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Differential Apoptotic Effect of Wogonin and Nor-Wogonin Via Stimulation of ROS Production in Human Leukemia Cells

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Abstract We investigate the roles of methoxyl (OCH_3) and hydroxyl (OH) substitutions at C8 of flavonoids on their apoptosis-inducing activities. Wogonin (Wog) and nor-wogonin (N-Wog) are structurally related flavonoids, and respectively contain an OH and OCH_3 at C8. In leukemia HL-60 cells, N-Wog exhibited more-potent cytotoxicity than Wog according to the MTT and LDH release assays, and the IC_{50} values of Wog and N-Wog in HL-60 cells were 67.5 ± 2.1 and 21.7 ± 1.5 μM, respectively. Apoptotic characteristics including DNA ladders, apoptotic bodies, and hypodiploid cells accompanied by the induction of caspase 3 protein processing appeared in Wog- and N-Wog-treated HL-60 cells. Interestingly, an increase in intracellular peroxide production was detected in N-Wog- but not Wog-treated HL-60 cells by the DCHF-DA assay, and the reduction of intracellular peroxide by catalase (CAT) induced by N-Wog significantly reduced the N-Wog- but not the Wog-induced cytotoxic effect according to the MTT assay in accordance with the blocking of DNA ladder formation and caspase 3 and PARP protein processing elicited by N-Wog. We further analyzed the effect of six structurally related compounds, including 5-OH, 7-OH, 5,7-diOH, 5,7-diOCH_3, 7,8-diOCH_3, and 7-OCH_3-8-OH flavones, on apoptosis induction in HL-60 cells. Results suggested that OH at C5 and C7 is essential for both the apoptosis-inducing activity of flavonoids, and OH at C8 may contribute to apoptosis induction ability. Evidence to support a distinct structure-activity relationship in apoptosis induction of flavonoids is provided for the first time in this study. J. Cell. Biochem. 103: 1394–1404, 2008. © 2007 Wiley-Liss, Inc.

Key words: wogonin; nor-wogonin; apoptosis; structure-activity relationship

Flavonoids occur in fruits, plants, and seeds, with the average daily diet estimated to contain about 1 g of flavonoids, which have been shown to be sufficient to reach pharmacologically significant concentrations in tissues. Flavonoids are natural antioxidants which scavenge free radicals. Several previous studies indicated that flavonoids possess antiinflammatory activities via blocking the production of cytokines, adhesion molecules, and matrix metalloproteases [Hougee et al., 2005; Kempuraj et al., 2005; Cimino et al., 2006; Piantelli et al., 2006]. The antitumor effects of flavonoids through apoptosis induction have extensively been reported. Phytochemicals such as curcumin, zerumbone, and tea polyphenols are known to induce apoptosis through downregulation of the apoptotic

Abbreviations used: ROS, reactive oxygen species; Bcl-2, B-cell lymphoma-2; CAT, catalase; Cyt c, cytochrome c; EtBr, ethidium bromide; DCHF-DA, dichlorodihydrofluorescein diacetate; NBT, nitro blue tetrazolium; BCIP, 5-bromo-4-chloro-3-indolyl-phosphate; Wog, wogonin; N-Wog, N-Wogonin; PARP, poly (ADP-ribose) polymerase

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suppressors, Bcl-2 and Bcl-XL, in several different tumor cell lines [Aggarwal et al., 2005, 2006; Takada et al., 2005; Nishikawa et al., 2006]. Our recent studies delineated how apoptosis induced by myricetin, baicalein, and quercetin is accompanied by stimulation of the mitochondrial pathway involving caspase 9 cleavage and cytochrome c (Cyt c) release [Shen et al., 2003; Chen et al., 2004; Ko et al., 2005]. Many studies have investigated the effects of flavonoids on oxidative stress-induced DNA damage, lipid oxidation, and cell death [Menegazzi et al., 2006; Schaefer et al., 2006]. Jeong et al. [2005] reported that flavonoids exhibited differential inhibitory effects on oxidized LDL-induced apoptosis. Heo and Lee [2004] indicated that quercetin exhibits effective activity against oxidative stress-induced neurodegeneration. Our previous studies also indicated that baicalein and quercetin, but not their respective glycosides, baicalin, rutin, and quercitrin, effectively protect glioma C6 cells and macrophages from H_2O_2-induced apoptosis via HO-1 induction [Chen et al., 2006a,b]. Although the apoptosis and antiapoptosis of flavonoids have extensively been investigated, the structure-activity relationships of flavonoids in apoptosis are still unclear.

Wogonin (5,7-diOH-8-OCH_3 flavone; Wog) is one of the active components in the root of *Scutellaria baicalensis* Georgi. Several biological activities of Wog have been investigated. Wog inhibits hemin-nitrite-H_2O_2-induced liver injury by blocking the oxidation of proteins [Zhao et al., 2006]. Bonham et al. [2005] indicated that Wog inhibits the proliferation of prostate cancer cells through inhibition of the androgen receptor-signaling pathway. In an ethanol-induced gastric mucosal damage model, Wog significantly reduced alcohol-induced gastropathy through antiinflammation and apoptosis induction [Park et al., 2004]. Shen et al. [2002] provided evidence to support the in vivo antiinflammatory effect of Wog against LPS-induced NO and PGE_2 production. Our previous studies showed that Wog effectively induces apoptosis in human leukemia cells and inhibits LPS-induced NO and PGE_2 production via blocking iNOS and COX-2 gene expression in RAW264.7 macrophage cells [Chen et al., 2001; Lee et al., 2002]. In addition, Wog addition effectively reduces LPS plus TPA-induced transformation in glioma C6 cells through inhibiting MMP-9 activation [Shen et al., 2006]. Although some biological effects of Wog have been shown, the importance of structural substitutions including OH at C5 and C7, and OCH_3 at C8 on the action of Wog is still undefined.

