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Studies on the Inhibitory Mechanisms of Baicalein in B16F10 Melanoma Cell Proliferation

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ABSTRACT

Baicalein induces the formation of superoxide and hydroxyl radicals via 12-lipoxygenase (12-LOX) in the B16F10 mouse melanoma cell line; baicalein also causes a reduction in cellular viability and induces cell apoptosis. In this study, we utilized ROS scavengers to evaluate the role of ROS in baicalein-induced cell death and used the 12-LOX downstream product, 12-hydroxyeicosatetraenoic acid (12-HETE), to counterbalance the 12-LOX-inhibitory action of baicalein. ROS scavengers had no effect on cell differentiation, but in the cellular viability (MTT) assay, ROS scavengers effectively reversed cell viability reduction induced by baicalein. A Western blot analysis revealed that the ROS scavengers had no effect on the cell apoptosis protein, active caspase-3. From the aspect of 12-LOX, 12-HETE had no effect on cell differentiation, but it effectively reversed the reduction in cellular viability caused by baicalein in B16F10 cells. 12-HETE also possessed an inhibitory effect on the increase in expression of active caspase-3 caused by baicalein. Combined pretreatment with ROS scavengers and 12-HETE minimized the damage caused by baicalein. The majority of cell death occurring in response to baicalein-induced ROS formation in B16F10 mouse melanoma was due to cell necrosis. Cell apoptosis due to 12-LOX suppression by baicalein only accounted for a small portion.

Key words: baicalein, 12-lipoxygenase, reactive oxygen species, B16F10 cells, apoptosis, necrosis

INTRODUCTION

Baicalein (5,6,7-trihydroxyflavone), a bioactive flavonoid extracted from the root of *Scutellaria baicalensis* or *Scutellaria radix*, is frequently used to treat chronic hepatitis in China and Japan. It possesses anti-inflammatory[1,2], antithrombotic[3] and antioxidant effects[4,5], and produces cell cycle arrest and suppression of proliferation in cancer cells[6-8].

Baicalein was confirmed to inhibit the activity of 12-lipoxygenase (12-LOX)[9] and is widely used to decrease 12-hydroxyeicosatetraenoic acid (12-HETE) generation in cell proliferation studies. Breast, colorectal, and prostate cancers, were reported to overexpress 12-LOX which was suggested to be a regulator of cancer cell growth[10-12]. 12-LOX levels are associated with the grade and stage of human prostate tumors[13]. The addition of 12-HETE in the presence of baicalein inhibited the loss of phosphorylated retinoblastoma (pRB) protein in PC3 cells, whereas 12-HETE alone induced pRB expression[14].

Reactive oxygen species (ROS) can cause membrane damage and lead to cell death via apoptosis or necrosis[15]. Although some natural herbal compounds have been reported to destroy tumor cells by the generation of ROS, the mechanisms concerning how ROS is induced have not been clarified[16].

Baicalein induces the apoptosis of Jurkat cells[17], HL60 cells[18], and mouse-rat hybrid retina ganglion cells[19], all of which are accompanied by intracellular ROS generation. In normal cells, our previous study demonstrated that baicalein induces hydroxyl radical formation in human platelets which contain a 12-LOX isoform (p12-LOX), but baicalein does not induce ROS in rat vascular smooth muscle cells and human umbilical vein endothelial cells[20]. In B16F10 cells, we also detected hydroxyl radicals and superoxide anion radicals generated by baicalein via 12-LOX, and found that baicalein caused a reduction in cellular viability. In addition, decreases in 12-LOX protein expression and free radical generation occurred in a 12-LOX small interfering RNA knockdown protein group compared with the baicalein control[21]. Those results suggested that ROS formation catalyzed by 12-LOX is one possible mechanism of baicalein-induced cell death,

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but the precise mechanism, apoptosis or necrosis, is still ambiguous.

Baicalein was recently shown to enhance TRAIL-induced apoptosis by the induction of a CCAAT/enhancer-binding protein homologous protein (CHOP). In contrast, baicalein increases TRAIL-R2 expression through a ROS-mediated mechanism independent of CHOP in human prostate cancer PC-3 cells[22]. However, baicalein barely induces apoptosis in normal cells. The mechanism of distinguishing tumor from normal cells by Baicalein remains unknown. In the present study, we demonstrated that B16F10 mouse melanoma cell death caused by baicalein was mainly due to ROS generation through 12-LOX, which suggests that the majority of cell death was due to cell necrosis. Cell apoptosis caused by 12-LOX suppression of baicalein accounted for only a small portion.

