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RESEARCH ARTICLE

Baicalein induces human osteosarcoma cell line MG-63 apoptosis via ROS-induced BNIP3 expression

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Abstract Baicalein, a flavonoid compound, is one of the active constituents of the root of *Scutellariae Radix*. Its antitumor effects have attracted widespread attention worldwide. One of its major functions is to induce the apoptosis of tumor cells, but the antitumor mechanism is currently unclear. In the present study, we found that baicalein increased MG-63 cell mortality in a dose-dependent manner. Meanwhile, baicalein activated apoptosis through induced intracellular reactive oxygen species (ROS) generation, and that ROS scavenger *N*-acetyl-cysteine (NAC), glutathione (GSH), and superoxide dismutase (SOD) apparently inhibited intracellular ROS production, consequently attenuating the baicalein-induced apoptosis. Baicalein also induce the mitochondrial fragmentation which precedes the cell apoptosis. This morphological alteration is accompanied by an increase in the expression of the protein BNIP3 as well as Mul1 and Drp1. Furthermore, we show that the inhibition of BNIP3 expression can inhibit cell apoptosis by baicalein treatment. Taken together, our results bring the evidence of a mechanism that links apoptosis and ROS-induced BNIP3 expression in MG-63 cells with bacalein treatment and suggest that baicalein has a good potential as an anti-osteosarcoma drug.

Keywords Apoptosis · Osteosarcoma · Baicalein · ROS · BNIP3

Guarantor of submission The corresponding author is the guarantor of submission.

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Abbreviations

ROS	Reactive oxygen species
NAC	*N*-acetyl-cysteine
GSH	Glutathione
SOD	Superoxide dismutase
BNIP3	BCL2/adenovirus E1B 19 kDa interacting protein 3
LDH	Lactate dehydrogenase

Introduction

Osteosarcoma is a primary malignant bone tumor that commonly affects childhood and adolescents. Surgical resection, chemotherapy, or chemoprevention are the most commonly used therapeutic strategies for osteosarcoma. However, any of these therapies alone cannot yield sufficient therapeutic

outcomes [1]. Recently, more attention has been focused on seeking for natural chemopreventive compounds capable of inhibiting or reversing the multistep carcinogenesis with minimal toxicity. Between 1981 and 2006, almost 73 % of all drugs approved for anti-cancer are either natural products or based on natural products [2].

Baicalein (5,6,7-trihydroxyflavone) is derived from the root of *Scutellaria baicalensis* Georgi, a medicinal plant traditionally used in Chinese herbal medicine [3]. Previous investigations have shown that baicalein is an antioxidant [4] and anti-inflammatory agent [5], possessing antioxidant activities [6] and cytoprotective effects [7]. On the other hand, it is reported that baicalein induced cellular abnormalities in various human cancer cell lines through multiple signaling pathways, involving cell proliferation pathway, cell apoptosis and caspase activation pathway, tumor suppressor pathway, death receptor pathway, mitochondrial pathway, and protein kinase pathway [8, 9], but the exact mechanism of apoptosis and its related pathways induced by baicalein is not clearly understood. BCL2/adenovirus E1B 19 kDa interacting protein 3 (BNIP3) is a BH3-only protein which is well known to participate in cell apoptosis and autophagy. It is a pro-apoptotic Bcl2 family member and can directly interact with anti-apoptotic protein Bcl2. In the present study, osteosarcoma MG-63 cells were treated with baicalein at different concentrations. The results showed that baicalein promoted cell mortality in a dose-dependent manner via intracellular reactive oxygen species (ROS) generation. Furthermore, BNIP3-mediated baicalein induced ROS production and cell apoptosis. We propose that BNIP3 translocation to mitochondria with simultaneous decrease of Bcl2 expression represents a mechanism of cell apoptosis in baicalein-treated cells.

Materials and methods

Chemicals and reagents

Baicalein (Catalog No. 94121), Dapi (Catalog No. D9542), *N*-acetyl-cysteine (NAC; Catalog No. A7250), glutathione (GSH; Catalog No. G6013), superoxide dismutase (SOD; Catalog No. S5639), and dimethyl sulfoxide (DMSO; Catalog No. D8418) were obtained from Sigma. Dulbecco's modified Eagle's medium (DMEM; Catalog No. 11965-092), fetal bovine serum (FBS; Catalog No. 26140-079), L-glutamine (Catalog No. 25030-081), penicillin–streptomycin (Catalog No. 10378-016), and trypsin–EDTA (Catalog No. 25300-062) were obtained from Gibco (Grand Island, NY, USA). Anti-Bcl-2, anti-Bax, anti-Cyto C, anti-BNIP3, and anti-actin were purchased from Cell Signaling Technology (Beverly, MA, USA). All secondary antibodies were purchased from Jackson ImmunoResearch (West Grove, PA, USA). All other reagents were purchased from Sigma (USA).