In the present study, we examined the effects of structurally related compounds of Wog including Nor-wogonin (5,7,8-triOH flavone, N-Wog), 5-OH flavone, 7-OH flavone, 5,7-diOH flavone, 5-OH-7-OCH_3 flavone, 7,8-diOCH_3 flavone, and 7-OCH_3-8-OH flavone on the viability of human leukemia HL-60 cells. The roles of OH at C5 and C7, and OCH_3 at C8 in apoptosis induction activity of Wog are elucidated.

MATERIALS AND METHODS

Cells

Human leukemia HL-60 cells were obtained from the American Type Culture Collection (ATCC). HL-60 cells were cultured in RPMI medium supplemented with antibiotics (100 U/ml of penicillin A and 100 U/ml of streptomycin) and 10% heat-inactivated fetal bovine serum (Gibco/BRL) and maintained in a 37°C humidified incubator containing 5% CO_2.

Agents

The structurally related flavonoids of Wog, N-Wog, and 5-OH, 7-OH, 5,7-diOH, 7, 8-diOCH_3, and 7-OCH_3-8-OH flavones, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were obtained from Sigma Chemical (St. Louis, MO). All chemical solvents were purchased from Merck. The antibodies for detecting the expressions of caspase 3, PARP, and α-tubulin protein were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Peptidyl inhibitors including Z-VAD-FMK and Ac-DEVD-FMK, and the peptidyl substrate of caspase 3 Ac-DEVD-pNA were purchased from Calbiochem (La Jolla, CA).

Cell Viability Assay

MTT was used as an indicator of cell viability as determined by its mitochondrial-dependent reduction to formazone. Cells were plated at a density of 10^5 cells/well in 96-well plates for 12 h, followed by treatment with different concentrations of each compound for a further 12 h. Cells were washed with PBS twice, and MTT (50 mg/ml) was added to the medium for 4 h. Then, the supernatant was removed, and the formazone crystals were dissolved using

0.04 N HCl in isopropanol. The absorbance was read at 600 nm with an ELISA analyzer (Dynatech MR-7000; Dynatech Laboratories).

Western Blotting

Total cellular extracts were prepared according to our previous papers [Lin et al., 2005], separated on 8–12% sodium dodecylsulfate (SDS)–polyacrylamide minigels, and transferred to Immobilon polyvinylidene difluoride membranes (Millipore). Membranes were incubated with 1% bovine serum albumin and then with the indicated antibodies (Santa Cruz Biotechnology) overnight at 4°C. Expression of the protein was detected by staining with nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) (Sigma).

DNA Integrity Assay

Cells under different treatments were collected, washed twice with PBS, lysed in 80 μl of lysis buffer [50 mM Tris (pH 8.0), 10 mM ethylenediaminetetraacetic acid (EDTA), 0.5% sodium sarkosinate, and 1 mg/ml proteinase K] for 3 h at 56°C, and then treated with 0.5 mg/ml RNase A for an additional 1 h at 56°C. DNA was extracted with phenol/chloroform/isoamyl alcohol (25/24/1, v/v) before loading. Samples were mixed with loading buffer [50 mM Tris, 10 mM EDTA, 1% (w/w) low-melting point agarose, and 0.025% (w/w) bromophenol blue] and loaded onto a pre-solidified 2% agarose gel containing 0.1 mg/ml ethidium bromide. The agarose gels were run at 50 V for 90 min in TBE buffer, after which they were observed and photographed under UV light.

Caspases Enzyme Activity Assay

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 ethyleneglycoltetraacetic acid (EGTA). Cell lysates were clarified by centrifugation at 20,000*g* for 3 min, and clear lysates containing 100 μg of protein were incubated with 100 μM enzymespecific colorimetric substrates including Ac-DEVD-pNA for caspase 3/CPP32 at 37°C for 1 h. Alternative activity of the indicated caspases was described as the cleavage of colorimetric substrate by measuring the absorbance at 405 nm.

Statistical Analysis

Values are expressed as the mean ± SE. The significance of the difference from the respective controls for each experimental test condition was assayed using Student's *t*-test for each paired experiment. A P value <0.05 or <0.01 was regarded as indicating a significant difference.

RESULTS

Nor-Wog Is a More-Effective Apoptotic Inducer Than Wog in Human Leukemia HL-60 Cells

The chemical structures of Wog (W; Wog; 5,7-diOH-8-OCH_3 flavone) and N-Wog (NW; N-Wog; 5,7,8-triOH flavone) are shown in Figure 1A. The difference between Wog and N-Wog is a methoxyl (OCH_3) group at C8 of Wog and a hydroxyl (OH) group at C8 of N-Wog. Data from the DNA fragmentation assay showed that both Wog and N-Wog induce DNA ladders in HL-60 cells in dose-dependent manners, and DNA ladders were detected in Wog- (80 μM) and N-Wog (40 μM)-treated HL-60 cells (data not shown). Wog (80 μM) and N-Wog (40 μM) time-dependently induced DNA ladders, and the initial time for DNA ladder formation was at 4 and 2 h after Wog or N-Wog treatment, respectively (Fig. 1B). The intensity of DNA ladder formation was more significant in N-Wog-treated than in Wog-treated HL-60 cells. Data from the MTT assay indicated that Wog and N-Wog dose-dependently decreased the viability of HL-60 cells, and the IC_{50} values of Wog and N-Wog were respectively 67.5 ± 2.1 and 21.7 ± 1.5 μM by the MTT assay (Fig. 1C). Additionally, data from the LDH release assay supported Wog and N-Wog being able to reduce the viability of HL-60 cells, with N-Wog being more potent than Wog (Fig. 1D). We further microscopically examined morphological changes in HL-60 cells in the presence of Wog or N-Wog treatment. As illustrated in Figure 1E, the occurrence of apoptotic bodies was detected in Wog- and N-Wog-treated cells, and the apoptotic bodies induced by N-Wog were observed at the doses of 20 and 40 μM; however, those induced by Wog were only observed at a dose of 80 μM. The ratio of hypodiploid cells (sub-G1) in Wog- or N-Wog-treated HL-60 cells was examined by a flow cytometric analysis with PI staining. Data from the time-dependent study showed that both Wog and N-Wog were able to increase the percentage of hypodiploid cells, and the ratio of hypodiploid cells in the N-Wog-treated group was much higher than that in the Wog-treated group at the