MATERIALS AND METHODS

I. *Materials*

Arachidonic acid (AA), aprotinin, 4-hydroxy-3-methoxyacetophenone (acetovanillone), catalase (CAT), dimethyl sulfoxide (DMSO), dithiothreitol (DTT), 5,5-dimethyl-1-pyrroline N-oxide (DMPO), N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), mannitol, tetrazolium dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), phenylmethylsulfonyl fluoride (PMSF) and quinacrine were purchased from Sigma Chemical (St. Louis, MO, USA). 1-Hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine HCl (CMH) was purchased from Alexis (San Diego, CA, USA). Baicalein was purchased from Aldrich Chemical (Milwaukee, WI, USA). Sodium dodecylsulfate (SDS), Triton X-100 and Tris-base were obtained from Amersham Life Sciences (Arlington Heights, IL, USA). Polyoxyethylenesorbitan monolaurate (Tween 20), N,N,N,N,-tetramethylethylethylenediamine (TEMED) and glycerol were purchased from Pharmacia Biotech (Piscataway, NJ, USA). Amphotericin B (fungizone), Dulbecco's modified Eagle medium (DMEM), porcine elastase, fetal calf serum (FCS), L-glutamine, Hank's balanced salt solution (HBSS), penicillin/streptomycin, sodium pyruvate, trypan blue stain and trypsin-EDTA were purchased from Gibco BRL (Grand Island, NY, USA). The 12-lipoxygenase primary antibody and cleaved caspase-3 rabbit (ASP175) antibody were purchased from Sigma Chemical, Abcam (Cambridge, UK) and Cell Signaling Technology (Beverly, MA, USA), respectively. Horseradish peroxidase (HRP)-conjugated sheep anti-mouse and anti-rabbit antibodies were obtained from Amersham (Bucks, UK), and mouse and rabbit control immunoglobulin G (IgG) was purchased from Organon Teknika-Cappel (Malvern, PA, USA).

II. *Tumor Cell Lines and Cell Culture*

B16F10 murine melanoma cells were obtained from the National Institute of Preventive Medicine, Department of Health, Executive Yuan (Taipei, Taiwan) and were cultured at 37°C under a humid atmosphere and 5% CO_2, in DMEM medium with 10 mM of HEPES, 24 mM of sodium bicarbonate, 40 mg/L of gentamycin (pH 7.2) and 10% FCS.

III. *Morphological Study*

After the cells had been incubated with baicalein (10, 25 and 50 μM) for 12 h, the cell morphology was observed and images were captured under a light microscope connected to a digital camera (Nikon TS100, Japan).

IV. *Proliferation Assay*

Cell viability was assessed using a standard MTT assay. B16F10 cells in the exponential growth phase were suspended in DMEM containing 10% FCS and cultured in flat-bottomed, 96-well plates (2×10^4 cells/well) for 24 h at 37°C, followed by the addition of baicalein. The final concentrations of the drug were 10, 25 and 50 μM, and plain DMEM was used as the negative control. The plates were incubated in a humidified incubator of 5% CO_2 at 37°C for 6, 12, 18 and 24 h. The supernatants were discarded after centrifugation. MTT (0.5 mg/mL) at 100 μL/well was added to the plates and incubation continued for 3 h. The supernatant was then carefully removed and 300 μL of DMSO was added to dissolve the formazan crystals. The optical density at 540 nm was read using an enzyme-linked immunosorbent assay (ELISA) reader. The percentage of cell viability was calculated as the absorbance of treated cells/control cells × 100%.

V. *Flow Cytometric Analysis*

B16F10 cells were seeded onto six-well plates at a density of 5×10^4 cells per well and incubated for 24 h before the experiment. The cells were washed with PBS, treated with media containing baicalein in DMSO and incubated for 24 h. Supernatants and collected cells were centrifuged, and cell pellets were suspended in 1× calcium buffer at a rate 10^5 cells/100 μL. The cells were stained with annexin (BD 556420) and propidium iodide (PI; Sigma P4864) in a dark room for 15 min. The DNA content was determined on a flow cytometer (Becton Dickinson, FACScan Syst., San Jose, CA, USA). Percentages of apoptotic, necrotic, and decompensated cells were calculated among all viable cells (100%). All experiments were repeated three times to ensure reproducibility.

VI. *Western Blotting*

To determine the expression of caspase-3, B16F10 cells were cultured on 24-well plates and treated with baicalein or an isovolumetric solvent control for 12 h. At the indicated times, the cells were washed with ice-cold PBS buffer (pH 7.3). Proteins were extracted with lysis buffer for 30 min. Lysates were centrifuged and the supernatant (60 - 80 μg

protein) was subjected to SDS-polyacrylamide gel electrophoresis (PAGE) and electrophoretically transferred onto polyvinylidene difluoride (PVDF) membranes (0.45 μm; Hybond-P; Amersham). Samples were mixed with sample buffer, boiled for 10 min, separated by 0.1% SDS, subjected to 10% polyacrylamide gel electrophoresis under denaturing conditions and electroblotted onto Immobilon-P membranes (Millipore). The membranes were blocked with TBS (5% non-fat dry milk, 0.1% Tween 20 in 50 mM of Tris-HCl buffer and 150 mM of NaCl; pH 7.5) overnight at 4°C. The membranes were incubated with an anti-12-lipoxygenase, anti-α-tubulin, anti-lamin A/C, anti-caspase-3 or anti-β-actin antibody (Sigma) for 3 h at room temperature. After four washes in TBS/0.1% Tween 20, the membranes were probed with a secondary goat anti-rat (Santa Cruz Technology, Santa Cruz, CA, USA) or goat anti-mouse (KPL) antibody, each conjugated to HRP. After washing four times with TBS and 0.1% Tween 20, the band with peroxidase activity was detected using film exposure with enhanced chemiluminescence detection reagents (ECL^+ system, Amersham). Densitometric analysis of specific bands was performed using a Photo-Print Digital Imaging System (IP-008-SD) with analytic software (Bio-1Dlight, V 2000).

