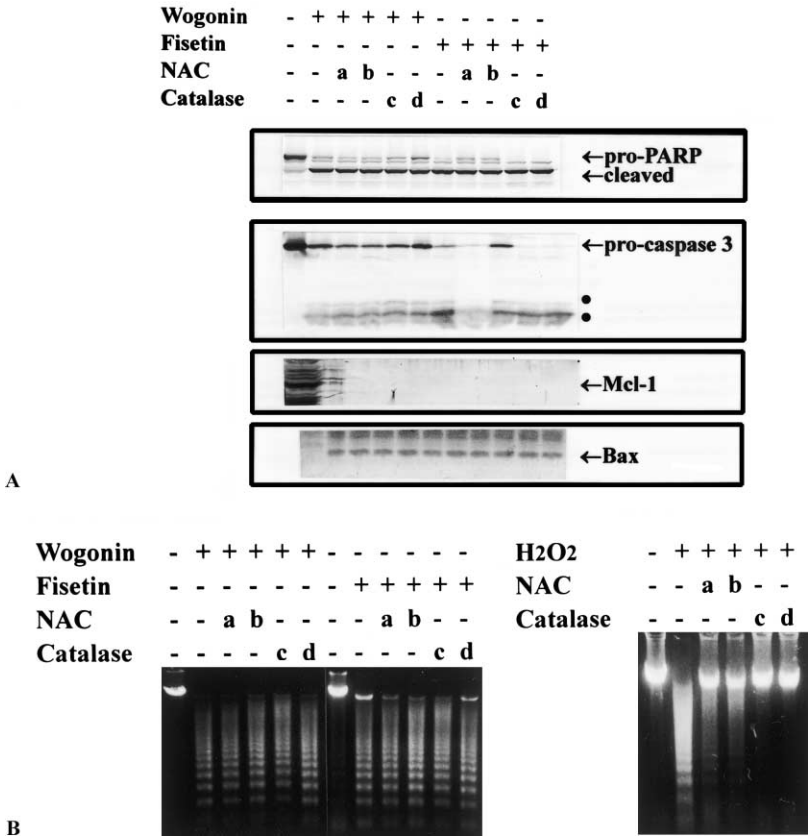

Fig. 8. Effects of NAC and catalase on wogonin- and fisetin-induced apoptosis. (A) HL-60 cells were treated with different concentrations of NAC (a: 20 mM; b: 40 mM) or catalase (c: 200 U/mL; d: 400 U/mL) for 1 hr followed by wogonin and fisetin (80 μM) treatment for 12 hr. Expressions of PARP, caspase 3, Mcl-1 and Bax were determined as described in Fig. 5. (B) HL-60 cells were treated with NAC or catalase for 1 hr followed by wogonin (80 μM), fisetin (80 μM), or H_2O_2 (200 μM) treatment for 12 hr. Integrity of DNA was analyzed by agarose electrophoresis as described in Section 2.

DNA ladders (Fig. 8B). However, both NAC and catalase show significant preventive effects on ROS (H_2O_2)-induced apoptosis (Fig. 8C). In this study, we also found that both NAC and catalase show a slight but obvious increase in wogonin- or fisetin-induced DNA ladder intensity and caspase 3 procession. We propose that the apoptosis-inducing effects of wogonin and fisetin might parallel the actions of NAC and catalase, and that proxidant activities might not be involved.

3.7. Decrease of intracellular ROS by wogonin and fisetin

In order to further demonstrate the role that ROS play in wogonin- and fisetin-induced apoptosis, the intracellular peroxide level was determined by fluorescence intensity of DCF. Changes in intracellular peroxide levels were determined by flow cytometry analysis. The results show that both wogonin and fisetin treatments significantly decreased the amount of intracellular peroxide levels from 496.3 ± 12.1 (control) to 265.9 ± 14.3 (wogonin) and 55.2 ± 9.5 (fisetin), respectively ($P < 0.01$, compared with control). Adding H_2O_2 (200 μM) into the medium with DCHF-DA elevated the intracellular fluorescence intensity to 1808.6 ± 21.7 as is described as a positive control here (Fig. 9).

4. Discussion

Polyphenols are common components which naturally existed in plants and have been demonstrated to show several biological properties including free radical scavenging and anti-inflammatory and anti-carcinogenesis activities. The present results show that wogonin and fisetin cause the potent and rapid induction of apoptosis, concurrent with DNA ladders, apoptotic appearance, and sub-G1 peak appearance in HL-60 cells. Activation of caspase 3, but not caspase 1, associated with PARP cleavage was detected in wogonin- and fisetin-treated HL-60 cells. Apoptotic events were reconstituted *in vitro* to demonstrate the role of endonuclease. The results indicate that the main endonuclease responsible for apoptotic DNA fragmentation induced by wogonin or fisetin might be a neutral Ca^{2+}/Mg^{2+}-dependent one. The diverse properties of the endonucleases responsible for DNA fragmentation have been described [32,33]. In most instances, a Ca^{2+}/Mg^{2+}-dependent endonuclease induced by agents is constitutively expressed in the nuclei isolated from various cells and is inhibited by EDTA or EGTA [34]. The optimal pH has been considered to be around 7–9 [35]. HL-60 cells have also been reported to possess a Ca^{2+}-dependent endonuclease in the nuclei [36], however, Kawabata *et al.* reported