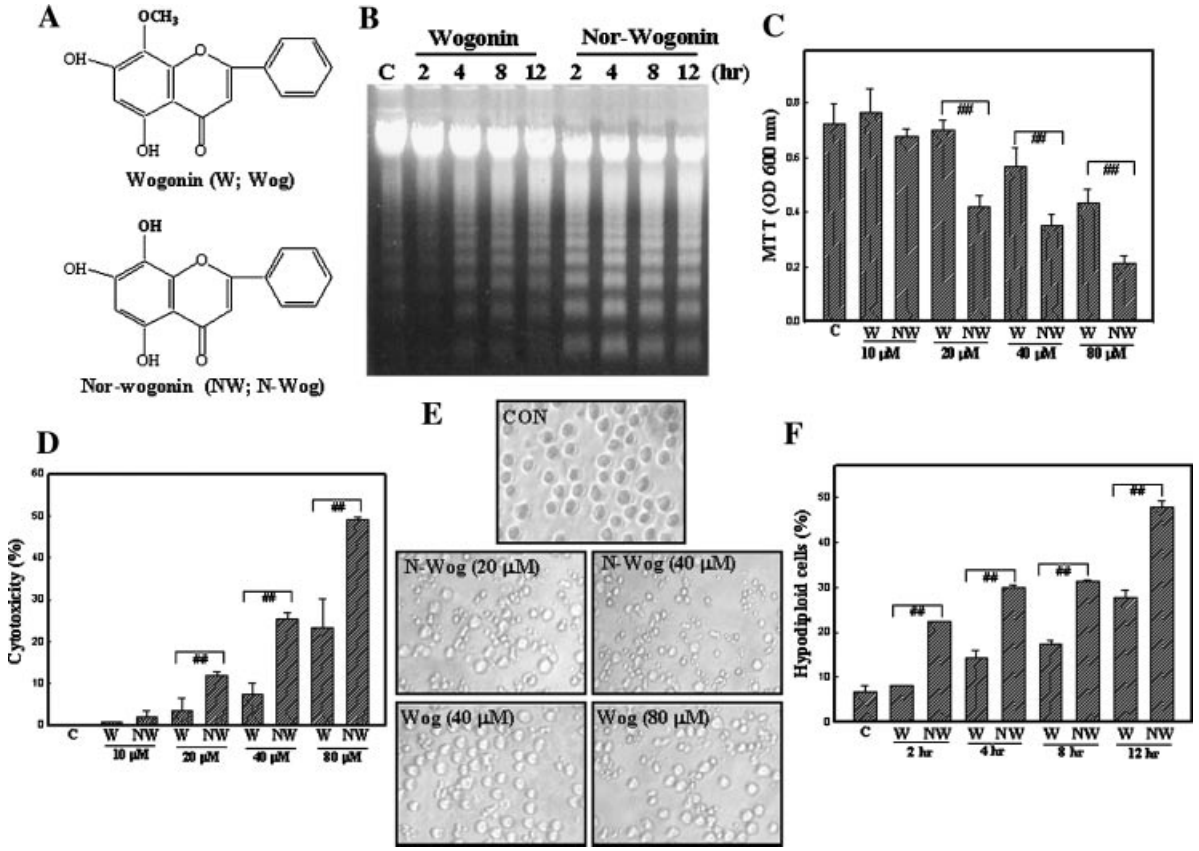

Fig. 1. Wogonin (Wog) and Nor-wogonin (N-Wog) induced apoptosis in human leukemia HL-60 cells. **A**: The chemical structures of Wog and N-Wog are depicted. The difference between Wog and N-Wog is the substitution of OCH_3 and OH (bold) at C8 of Wog and N-Wog. **B**: Induction of DNA ladders by Wog and N-Wog in a time-dependent manner. HL-60 cells were treated with Wog (80 μM) or N-Wog (40 μM) for different times (2, 4, 8, and 12 h), and the integrity of the DNA was analyzed by agarose electrophoresis. **C**: Wog and N-Wog reduced the viability of HL-60 cells. Cells were treated with different doses (10, 20, 40, and 80 μM) of Wog or N-Wog for 12 h, and the viability of cells was analyzed by the MTT assay as described in ''Materials and Methods.'' **D**: As described in (C), the amount of LDH in the medium of the indicated groups was examined. The cytotoxicity index (%) was calculated by the equation, [(Treated group−Control group)/(Triton X-100-treated group−Control group)] × 100% as described in ''Materials and Methods.'' **E**: The appearance of apoptotic bodies in Wog- (40 and 80 μM) and N-Wog (20 and 40 μM)-treated HL-60 cells. Cells were treated with Wog or N-Wog, and the apoptotic bodies were detected under a microscope. **F**: Wog and N-Wog induced hypodiploid cells according to the flow cytometry analysis. HL-60 cells were treated with Wog (80 μM) or N-Wog (40 μM) for different times (2, 4, 8, and 12 h), and the ratio of hypodiploid cells was determined by a flow cytometric analysis with PI staining. $^{\#\#}P < 0.01$ indicates a significant difference between the indicated groups, as analyzed by Student's *t*-test.

indicated time points (Fig. 1F). These data provide evidence to indicate that N-Wog is a more-effective apoptotic inducer than Wog in human leukemia cells HL-60 cells.