VII. *Statistical Analysis*

Experimental results are expressed as the mean ± SEM and are accompanied by the number (*n*) of observations. Data were assessed using an analysis of variance (ANOVA). If this analysis indicated significant differences among the group means, then each group was compared using the Newman-Keuls method. A p value of less than 0.05 was considered statistically significant.

RESULTS

I. *Effect of ROS Scavengers on Baicalein-Induced Inhibition of Proliferation of B16F10 Melanoma Cells*

We compared the effects of the spin-trappers, CMH and DMPO, with the extracellular ROS scavengers, mannitol and catalase, on baicalein-induced proliferation inhibition in B16F10 melanoma cells in this study. We first evaluated morphological changes induced by baicalein and the effects of ROS scavengers on baicalein-induced morphological changes. B16F10 melanoma cells were treated with 50 μM of baicalein for 24 h. ROS scavengers were administered 1 h before baicalein. As shown in Figure 1, B16F10 cells became much more spindly in shape after 24 h, and some of the cells seemed to have broken down (Figure 1B). Both pretreatment with 25 mM of mannitol (Figure 1C) and 300 unit/mL of catalase (Figure 1D) both increased the number of cells without obvious changes to the cell morphology. Similar results were observed in groups pretreated with spin-trappers, CMH (Figure 1E) and DMPO (Figure 1F).

II. *Effect of Adding 12-HETE Back on Baicalein-Induced Inhibition of Proliferation of B16F10 Melanoma Cells*

We evaluated the effects of 12-HETE on baicalein-induced morphological changes. B16F10 melanoma cells were treated with 50 μM of baicalein for 24 h. 12-HETE was administered 1 h before baicalein. As shown in Figure 1, both pretreatments with 500 nM (Figure 1G) and 1000 nM of 12-HETE (Figure 1H) showed no obvious changes in the cell morphology.

III. *Effects of ROS Scavengers and 12-HETE on Baicalein-Induced Inhibition of Proliferation of B16F10 Melanoma Cells*

To elucidate the mechanisms of inhibition of B16F10 cell growth by baicalein, we investigated whether ROS scavengers and 12-HETE could reverse the cell shape change effects of baicalein. The scavengers and 12-HETE were added 1 h before the addition of baicalein (50 μM for 24 h). The shape changes obviously decreased (Figure 2) compared to ROS scavenger-treated or 12-HETE-treated groups (Figure 1).

The effects of ROS scavengers on the proliferation of B16F10 melanoma cells were determined by a MTT colorimetric survival assay (Figure 3A). All values were normalized to percentage of the control and represented the average of three independent incubations. Proliferation of groups of melanoma cells treated with 1 mM of CMH, 10 mM of DMPO, 25 mM of mannitol and 300 unit/mL of catalase were higher than the baicalein-treated (50 μM for 24 h) group, and results were 49.8 ± 7.8%, 72.8 ± 6.8%, 71.3 ± 3.9%, 57.8 ± 4.8% and 80.1 ± 3.3%, respectively. The effects of 12-HETE on the proliferation of B16F10 melanoma cells were shown in Figure 3B. Proliferation levels in the 500 nM and 1000 nM 12-HETE-treated groups of melanoma cells were higher than that of the baicalein-treated (50 μM for 24 h) group, but only the effect of 1000 nM of 12-HETE was significant, and results were 55.1 ± 4.5%, 62.2 ± 3.8% and 75.3 ± 2.8%, respectively. The combined effects of ROS scavengers and 12-HETE on the proliferation of B16F10 melanoma cells were shown in Figure 3C. Proliferation of groups of melanoma cells treated with 1000 nM of 12-HETE and 1 mM of CMH, 1000 nM of 12-HETE and 10 mM of DMPO, 1000 nM of 12-HETE and 25 mM of mannitol, and 1000 nM of 12-HETE and 300 units/mL of catalase were significantly higher than the baicalein-treated (50 μM for 24 h) group ($p < 0.001$), and results were 48.6 ± 3.8%, 91.0 ± 2.6%, 88.9 ± 4.6%, 85.9 ± 2.1% and 92.2 ± 5.7%, respectively. This indicates that 12-HETE and ROS generation are involved in baicalein-induced cell death. In addition, proliferation of groups of melanoma cells treated with test compounds only showed no significant difference from the control group (Figure 3D).