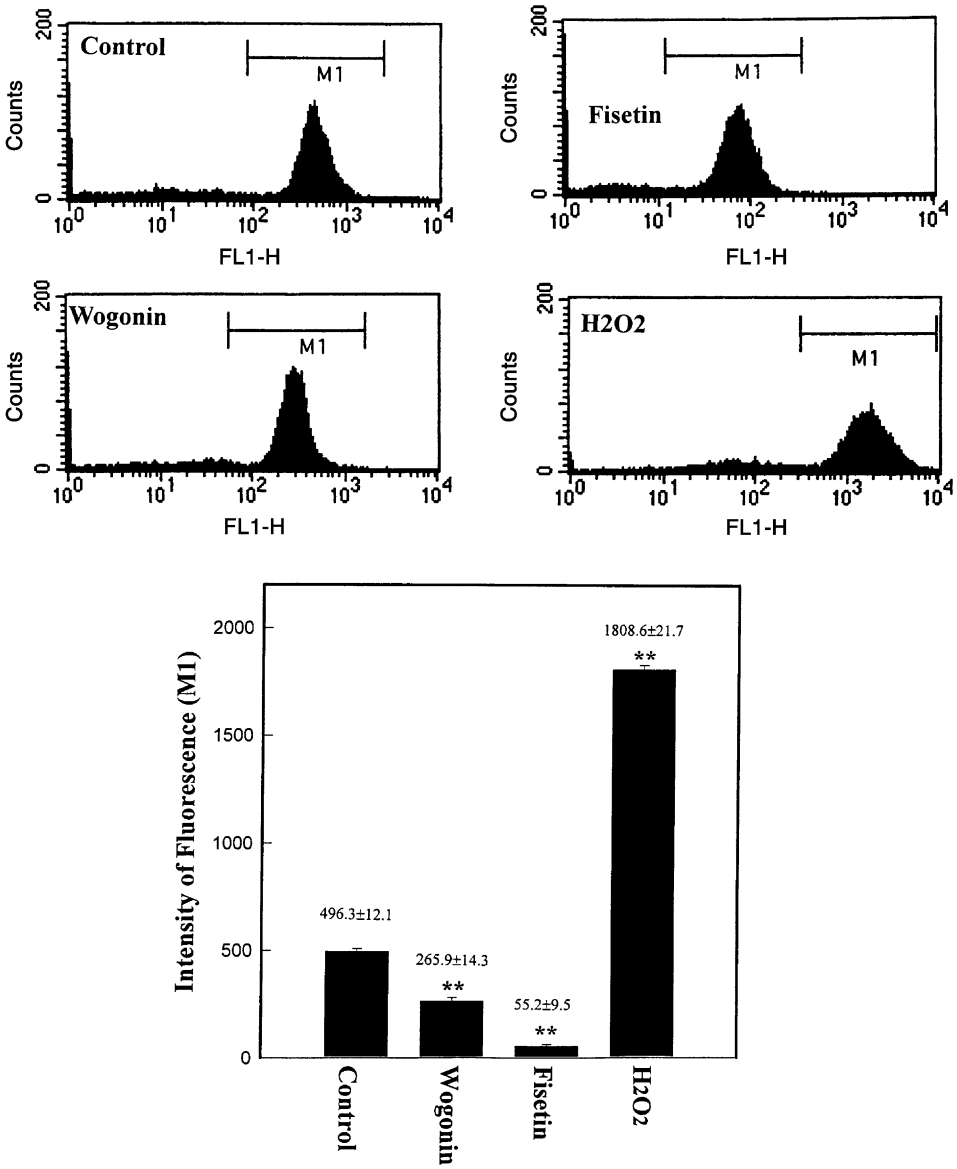

Fig. 9. Inhibition of intracellular ROS by wogonin and fisetin in the DCHF-DA assay. HL-60 cells were treated with wogonin (80 μM) or fisetin (80 μM) for 1 hr followed by the addition of DCHF-DA (100 μM) for a further hour. The fluorescence intensity of cells was measured by flow cytometry analysis. Each value is presented as the mean ± SE of three independent experiments. (**) $P < 0.01$, significantly different from the control, as analyzed by Student's *t*-test.

that Ca^{2+}-independent and Mg^{2+}-dependent endonuclease activity in nuclei of HL-60 cells [37]. In this study, we demonstrate that a Ca^{2+}-dependent endonuclease is expressed in wogonin- and fisetin-treated HL-60 cells, and that the characteristics of wogonin- and fisetin-activated endonuclease are identical to those of gamma-ray-, A23187- or UV-treated thymocytes [36,38]. Our previous study demonstrated that a 37-kDa Ca^{2+}-dependent endonuclease was activated in the process of spontaneous apoptosis in colorectal carcinoma cells [29]. Thus, this suggests that activation of Ca^{2+}-dependent endonucleases was involved in wogonin- and fisetin-induced apoptosis.