Cell culture

The MG-63 and hFOB 1.19 cell lines were obtained from American Type Culture Collection (ATCC, Rockville, MD, USA). Cells were plated in 10-cm^2 tissue culture dishes and grown at 37 °C under humidified 5 % CO_2/95 % air at one atmosphere in DMEM medium supplemented with 10 % FBS, 1 % penicillin–streptomycin (100 U/mL of penicillin G and 100 mg/mL of streptomycin), and 1 % glutamine. hFOB1.19 cells were plated into 10-cm^2 tissue culture dishes and allowed to grow until 80 % confluence.

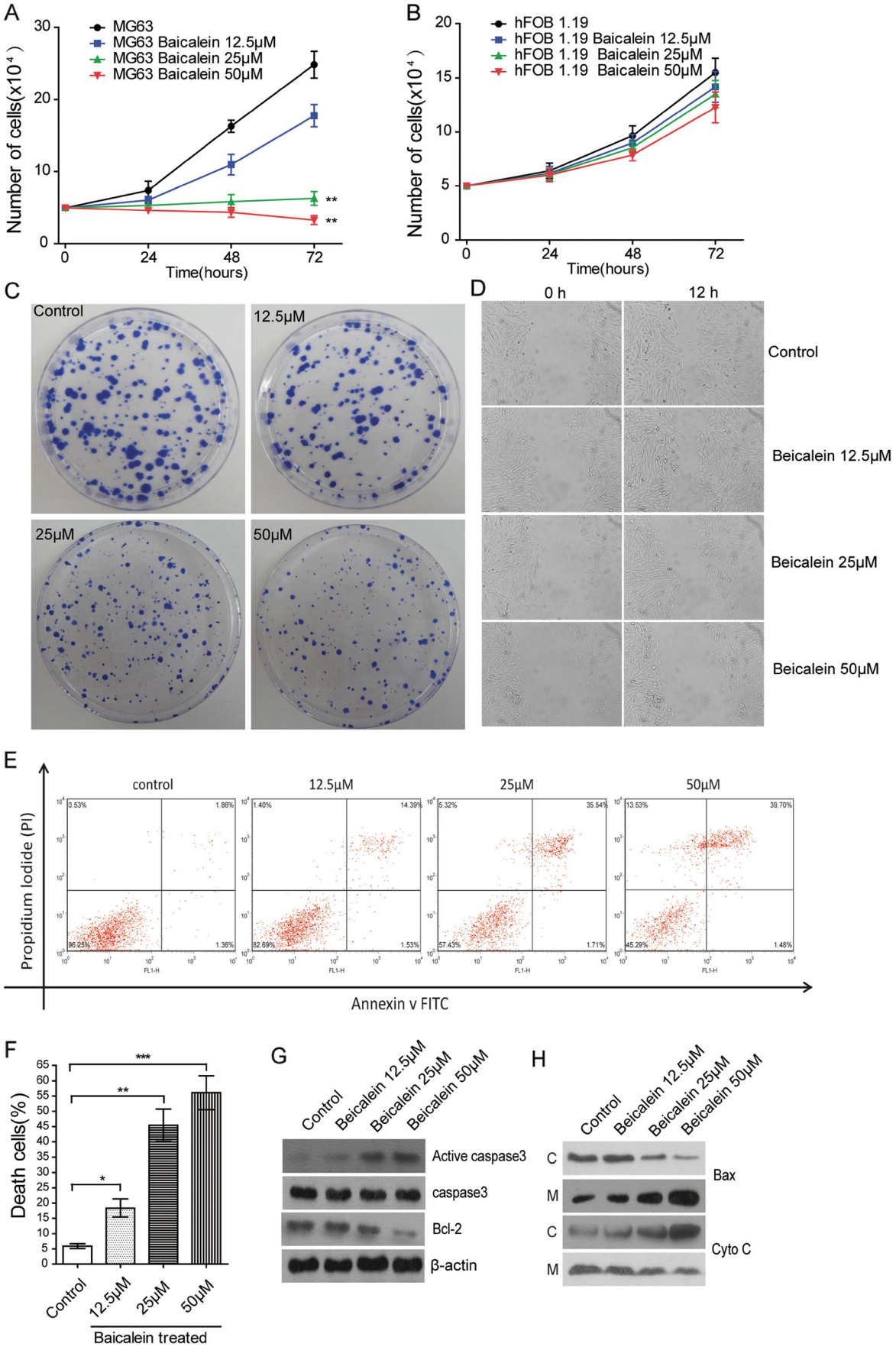
Cell viability assay

Cell viability was assessed by 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. MG-63 cells plated in 96-well plates were treated with baicalein at different concentrations (12.5, 25, and 50 μM) for 48 h; the cell number of each well is 5×10^4. Then, cells were washed twice with phosphate-buffered saline (PBS). Subsequently, 20 μL of 0.5 mg/mL MTT solution was added to each well. The plates were incubated at 37 °C for another 4 h. The medium was carefully aspirated. A 50 μL aliquot of DMSO was added, and the absorbance at 540 nm was measured for each well in an ELISA reader (Synergy HT; Bio-TEK, USA) [10].

Colony formation and cell migration

After treatment with various concentrations of baicalein (12.5, 25, and 50 μM), 300 cells were seeded in a 10-cm^2 plate and incubated for 10 days to allow colony formation. Cell colonies were visualized by 5 % crystal violet staining. Cell migration

Fig. 1 Baicalein inhibited in vitro tumor properties and cell apoptosis of human osteosarcoma cells. **a** The cytotoxicity of baicalein in MG-63 cells was determined using the MTT assay as described. Baicalein significantly inhibited MG-63 cells growth in a time- and dose-dependent manner. Each point showed the means±SD of three experiments. *$P<0.05$ compared with control. **b** MTT assay of hFOB1.19 cells with Baicalein treatment was determined. **c** Baicalein treatment inhibited colony formation in MG-63 cells. **d** Baicalein treatment reduced cell migration in MG-63 cells. MG-63 cells were