Activation of the Caspase 3 Enzyme and Protein Processing in Wog- and N-Wog-Induced Apoptosis

Activation of the caspase 3 cascade has been shown in Wog-treated HL-60 cells; however, the role of caspase 3 in N-Wog-induced apoptosis is still unclear. As illustrated in Figure 2A, Wog and N-Wog dose-dependently induced the processing of caspase 3 and its downstream substrate, PARP protein, which was characterized by the appearance of cleaved fragments of caspase 3, and PARP protein. The cleaved fragments of PARP, and caspase 3 were observed by Western blotting in the presence of Wog (80 μM) or N-Wog (40 μM) treatment for different times (Fig. 2B). The enzyme activity of caspase 3 was analyzed using the peptidyl caspase 3 colorimetric substrate, Ac-DEVD-pNA. Data in Figure 2C show that Wog and N-Wog dose-dependently induced the activity of the caspase 3 enzyme in HL-60 cells. Similarly, Wog (80 μM) or N-Wog (40 μM) time-dependently increased the enzyme activity of caspase 3

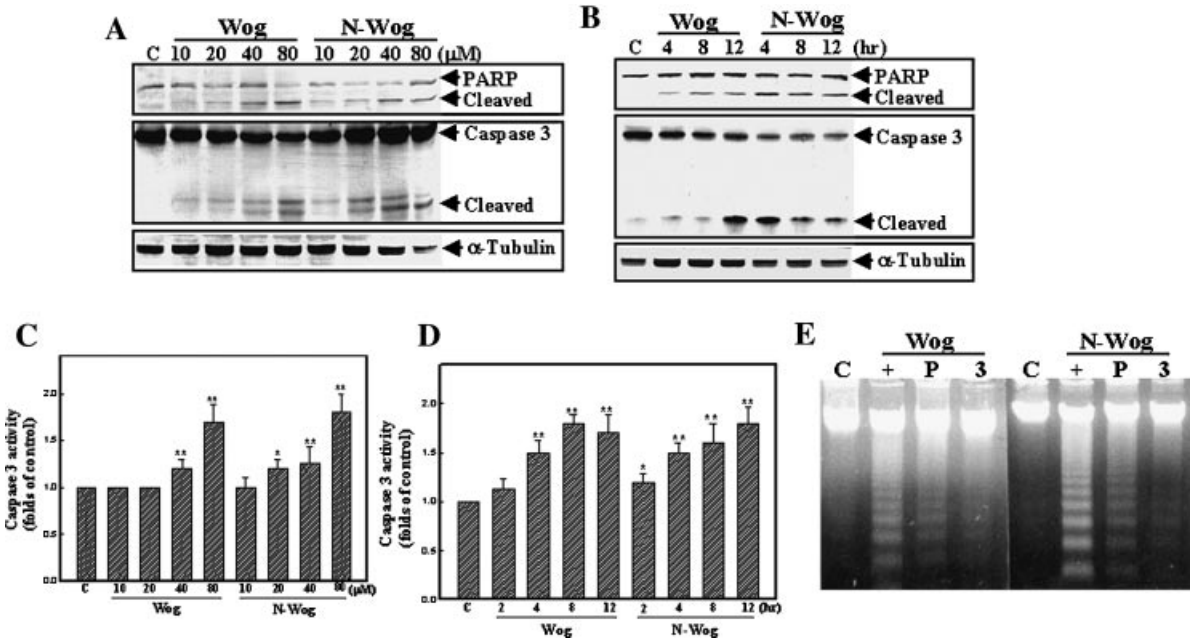

Fig. 2. Activation of caspase 3 in Wog or N-Wog-induced apoptosis. **A**: Wog and N-Wog induced the cleavage of caspase 3 and the PARP protein in HL-60 cells. Cells were treated with different doses (10, 20, 40, and 80 μM) of Wog or N-Wog for 12 h, and the expressions of PARP, caspase 3, and α-tubulin protein were detected by Western blotting. **B**: As described in (A), cells were treated with Wog (80 μM) or N-Wog (40 μM) for different times (4, 8, and 12 h), and the expression of the indicated protein was detected by Western blotting. **C**: Wog and N-Wog dose-dependently increased caspase 3 enzyme activity in HL-60 cells. Cells were treated as described in (A), and the activity of caspase 3 enzyme in each group was detected using Ac-DEVD-pNA as the substrate. **D**: Wog and N-Wog time-dependently increased caspase 3 enzyme activity in cells. As described in (B), the enzyme activity of caspase 3 in each sample was examined. **E**: The addition of a peptidyl caspase 3 inhibitor (Ac-DEVD-FMK) or Pan inhibitor (Z-VAD-FMK) significantly inhibited Wog- and N-Wog-induced DNA ladders in HL-60 cells. Cells were treated with Z-VAD-FMK and Ac-DEVD-FMK (200 μM) for 1 h followed by the addition of Wog (80 μM) or N-Wog (40 μM) for 12 h. The integrity of DNA in each group was analyzed by agarose electrophoresis. $^{**}P < 0.01$ indicates a significant difference from the control group, as analyzed by Student's *t*-test.

in HL-60 cells (Fig. 2D). Additionally, DNA ladders induced by Wog and N-Wog were blocked by adding the broad caspase inhibitor, Z-VAD-FMK, or the specific caspase 3 peptidyl inhibitor, Ac-DEVD-FMK (Fig. 2E). This indicates that activation of caspase 3 participates in the induction of apoptosis by Wog and N-Wog.

Catalase Inhibits N-Wog- But Not Wog-Induced Apoptotic Events Including DNA Ladders and Caspase 3 Activation in HL-60 Cells

Catalase (CAT) has been shown to scavenge ROS through conversion of H_2O_2 to H_2O and O_2. Results of the DNA fragmentation assay showed that CAT treatment significantly prevented HL-60 cells from N-Wog- but not Wog-induced DNA ladder formation. However, TPA (50 and 100 ng/ml) treatment exhibited no effect on DNA ladder formation induced by N-Wog or Wog (Fig. 3A). The reduction in H_2O_2 (200 μM)-induced DNA ladder formation by CAT was observed as a positive control. Additionally, N-Wog-induced the processing of caspase 3 and its downstream substrate, PARP protein, was prevented by the addition of CAT; however, those events induced by Wog were not affected by the addition of CAT (Fig. 3B). Data from the MTT assay supported CAT treatment protecting HL-60 cells from N-Wog- but not Wog-induced cytotoxicity (Fig. 3C). Analyzing the enzyme activity of caspase 3 indicated that the activity of the caspase 3 enzyme induced by N-Wog was significantly attenuated by the addition of CAT; however, CAT produced no effect on Wog-induced caspase 3 enzyme activity (Fig. 3D).