IV. *Effects of ROS Scavengers on Cleaved Caspase-3 Protein Expression Induced by Baicalein in B16F10 Melanoma Cells*

Caspase-3 plays an important role as an executioner

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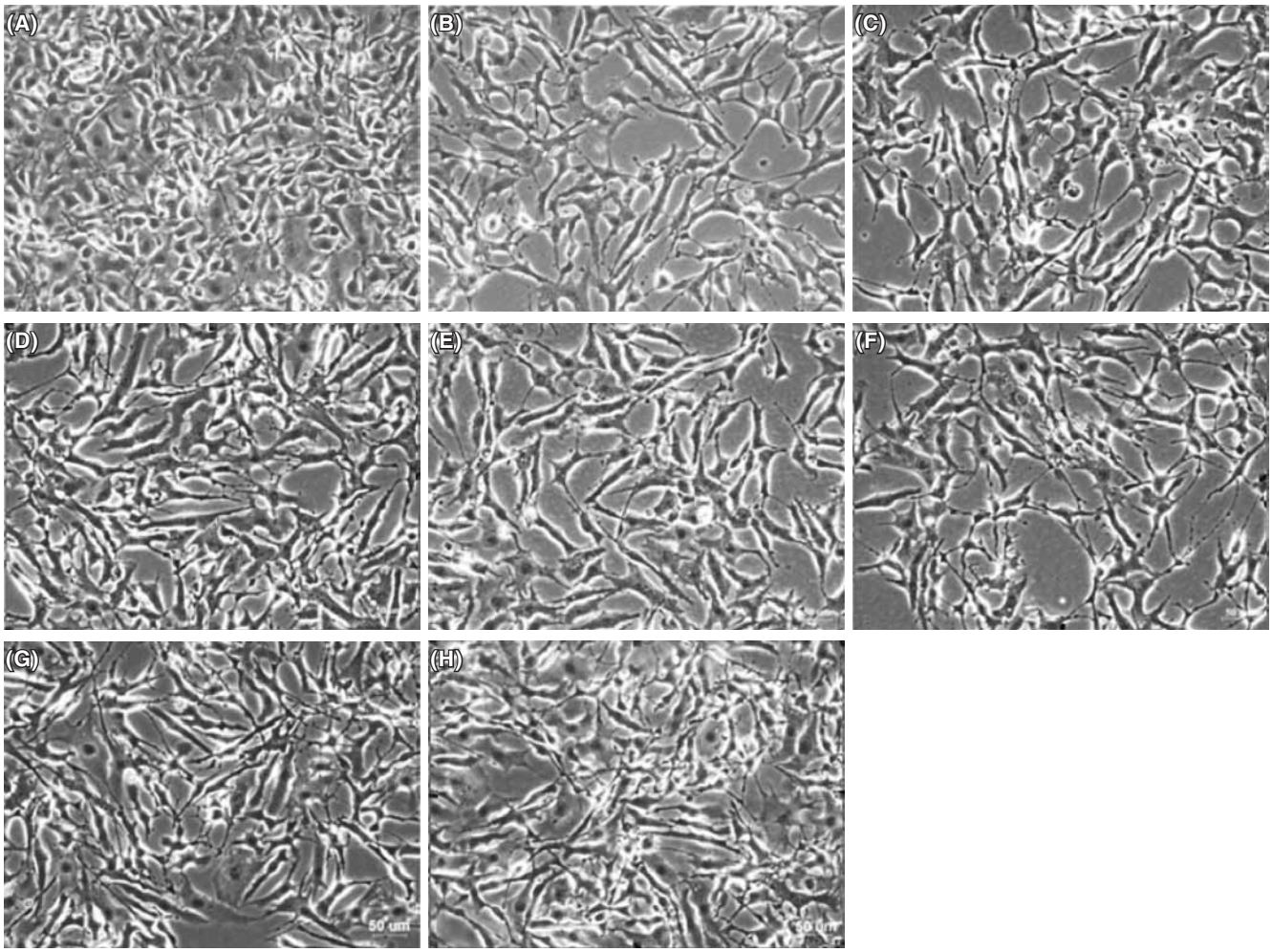

Figure 1. Effects of ROS scavengers and 12-HETE, given separately, on baicalein-induced morphological changes in B16F10 melanoma cells. B16F10 cells were treated with 50 μM of baicalein for 24 h. ROS scavengers were administered for 1 h before baicalein. The cells were washed with PBS three times before viewing under microscopy. (A) Resting, (B) 50 μM of baicalein, (C) 50 μM of baicalein + 25 mM of mannitol, (D) 50 μM of baicalein + 300 units/mL of catalase, (E) 50 μM of baicalein + 1 mM of CMH, (F) 50 μM of baicalein + 10 mM of DMPO, (G) 50 μM of baicalein + 500 nM of 12-HETE, (H) 50 μM of baicalein + 1000 nM of 12-HETE (magnification: ×100).

in the apoptotic process. During apoptosis, the Mr-32,000 procaspase-3 is cleaved into Mr-17,000 and Mr-11,000 forms of active caspase-3[23]. Our results showed that baicalein induced marked caspase-3 activation in B16F10 melanoma cells after 24 h of treatment, while pretreatment with 25 mM of mannitol, 300 unit/mL of catalase, 1 mM of CMH and 10 mM of DMPO showed no significant effect (Figure 4). This result indicated that ROS are involved in baicalein-induced cell death but not cell apoptosis.