seeded in a 10-cm^2 plate and incubated for 12 h to allow monolayer cell formation. Cells were wounded by a micropipette tip and then incubated in the presence of 1 % FBS culture medium for 12 h. Cell migration toward the wounded area was observed and photographed. **e** Analysis of baicalein-driven apoptosis was determined by flow cytometry. **f** Statistic graph indicated the percentage of apoptotic cells in various concentration treatment of baicalein. The results revealed that baicalein induced a dose-dependent apoptosis in MG-63 cells. Data is expressed as means±SD. ($n=3$). **$P<0.01$ compared with control cells. **g** Changes in the expression of apoptosis-related proteins in response to treatment with baicalein. MG-63 cells were treated with 12.5, 25, and 50 μg/mL of baicalein for 48 h. Cell extracts were subjected to Western blotting to determine the levels of uncleaved (inactive) and cleaved (active) caspase-3, apoptosis-related proteins BAX, Bcl-2, and Cyto C. β-Tubulin was used as loading control. Representative Western blots were shown


A
MG63
MG63 Baicalein 12.5μM
MG63 Baicalein 25μM
MG63 Baicalein 50μM
Number of cells(x10⁴)
Time(hours)
B
hFOB 1.19
hFOB 1.19 Baicalein 12.5μM
hFOB 1.19 Baicalein 25μM
hFOB 1.19 Baicalein 50μM
Number of cells(x10⁴)
Time(hours)
C
Control
12.5μM
25μM
50μM
D
0 h
12 h
Control
Beicalein 12.5μM
Beicalein 25μM
Beicalein 50μM
E
control
12.5μM
25μM
50μM
Propidium Iodide (PI)
Annexin v FITC
F
Death cells(%)
Control
12.5μM
25μM
50μM
Baicalein treated
G
Control
Beicalein 12.5μM
Beicalein 25μM
Beicalein 50μM
Active caspase3
caspase3
Bcl-2
β-actin
H
Control
Beicalein 12.5μM
Beicalein 25μM
Beicalein 50μM
C
M
Bax
Cyto C

was evaluated using in vitro wound healing experiment. MG-63 cells were seeded in a 10-cm^2 plate and incubated for 12 h to allow monolayer cell formation. Cells were wounded by a micropipette tip and then incubated in the presence of 1 % FBS culture medium with various concentrations of baicalein (12.5, 25, and 50 μM) for 12 h. Cell migration toward the wounded area was observed and photographed.