N-Wog But Not Wog Induces Apoptosis Through Elevation of Intracellular ROS Production

We investigated if ROS production is involved in the differential apoptotic potency of N-Wog and Wog in HL-60 cells. DCHF-DA has extensively been used to examine intracellular peroxide levels. Data on DCHF-DA showed that

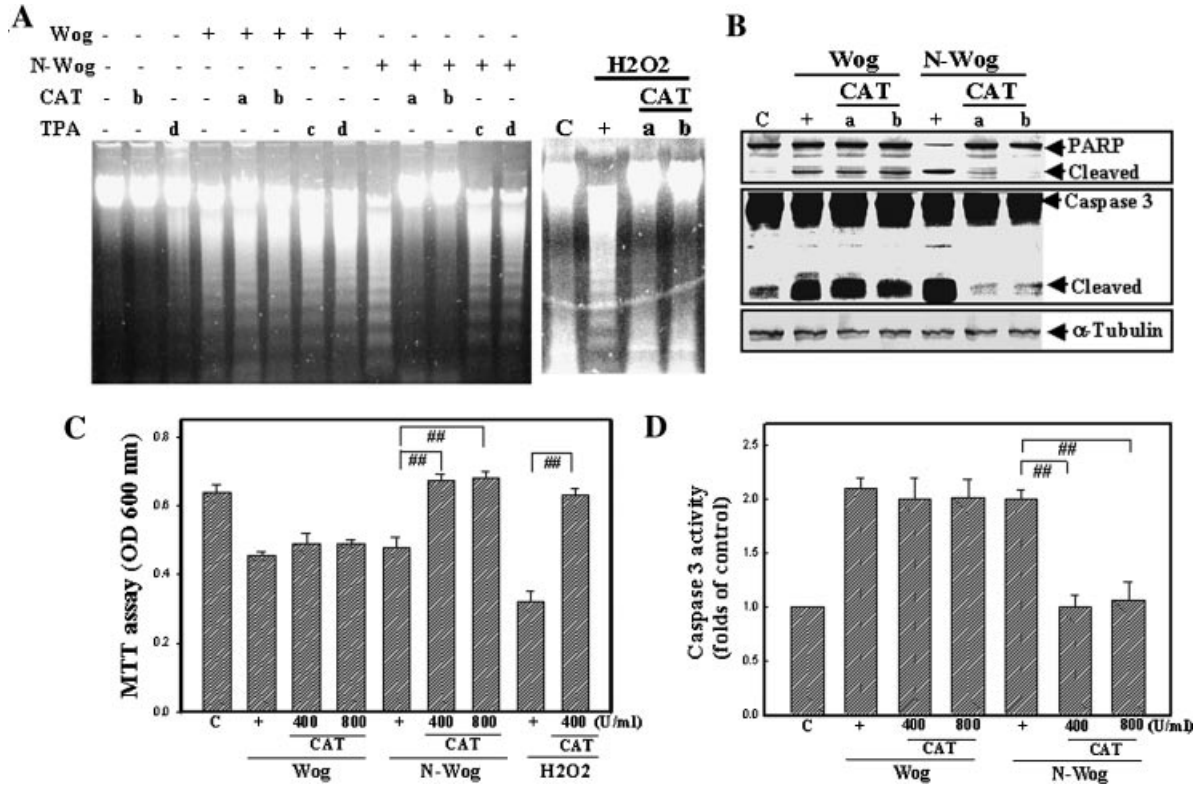

Fig. 3. Catalase (CAT) addition inhibits DNA ladders and caspase 3 activation in N-Wog- but not Wog-treated HL-60 cells. **A**: HL-60 cells were treated with CAT (a: 400 U/ml; b: 800 U/ml) or 12-*O*-tetradecanoylphorbol-13-acetate (TPA) (c: 50 ng/ml; d: 100 ng/ml) for 30 min followed by the addition of Wog (80 μM), N-Wog (40 μM), or H_2O_2 (200 μM) for an additional 12 h. The integrity of DNA in each group was analyzed by agarose electrophoresis followed by ethidium bromide straining. **B**: The addition of CAT inhibited N-Wog- but not Wog-induced cleavage of caspase 3 and the PARP protein. As described in (A), cells were treated with CAT for 30 min followed by incubation with Wog (80 μM) or N-Wog (40 μM). The expressions of caspase 3, PARP, and α-tubulin were examined by Western blotting using specific antibodies. **C**: CAT protected N-Wog- but not Wog-induced cytotoxicity in HL-60 cells. As described in (A), the viability of cells under different treatments was analyzed by the MTT assay. **D**: CAT inhibited N-Wog- but not Wog-induced caspase 3 enzyme activity. As described in (B), the activity of caspase 3 enzyme in the indicated groups was analyzed using Ac-DEVD-pNA as a substrate.

Wog treatment slightly increased the intracellular peroxide level. Interestingly, a significant increase in the intracellular peroxide level was detected in N-Wog-treated HL-60 cells, which was blocked by the addition of CAT (Fig. 4A). The H_2O_2 (200 μM)-induced an increase in intracellular peroxide was inhibited by the addition of CAT, and this served as a positive control. An increase in intracellular peroxide by N-Wog was also observed under fluorescence microscopy after DCHF-DA staining (data not shown). Quantitative data derived from three independent experiments are shown in Figure 4B, which indicates that intracellular peroxide induced by N-Wog and H_2O_2 was reduced by the addition of CAT; however, no significant alteration in intracellular peroxide levels was detected in Wog-treated HL-60 cells.