Recent evidence suggests that the proteolytic degradation of specific substrates is responsible for many of the morphologic and biochemical features of apoptosis [39,40]. Caspase 3 is a member of the caspase family and requires proteolytic cleavage for its activation. Activated caspase 3 cleaves PARP to generate 85 and 31 kDa fragments during apoptosis, and several previous studies including ours demonstrated that activation of caspase 3 and induction of PARP cleavage are important for the occurrence of apoptosis [21,26]. Our *in vitro* results in HL-60 cells indicate the concurrent occurrence of apoptotic DNA fragmentation, caspase 3 activation, and PARP cleavage in wogonin- and fisetin-treated HL-60 cells. In wogonin- and fisetin-treated HL-60 cells, elevation of caspase 3 activity, but not caspase 1 activity, was detected. Wogonin- and fisetin-induced apoptotic changes and DNA fragmentation are attenuated by the caspase 3 inhibitor, Ac-DEVD-CHO, but not by the caspase 1 inhibitor, Ac-YVAD-CHO. Therefore, activation of caspase 3 but not caspase 1 plays an important role in executing apoptosis in

wogonin- and fisetin-treated HL-60 cells. The relationship between caspase 3 activation and endonuclease activation was also examined in this study. In Ac-DEVD-CHO-, but not Ac-YVAD-CHO-treated HL-60 cells, wogonin- and fisetin-induced DNA ladders, PARP cleavage and caspase 3 activation were blocked, and these were associated with decrease in endonuclease activity. These data indicate that caspase 3 activation was involved in endonuclease activation, and located upstream of endonuclease activation in wogonin- and fisetin-induced apoptosis. In this study, Ac-DEVD-CHO was able to attenuate wogonin- or fisetin-induced caspase 3 protein procession. Caspase 3 is an excutioner caspase and relies on the action of the initiator caspases including caspase 8 and caspase 9 for its action. Srinivasula *et al.* reported feedback activation by caspase 3 onto caspase 9 [41]. We proposed that inhibition of wogonin- and fisetin-induced capase 3 activity by Ac-DEVD-CHO might be able to attenuate upstream caspase 9 activation through a feedback regulation, and indirect suppress caspase 3 protein procession.

Bcl-2 family genes including *Bcl-2*, *Bax*, *Bcl-XL*, *Bad*, *Mcl-1*, *Bak* and *Bag* have been demonstrated to be involved in the process of apoptosis [21,42]. Two different types of Bcl-2 family proteins have been identified, one is proapoptotic such as Bax, Bak, and Bcl-Xs and the other is antiaopoptotic such as Bcl-2, Bcl-XL, and Mcl-1. Previous studies indicated that an increase in proapoptotic Bcl-2 family proteins and a decrease in anti-apoptotic Bcl-2 family proteins are involved in the process of apoptosis [43,44]. This study showed an increase in bax and a decrease in Mcl-1 proteins detected in wogonin- and fisetin-treated HL-60 cells. We propose that both bax and Mcl-1 proteins participate in wogonin- and fisetin-induced apoptosis in HL-60 cells.

Wogonin is a popular flavonoid isolated from the Chinese herb *Scutellaria baicalensis*. Previous study demonstrated that *S. baicalensis* showed the anti-proliferative activities on cancer cells through inducing apoptosis, and increasing the production of NO by stimulation of NOS activities in macrophages [45]. Several biological activities of wogonin have also been proposed including an anti-proliferative effect on vascular smooth muscle cells, inhibition of LPS-induced NO and PGE2 productions and antioxidative activity [46]. Our unpublished data indicate that wogonin also show effective apoptosis-inducing activities on colorectal carcinoma (COLO205) and hepatocellular carcinoma cells (SK-Hep) through accumulating p53 and p21 protein in cells; activation of caspase 3 and endonuclease were detected in these two cell lines (data not shown). In contrast to the p53 status of COLO205 and SK-Hep cells, the HL-60 cell lines used in the present study consist of leukemia cells without detectable p53 protein [47]. These results indicate that induction of apoptosis by wogonin might be mediated by a p53-dependent or p53-independent pathway followed by stimulating the activities of caspase 3 and endonuclease. The cross-linking between p53-dependent and p53-independent pathways in wogonin-induced apoptosis is still unclear.

In conclusion, flavonoids have been found to be toxic due to their prooxidant activities, and in the presence of Cu^{2+} or Fe^{2+}, flavanoids act as prooxidants rather than antioxidants. The more OH substitutions these are on their structures the stronger the prooxidant and antioxidant activities. However, the prooxidant and antioxidant behaviors of flavonoids are still unclear. In this study, pretreatment of two free radical scavengers, NAC and catalase, had no effects on wogonin- and fisetin-induced apoptosis and gene expressions, but significantly inhibited H_2O_2-induced DNA ladders. Direct measurement of intracellular ROS levels by DCHF-DA assay indicated that both wogonin and fisetin treatment suppressed the intracellular ROS levels in HL-60 cells. These results provided an interesting correlation between antioxidative activities and flavonoids-induced apoptosis, and deserved to be pursued further.

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