Analysis of apoptosis by flow cytometry

Following maintenance in culture by different treatments, the cells were harvested and incubated with the conjugate annexin V Alexa-Fluor-488 for 15 min in annexin V-binding buffer. Propidium iodide (PI; 0.5 μg/mL) was added 5 min before imaging. After treatment, annexin V and PI-positive cells were excited at 488 and 568 nm. The cells were then analyzed on a FACSCalibur flow cytometer (BD Biosciences).

ROS detection using DCFDA

MG-63 cells cultured in DMEM supplemented with 10 % FBS and 1 % P/S were treated with baicalein for 24 h. Cells were washed with PBS, trypsinized, and neutralized with FBS. After washing with PBS again, cells were incubated in PBS containing 10 μM 2′,7′-dichlorofluorescein diacetate (DCFDA) for 30 min. Then, the 2′,7′-dichlorofluorescin (DCF) intensity was detected by flow cytometry with maximum excitation and emission spectra of 495 and 529 nm, respectively.

LDH release detection

Cells were treated with baicalein at different concentrations (12.5, 25, and 50 μM) for 48 h. Cell culture medium was collected and centrifuged at 4000×*g* for 5 min in order to remove the detached dead cells and cell debris. Cells were lysed in a lysis buffer (0.1 % Triton X-100 in PBS) for 10 min at 4 °C. Lactate dehydrogenase (LDH) activity was determined by the CytoTox 96® Non-Radioactive Cytotoxicity Assay (Promega) according to the manufacture's instruction and quantified by measuring absorbance at 490 nm.

Western blotting assay and mitochondria/cytosol separation

Western blotting assay was done essentially as described previously [11]. The mitochondria/cytosol proteins were separated with mitochondria/cytosol fractionation kit (Abcam, ab65320) according to the protocol, and the enriched mitochondrial and cytosolic fractions were used to detect Cyto C production by Western blotting.

Gene silencing

To knockdown the expression of BNIP3, two human BNIP3-specific small interfering RNA (siRNA) and control siRNA (Guangzhou RiboBio Co., Ltd., Q000000665-1-B) were used. MG-63 cells were transiently transfected with the siRNAs using Lipofectamine 2000 (Invitrogen) for approximately 48 h, and cells were then harvested for Western blotting.

Statistical analysis

The statistical analysis was performed using GraphPadPrism (Version 5.0 GraphPadsoftware Inc., California, USA). All data were presented as the mean±SD. Statistical analysis was performed with Student's paired *t* test. Differences were considered statistically significant if the *P* value was less than 0.05.

Results

Effect of baicalein on tumor properties of MG-63 cells

The results showed that baicalein significantly inhibited MG-63 cell growth in a time- and dose-dependent manner (Fig. 1a). Baicalein treatment of MG-63 cells was conducted, and the percentage of viable cells was determined by an MTT reduction assay. When cultured with various concentrations of baicalein (12.5, 25, and 50 μM) for 24, 48, and 72 h, the cellular effects of baicalein were next examined. The treatment of baicalein resulted in a gradual and significant decrease in cell growth in MG-63 cell lines. To examine whether baicalein inhibits normal human osteoblast cell growth, hFOB1.19 cells were used as a control. MTT assay (Fig. 1b) showed that baicalein influenced little growth reduction of hFOB1.19 cells. Baicalein treatment also inhibited colony formation of MG-63 cells. As Fig. 1c showed, cell number over 50 in a cluster was defined as a signal colony. Consistently, cellular colony formation was significant suppression by baicalein treatment. Compared to the control, the numbers of cell colonies of baicalein treatment (12.5, 25, and 50 μM) were reduced to 92, 47, and 29 %, respectively. Colony size and cell density were also lower than that in the control. Baicalein apparently reduced cell growth and colony-forming ability in MG-63 cells.

Next, we examined whether baicalein treatment also influenced cell migration using in vitro wound healing. The wounded area was almost completely covered in control cells, whereas only a few cells moved into the area in 50 μM baicalein treatment (Fig. 1d). These results indicated that baicalein treatment reduced cell migration in MG-63 cells.