V. *Effects of 12-HETE and Scavengers on Cleaved Caspase-3 Protein Expression Induced by Baicalein in B16F10 Melanoma Cells*

As shown in Figure 5, we observed that baicalein induced marked caspase-3 activation in B16F10 melanoma cells after 24 h of treatment, and pretreatment with 500 nM and 1000 nM of 12-HETE decreased this activation, but only the effect of 1000 nM of 12-HETE was significant. This reveals that 12-HETE is involved in baicalein-induced B16F10 cell apoptosis.

VI. *Induction of Apoptosis and Necrosis by Baicalein in B16F10 Cells*

A flow cytometric assay was used to quantify apoptotic and necrotic cells treated with 50 μM of baicalein. Treated and untreated B16F10 cells were stained with annexin V-FITC and PI. Annexin V-FITC staining is used in conjunction with the dye PI to distinguish between cells that are early in apoptosis from those late in apoptosis. Figure 6 shows the cytotoxic response in B16F10 cells treated with baicalein. The apoptotic index is the sum of the percentage of cells that were positive for annexin-V-FITC alone (pre-apoptotic) and cells positive for both annexin-V-FITC and PI (late-apoptotic) within a population of cells.

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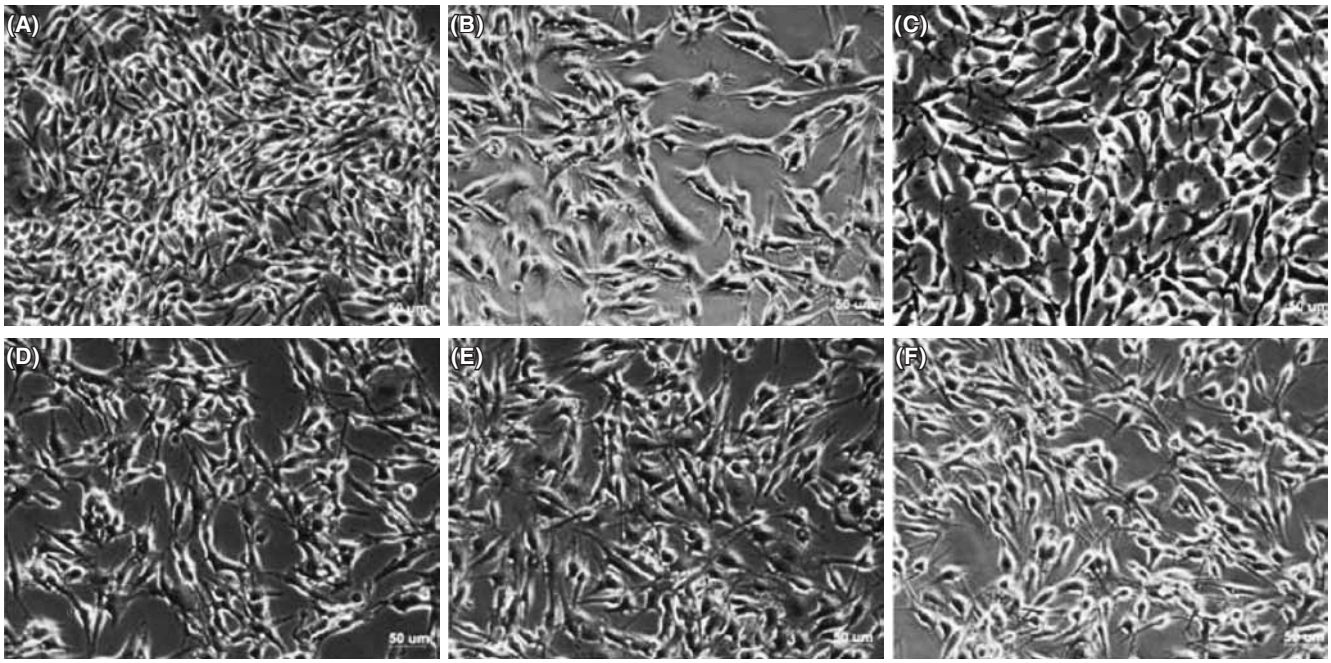

Figure 2. Effects of ROS scavengers plus 12-HETE on baicalein-induced morphological changes in B16F10 melanoma cells. B16F10 cells were treated with 50 μM of baicalein for 24 h. ROS scavengers and 12-HETE were added 1 h before treatment with baicalein. The cells were washed with PBS three times before examination by microscopy. (A) Resting, (B) 50 μM of baicalein, (C) 50 μM of baicalein + 1000 nM of 12-HETE + 1 mM of CMH, (D) 50 μM of baicalein + 1000 nM of 12-HETE + 10 mM of DMPO, (E) 50 μM of baicalein + 1000 nM of 12-HETE + 25 mM of mannitol, (F) 50 μM of baicalein + 1000 nM of 12-HETE + 300 unit/mL of catalase (magnification: ×100).