ROS-Dependent Mitochondrial Dysfunction in N-Wog-Treated HL-60 Cells

DiOC6(3) is a fluorescent dye for detecting the function of mitochondria, and a decrease in the fluorescent intensity of DiOC6(3) is shown in cells with a loss of the mitochondrial membrane potential. Data of the flow cytometry analysis indicated that Wog and N-Wog treatments induced mitochondrial dysfunctions in HL-60 cells, and the addition of CAT significantly prevented HL-60 cells from N-Wog- but not Wog-induced mitochondrial dysfunction (Fig. 5A). Suppression of H_2O_2 (200 μM)-induced mitochondrial dysfunction by CAT was used as a positive control. Furthermore, we examined the expression of the mitochondrial proteins, Bcl-2, Bax, and Cyt c, in HL-60 cells under different treatments. As illustrated

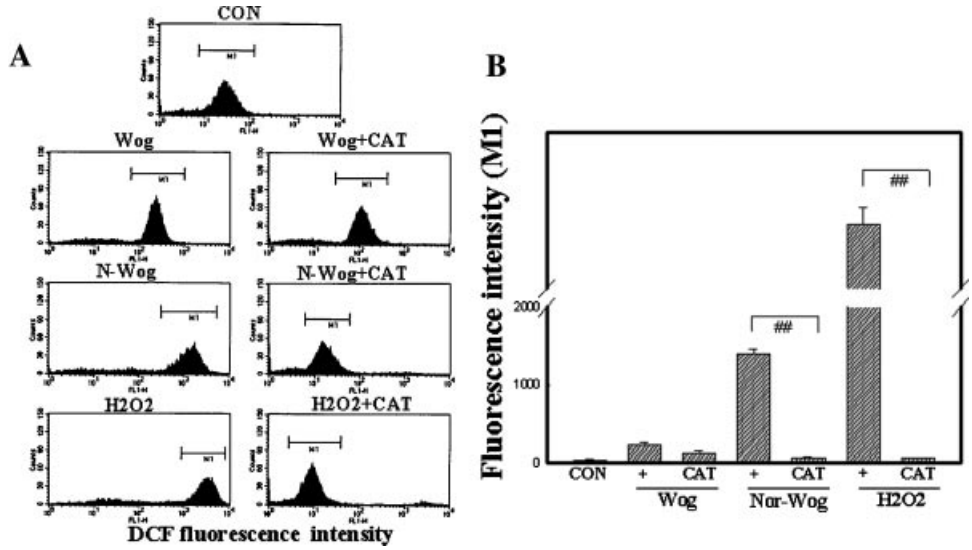

Fig. 4. An increase in intracellular peroxide level in N-Wog- but not Wog-treated HL-60 cells. Cells were treated with Wog (80 μM) or N-Wog (40 μM) or H_2O_2 (200 μM) for 1 h with or without a prior treatment with CAT (400 U/ml) for 30 min. At the end of the reaction, the level of intracellular peroxide was examined by adding DCHF-DA for an additional 30 min followed by a flow cytometric analysis. **A**: A representative example of the flow cytometric analysis is shown. **B**: Data derived from three independent experiments were calculated statistically, and results are presented as the mean ± SE. $^{\#\#}P < 0.01$ indicates a significant difference between the indicated groups as analyzed by Student's *t*-test.

in Figure 5B, an increase in Bax protein expression accompanied by an increase in the level of cytosolic Cyt c protein was detected in both N-Wog- and Wog-treated HL-60 cells, and the addition of CAT inhibited N-Wog- but not Wog-induced Bax and cytosolic Cyt c protein expressions. No alteration in Bcl-2 and α-tubulin protein levels was detected in HL-60 cells under different conditions. In the lower panel of Figure 5B, the ratio of Bax/Bcl-2 protein increased in Wog- and N-Wog-treated cells, and the N-Wog-induced Bax/Bcl-2 protein ratio was blocked by the addition of CAT. Data presented were derived from three independent experiments, and results are expressed as the mean ± SE.

Hydroxylation at C5, C7, and C8 Is Important for Apoptosis Induction in Human Leukemia HL-60 Cells by Flavonoids

In order to elucidate the importance of structural substitutions on Wog- and N-Wog-induced apoptosis, we analyzed the effects of 5-OH, 7-OH, 5,7-diOH, 5,7-diOCH_3, 7,8-diOCH_3, and 7-OCH_3-8-OH flavones on the viability of HL-60 cells. As illustrated in Figure 6A, the addition of 5,7-diOH or 7-OCH_3-8-OH flavone significantly induced apoptotic bodies in HL-60 cells under microscopic observations among six tested compounds. Data of the DNA fragmentation assay showed that 5,7-diOH and 7-OCH_3-8-OH flavone treatment induced DNA ladder formation in HL-60 cells, however, neither 5-OH, 7-OH, nor 7,8-diOCH_3 flavone induced DNA fragmentation in HL-60 cells (Fig. 6B). Furthermore, 5,7-diOH and 7-OCH_3-8-OH flavones induced the cleavage of caspase 3 and the PARP protein according to the Western blot analysis (Fig. 6C,D). These data suggest that OH at C5, C7, and C8 may be important for the apoptosis-inducing activity of flavonoids.

DISCUSSION

We provide evidence to support the differential roles of OH and COH_3 substitution in apoptosis induction by flavonoids. Data of the present study indicate that OH substitution at C5 and C7 is essential for apoptosis induction by flavonoids in human leukemia HL-60 cells. Interestingly, substitution of OCH_3 by OH at C8 may enhance the apoptotic activity of Wog. This suggests that OH at C8 may promote the apoptotic effect of flavonoids through stimulating ROS production. The importance of OH or OCH_3 substitution at C8 in the apoptosis properties of flavonoids is proposed.