It is evident from our results that MG-63 cells treated with various concentrations of baicalein (25 and 50 μM) for 72 h

had an increased cell death, so whether baicalein-induced apoptosis in MG-63 cells was detected. Briefly, cells were stained with Annexin V-FITC (Ann V) and propidium iodide (PI); apoptosis is defined by Ann V +/ PI + staining as determined by FACS; the results revealed that baicalein induced a dose-dependent apoptosis in MG-63 cells (Fig. 1e, f). Compared to the control groups (5.9±0.79 %), the percentage of apoptotic MG-63 cells with baicalein treatment (12.5, 25, and 50 μM) arises to 18.4±3.0, 45.4±5.3, and 56.1±5.5 %, respectively. Next, we examined the effects of baicalein on the apoptotic protein expression, including caspase-3, Cyto C, Bax, and Bcl-2. Baicalein treatment markedly increased active caspase-3 expression while decreasing the expression of Bcl-2 after 48 h depending on the dose of baicalein (Fig. 1g). Upon induction of apoptosis, Bax underwent a conformational shift and mainly distributed in the mitochondrial membrane, which results in the release of cytochrome c from the mitochondria. Next, the mitochondria/cytosol proteins were separated. The enriched mitochondrial and cytosolic fractions were used to detect Bax and Cyto C production by Western blotting. Baicalein induced mitochondrial localization of the Bax and the release of cytochrome c from mitochondrial to cytosol (Fig. 1h).