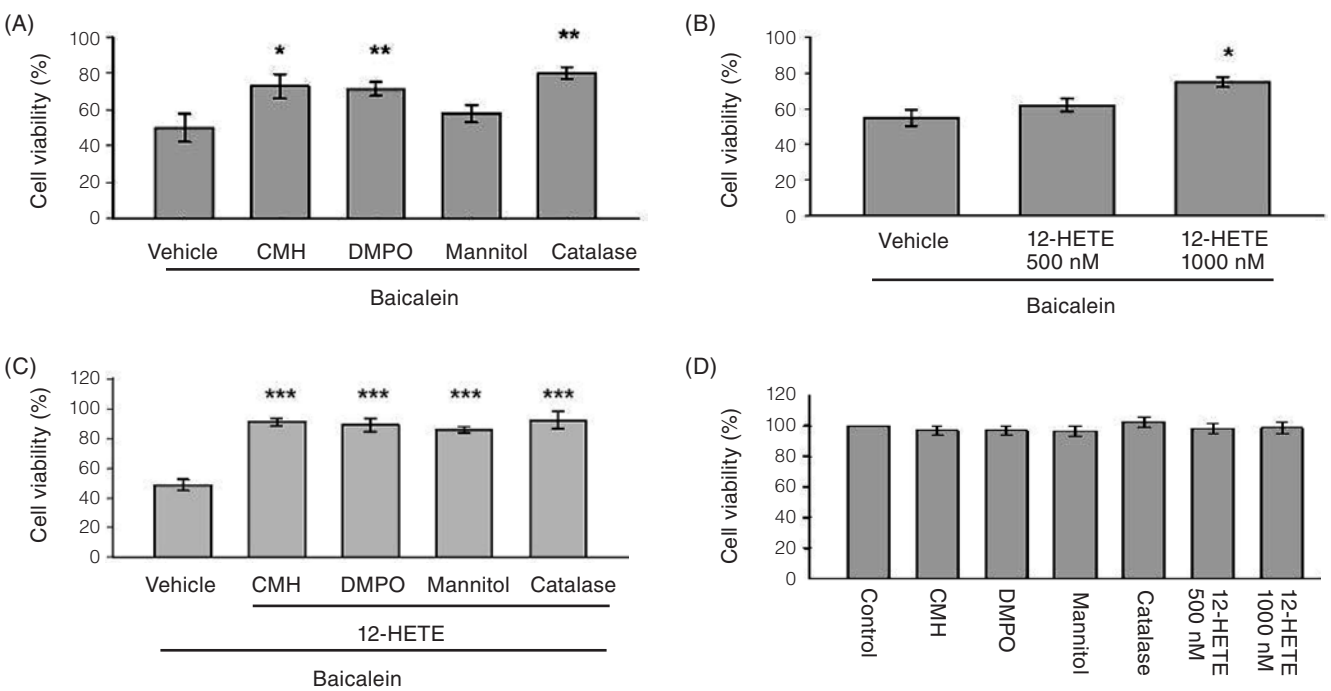

Figure 3. Effects of ROS scavengers plus 12-HETE on baicalein-induced inhibition of proliferation of B16F10 melanoma cells. B16F10 melanoma cells (5×10^4 cells/mL) were dispensed on 96-well plates until 80 - 90% confluent. (A) Cells were treated with 1 mM of CMH, 10 mM of DMPO, 25 mM of mannitol or 300 units/mL of catalase for 1 h; (B) cells were treated with 500 nM or 1000 nM of 12-HETE for 1 h; (C) cells were treated with 1000 nM of 12-HETE and ROS scavengers, and 10 mM of DMPO, 1 mM of CMH, 25 mM of mannitol or 300 units/mL of catalase for 1 h, before treatment with 50 μM baicalein for 24 h; (D) cells were treated with the indicated concentration of test compounds only. The percentage of viable cells is presented as the mean ± SEM of three independent experiments. ***$p < 0.001$; **$p < 0.01$; *$p < 0.05$, compared to baicalein treatment only.