Apoptosis has extensively been investigated, and activation of caspase cascades has been

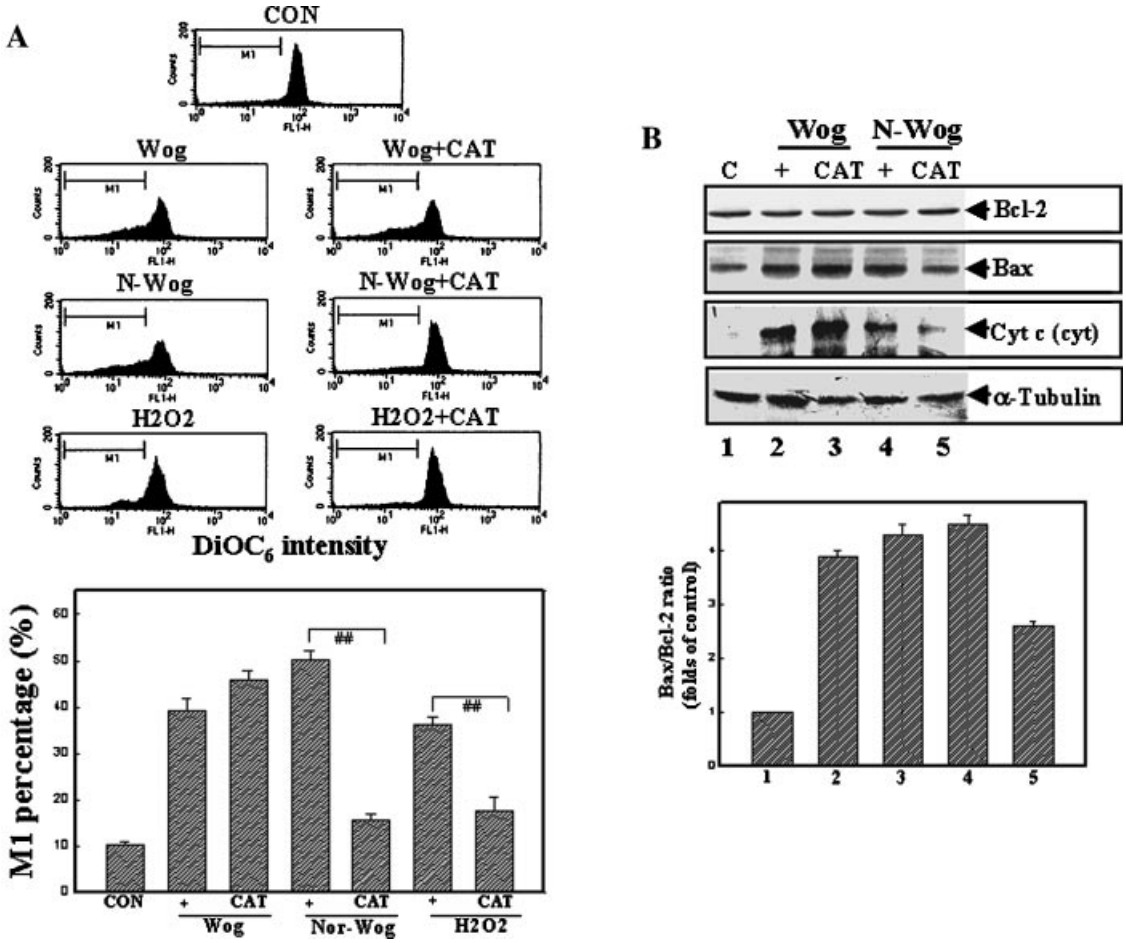

Fig. 5. Catalase prevented N-Wog- but not Wog-induced mitochondria dysfunction, the release of cytochrome c from mitochondria to the cytosol, and the ratio of the Bax/Bcl-2 proteins. Cells were treated with Wog (80 μM), N-Wog (40 μM), or H_2O_2 (200 μM) for 8 h in the presence or absence of a prior CAT (400 U/ml) treatment for 30 min. At the end of the incubation, the mitochondrial membrane potential was analyzed by adding DiOC6(3) as a fluorescent substrate. **A**: A representative example of the flow cytometric analysis is shown (**upper panel**). Data derived from three independent experiments were analyzed statistically, and results are expressed as the mean ± SE (**lower panel**). **B**: CAT inhibited N-Wog-induced Bax and cytosolic cytochrome c (Cyt c) protein expression in HL-60 cells. Cells were treated with CAT (400 U/ml) for 30 min followed by incubation with Wog (80 μM) or N-Wog (40 μM) for 8 h, and the expression of Bax, Bcl-2, α-tubulin, and Cyt c protein was detected by Western blotting (**upper panel**). The intensities of the Bax and Bcl-2 proteins were detected by a densitometric analysis, and the ratio of the Bax/Bcl-2 proteins was expressed as the mean ± SE from three independent experiments (**lower panel**). $^{\#\#}P < 0.01$ indicates a significant difference between indicated groups as analyzed by Student's *t*-test.

shown to occur in cells undergoing apoptosis. Caspase 3 is a downstream caspase, and activation of caspase 3 via induction of protein cleavage has been shown in chemical-induced apoptosis. Our previous study showed that Wog induces apoptosis through activation of caspase 3, which is independent of ROS production [Lee et al., 2002]. However, the apoptotic effect of N-Wog is still undefined. In the present study, N-Wog produced a more-potent cytotoxic effect than Wog, and activation of caspase 3 with cleavage of its downstream substrates, PARP and D4-GDI, was identified. N-Wog-induced apoptosis was significantly prevented by adding Ac-DEVD-FMK, a peptidyl caspase 3 inhibitor. This suggests that apoptosis is induced by Wog and N-Wog through activation of the caspase 3 cascade.