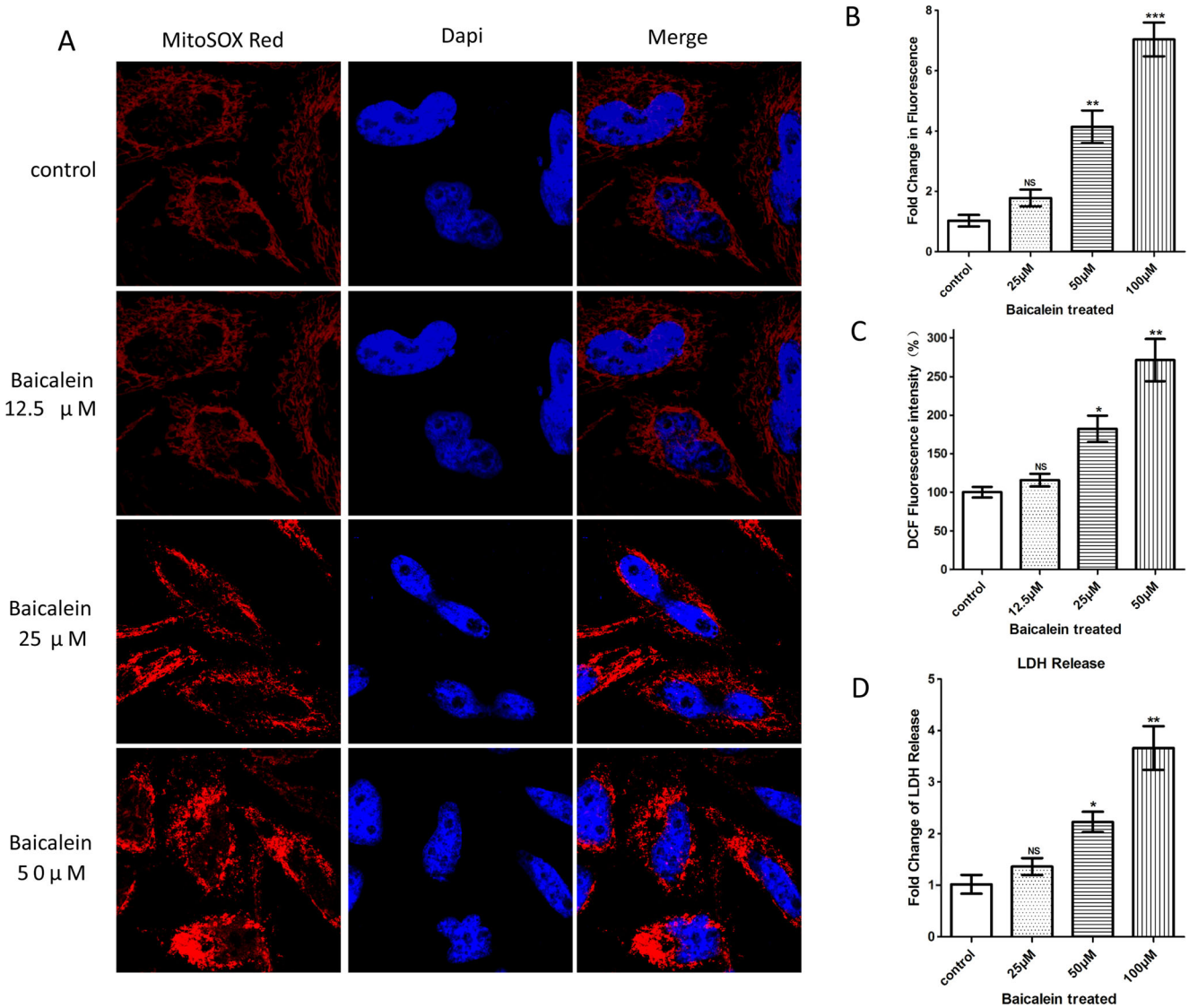


Fig. 2 Induction of ROS and LDH release by Baicalein in MG-63 cells. **a**, **b** Dose-dependent changes of MitoSOX fluorescence in MG-63 cells following baicalein treatment for 24 h. MitoSOX™ Red staining of baicalein-treated cells as detected using Leica confocal laser microscope (for MitoSOX™ Red: excitation at 420 nm and red photomultiplier channel). Dapi (blue fluorescence) was used for nuclear staining. *Scale bars*=25 μM. **c** Identification of ROS using DCFDA and flow cytometry. Data are expressed as means±SD. (n=3). **P<0.01 compared with control cells. **d** Dose-dependent LDH release in MG-63 cells treated with baicalein. MG-63 cells were incubated for 48 h and with different concentrations of baicalein. Release of LDH in the medium was measured at 490 nm. Results were presented as fold change compared to control cells

Taken together, these results showed that baicalein induces apoptosis in MG-63 cells.

Induction of ROS and LDH release by baicalein in MG-63 cells

It is reported that baicalein induced ROS production and LDH release in HL-60 cells [12], so the production of ROS in baicalein-treated MG-63 cells were tested with MitoSOX™ Red staining. MG-63 cells were treated with baicalein for 24 h and stained with MitoSOX™ Red. There was stronger perinuclear MitoSox staining (red fluorescence) in baicalein-treated MG-63 cells than that in controls (Fig. 2a, b). The cell permeant reagent was DCFDA, a fluorogenic dye that measures reactive oxygen species (ROS) activity within the cell. After diffusion into the cell, DCFDA is deacetylated by cellular esterases to a non-fluorescent compound, which is later oxidized by ROS into DCF. DCF is a highly fluorescent compound which can be detected by fluorescence spectroscopy with maximum excitation and emission spectra of 495 and 529 nm, respectively. Analysis of baicalein-driven ROS production was determined by estimating fluorescence intensity by flow cytometry (Fig. 2c); compared with the untreated control, baicalein treatment (25 and 50 μM) elicited substantial increases in mitochondrial ROS generation in MG-63 cells (1.7- and 2.7-folds, respectively, $P<0.05$).

Furthermore, lactate dehydrogenase (LDH) is a soluble cytosolic enzyme that is released into the culture medium following loss of membrane integrity resulting from either apoptosis or necrosis. LDH activity, therefore, can be used as an indicator of cell membrane integrity and serves as a general means to assess cytotoxicity resulting from chemical compounds or environmental toxic factors. We detected the content of LDH release in each group (12.5, 25, and 50 μM) using LDH assay (Fig. 2d). Compared with control group, baicalein treatment groups (25 and 50 μM) induced increases of LDH release (2.2- and 3.6-folds) which showed the cytotoxicity of baicalein. These results showed that baicalein induced ROS production and LDH release in MG-63 cells.

ROS scavengers attenuate baicalein-induced ROS production and apoptosis in MG-63 cells