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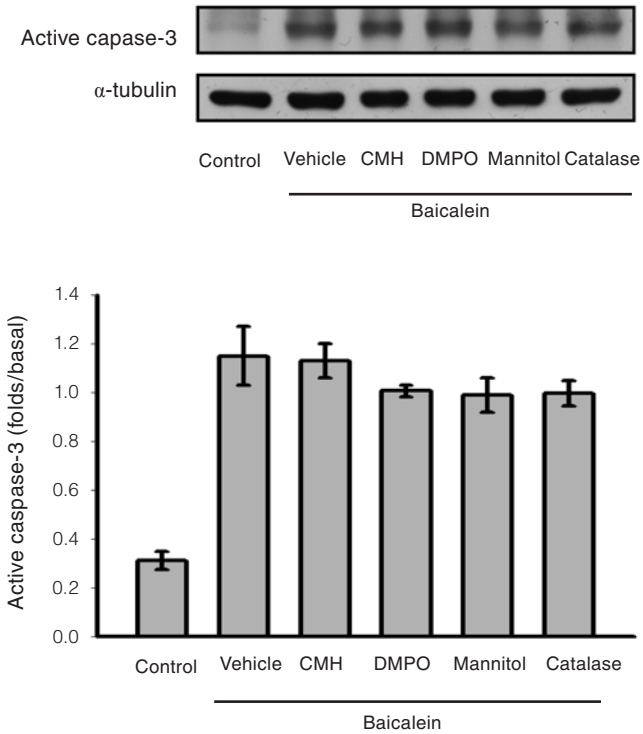

Figure 4. Effects of ROS scavengers and baicalein on cleaved caspase-3 protein in B16F10 melanoma cells. The cells were treated with the indicated concentration of 1 mM of CMH, 10 mM of DMPO, 25 mM of mannitol or 300 unit/mL of catalase for 1 h before treatment with 50 μM of baicalein for 24 h. The bar graph depicts the ratios of quantitative results obtained by scanning the anti-cleaved caspase-3 and anti-α-tubulin reactive bands and quantifying the optical density using Bio-1D version 99 image software. Data are presented as the mean ± SEM (n = 3).

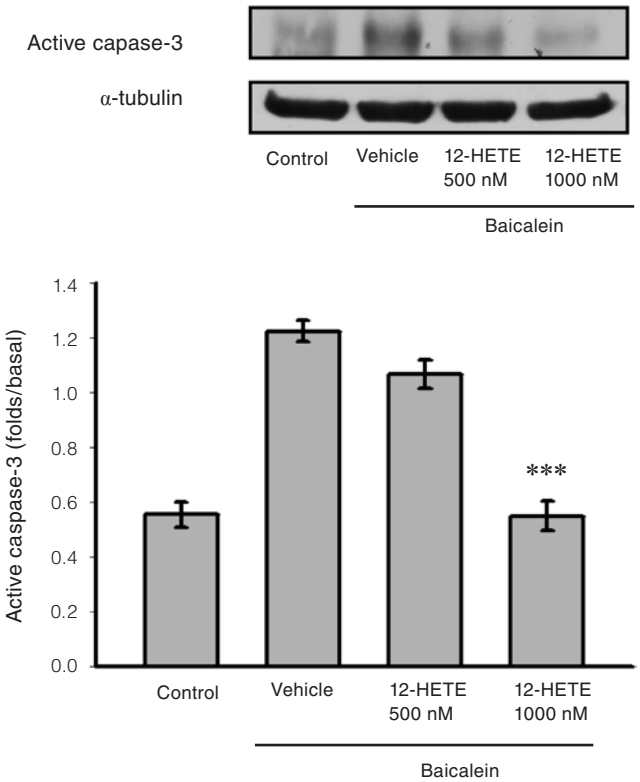

Figure 5. Effects of 12-HETE on baicalein-induced cleaved caspase-3 protein expression in B16F10 melanoma cells. The cells were treated with the indicated concentration of 500 nM or 1000 nM of 12-HETE for 1 h before treatment with 50 μM of baicalein for 24 h. The bar graph depicts the ratios of quantitative results obtained by scanning the anti-cleaved caspase-3 and anti-α-tubulin reactive bands and quantifying the optical density using Bio-1D version 99 image software. Data are presented as the mean ± SEM (n = 3). ***$p < 0.001$, compared to baicalein treatment only.