Flavonoids are generally recognized as antioxidants, but several studies have indicated that flavonoids exert prooxidant activities, and the ROS-producing activity is proposed to be proportional to the number and locations of OH groups in their chemical structures [Cao et al., 1997; Heijnen et al., 2002]. Heim et al. [2002] reported that OH groups in the B ring

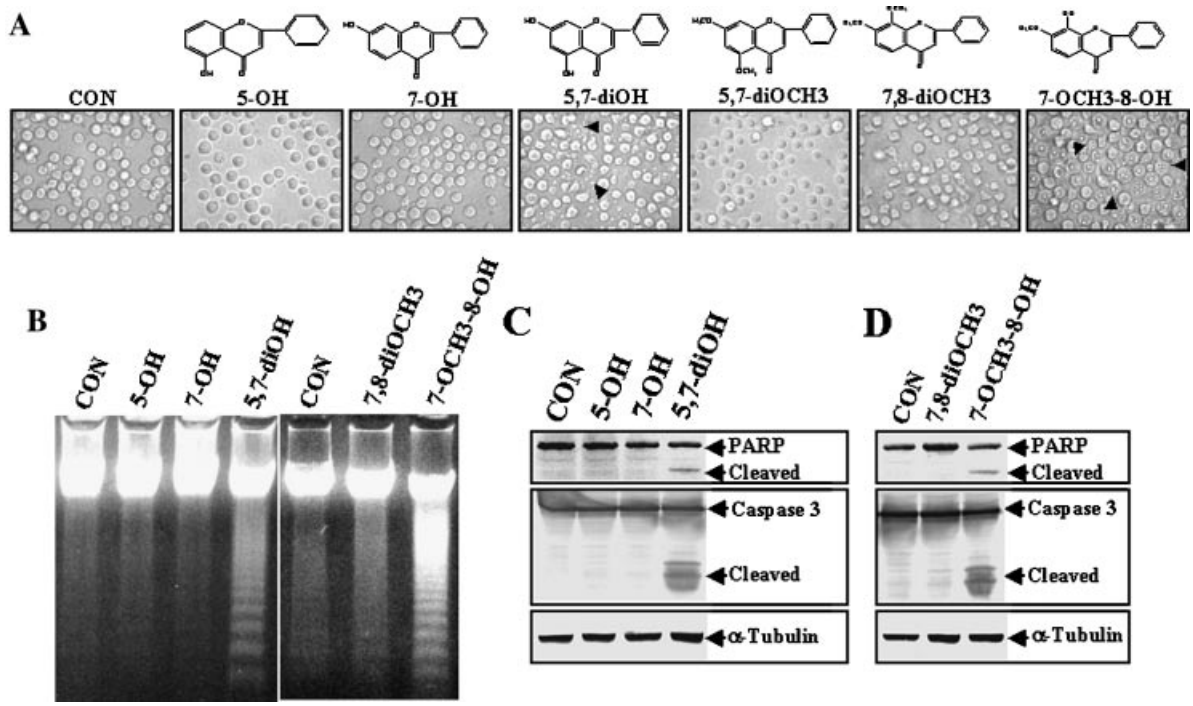

Fig. 6. Differential apoptotic effects of 5-OH, 7-OH, 5,7-diOH, 5,7-diOCH$_3$, 7,8-diOCH$_3$, and 7-OCH$_3$-8-OH flavones on the viability of HL-60 cells. **A**: 5,7-diOH and 7-OCH$_3$-8-OH flavones induced chromatin condensation in HL-60 cells. Cells were treated with the indicated compounds (40 μM) for 12 h, and the condensed chromatin was detected under microscopic observation. **B**: 5,7-diOH and 7-OCH$_3$-8-OH flavones induced DNA fragmentation in HL-60 cells. As described in (A), the integrity of DNA in each group was analyzed by agarose electrophoresis. Additionally, 5,7-diOH flavone (**C**) and 7-OCH$_3$-8-OH flavone (**D**), at a dose of 40 μM, induced the cleavage of caspase 3 and PARP protein in HL-60 cells. As described in (A), the expression of caspase 3, PARP, and α-tubulin protein was detected by Western blotting.

contributed to the production of hydroxyl radicals by flavonoids through the Fenton reaction. More evidence was given to support the importance of OH numbers in the antioxidant activities of flavonoids, and they indicated that increasing the number of OH substations might enhance the ROS-scavenging activity of flavonoids [Noroozi et al., 1998]. In the present study, we found that N-Wog performed more potent apoptotic effect than Wog. In addition, an increase in intracellular ROS was detected in N-Wog-treated cells, and N-Wog-induced apoptosis was significantly suppressed by adding CAT. These data suggest that OH substitution at C8 may contribute to the apoptotic action of flavonoids via prooxidant effects.

Several biological effects of Wog have been reported. Ma et al. [2005] indicated that Wog possesses the ability to induce apoptosis in human myeloma cells. In human leukemia HL-60 cells, Wog inhibited N-acetyltransferase activity and gene expression [Yu et al., 2005]. Park et al. [2004] suggested a preventive effect of Wog against ethanol-induced gastric mucosal damage in rats. Our recent studies indicated that Wog effectively suppresses NO production in macrophages induced by LPS [Shen et al., 2002], and it also induces apoptosis in human leukemia HL-60 cells [Lee et al., 2002]. Although several biological activities of Wog have been reported, the functions of N-Wog, and the roles of OH at C5 and C7, and OCH$_3$ at C8 on the action of Wog are still unclear. Data of the present study showed that (a) N-Wog was more effective than Wog in inducing apoptosis in HL-60 cells; (b) 5, 7-diOH flavone induced apoptosis in HL-60 cells; (c) 7-OCH$_3$-8-OH flavone but not 7,8-diOCH$_3$ flavone induced apoptosis in HL-60 cells. This evidence supports the notion that OH substitutions at C5, C7, and C8 contribute to the apoptosis induction of flavonoids, and activation of prooxidant effect is involved.

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