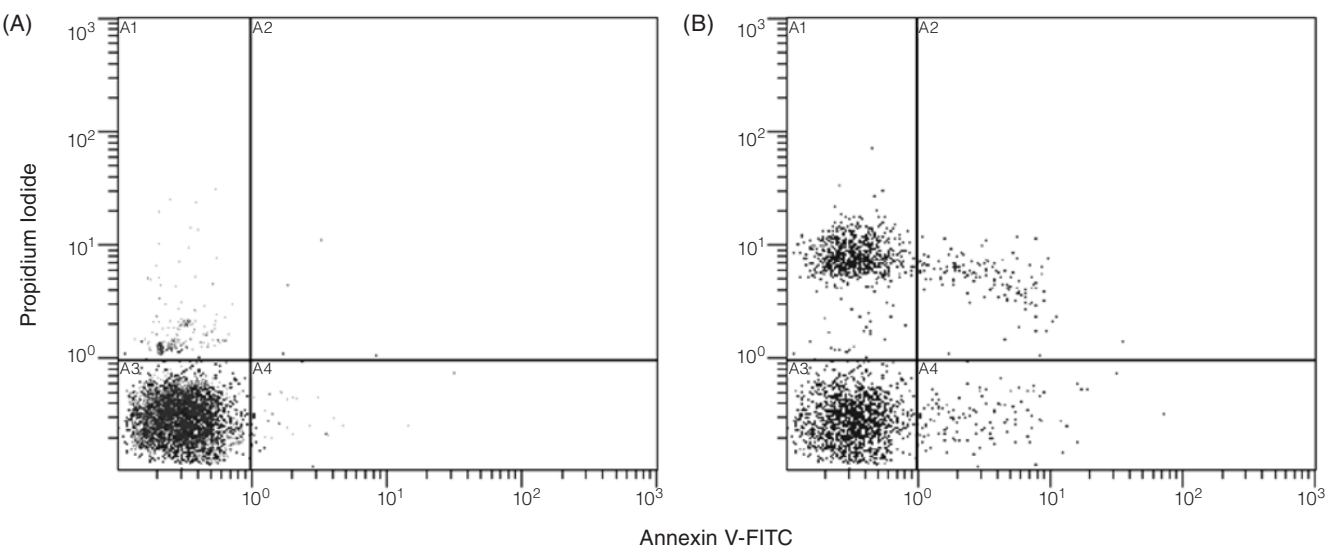

Figure 6. Flow cytometric analysis of apoptosis in B16F10 melanoma cells following treatment with baicalein. (A) Control cells; (B) cells treated with 50 μM of baicalein.

The necrosis index is the sum of the percentage of cells that were positive for PI within a population of cells. Untreated cells were primarily annexin V-FITC and PI negative (lower left quadrant, panel A), indicating that the cells were viable. In comparison with untreated B16F10 cells which showed 4.25% background cell death (upper left quadrant and upper right quadrant, panel A), cells treated with 50 μM of baicalein showed 41.24% cell death, including 8.98% by apoptosis (upper right quadrant, panel B) and 32.26% by necrosis (upper left quadrant, panel B). This indicated that the majority of cell death induced by baicalein is through cell necrosis.

DISCUSSION

Based on several studies, baicalein can induce apoptosis in various cancer cells and the mechanisms of action are associated with caspase activation and mitochondrial dysfunction[14,24-26]. However, there is no available information to address the roles of ROS in the induction of cell death caused by baicalein. Li *et al.* suggested that ROS and cellular Ca^{2+} modulate baicalein-induced apoptosis via a Ca^{2+}-dependent mitochondrial death pathway in mouse-rat hybrid retina ganglion cells and demonstrated release of Cyt c from mitochondria into the cytosol and activation of caspase-3[19]. In our previous study, we demonstrated that baicalein induced the formation of ROS via 12-LOX in the B16F10 mouse melanoma cell line[21]. In the present investigation, we utilized extracellular ROS scavengers, mannitol and catalase, and intracellular ROS scavengers, DMPO and CMH, to evaluate the role of ROS in baicalein-induced cell death. Our data demonstrated that ROS scavengers decreased baicalein-induced cell death but did not affect the level of activated caspase-3, providing further evidence that ROS are not associated with baicalein-induced apoptosis in B16F10 cells. One interpretation of this observation is that ROS-induced cell death may be due to cell necrosis.

AA release from cell membranes is necessary for cancer cell proliferation. Moreover, LOX catalyzes AA into hydroperoxyeicosatetraenoic acid (HPETE) and finally to hydroxyeicosatetraenoic acid (HETE) or to leukotrienes. Many cancer cell lines express 12-LOX and produce 12-HETE[27,28], which can stimulate cancer growth via activation of the p44/42 mitogen-activated protein kinase and PI3/Akt kinase pathways[29]. Furthermore, 12-HETE can facilitate the invasion and metastasis of cancer cells[30,31]. The addition of exogenous 12-HETE increased the incorporation of thymidine into DNA in a dose-dependent manner in A431 human epidermoid carcinoma cells[32,33]. Prostate[14] and gastric cancer cells[26] were also reported to increase proliferation in response to 12-HETE. In this study, we found that 12-HETE had no effect on cell growth differentiation, but it effectively reversed the reduction in cellular viability caused by baicalein in B16F10 cells. This result was similar to that with ROS scavengers. In addition, 12-HETE also possessed an inhibitory effect on the increase in expression of active caspase-3 caused by baicalein. Finally, we pretreated ROS scavengers and 12-HETE at the same time and the results showed that the combined treatment minimized the damage caused by baicalein.

Our results conclusively demonstrate that B16F10 mouse melanoma cell death caused by baicalein is related to both 12-LOX suppression and ROS generation, but apoptosis only occurs because of 12-LOX suppression by baicalein. Moreover, we used cytometric analysis to examine cell death caused by baicalein and found that cell necrosis was the major mode of cell death and that apoptosis plays only a minimal role. Taking together, we concluded that the majority of cell death occurring in response to baicalein-induced ROS formation in B16F10 mouse melanoma cells was due to necrosis. Cell apoptosis caused by 12-LOX suppression of baicalein only accounted for a small portion.

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