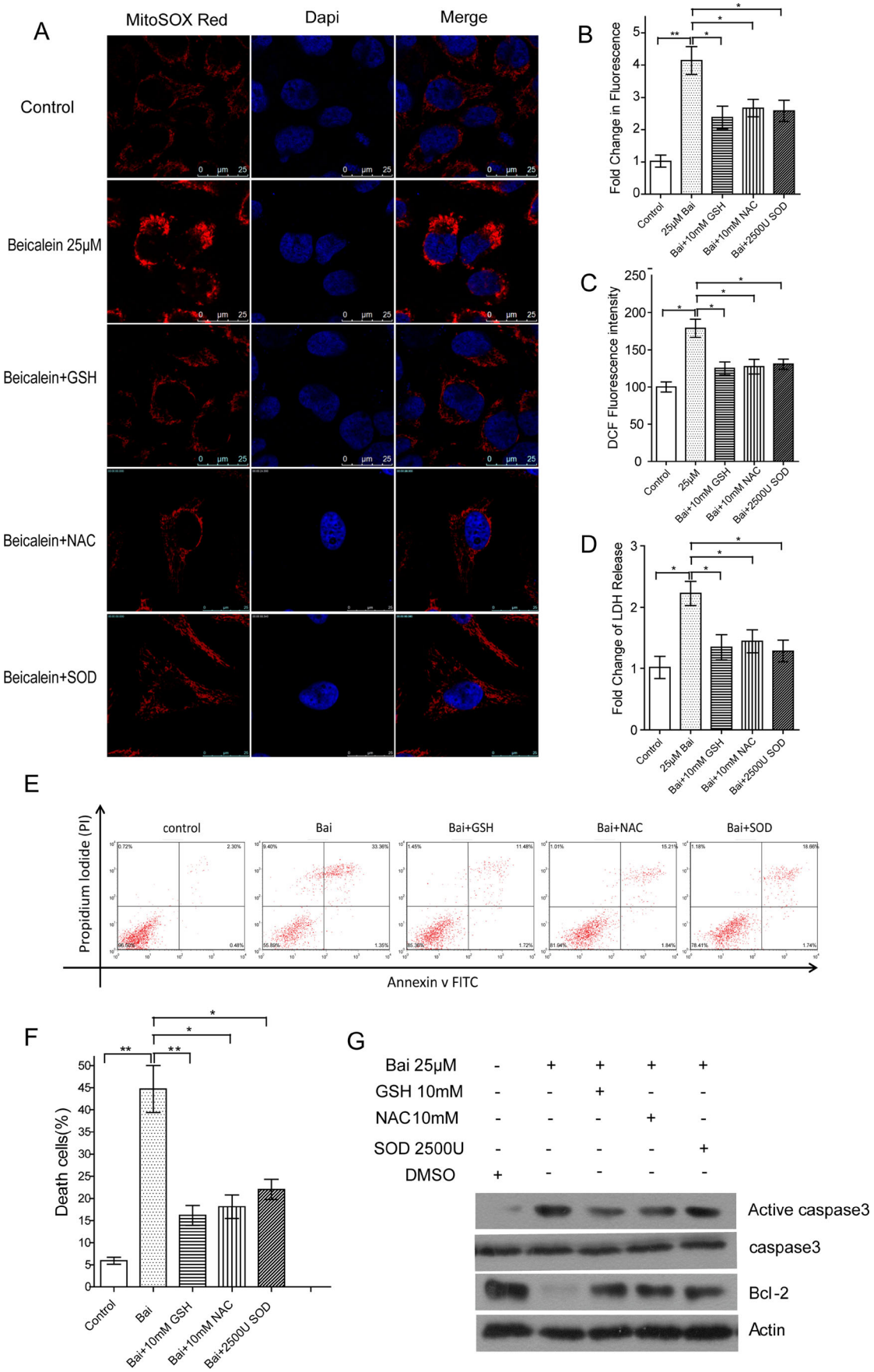
Previously, we found that baicalein induced apoptotic MG-63 cell death and increased the ROS production, so we wanted to know the relationship of baicalein-induced apoptosis and ROS production. Three ROS scavengers (GSH, NAC, and SOD) were supplemented in baicalein-treated MG-63 cells. Cell apoptosis and ROS production were detected by previous procedures. Fluorescent images showed that perinuclear MitoSox staining was weaker in ROS scavenger treatment (Fig. 3a, b). ROS scavenger significantly reduced ROS production induced by baicalein using DCFDA and flow cytometry (Fig. 3c). The content of LDH release induced by baicalein was also attenuated by GSH, NAC, or SOD treatment (Fig. 3d).

Fig. 3 ROS scavenger attenuated baicalein-induced ROS production and apoptosis in MG-63 cells. **a**, **b** Detect MitoSOX fluorescene intensity after treatment of baicalein with or without ROS scavenger. The MitoSOX fluorescence demonstrates the production of ROS decreased with ROS scavenger (GSH, NAC, and SOD). **c** Identification of ROS using DCFDA and flow cytometry after treatment of baicalein with or without ROS scavenger. Data are expressed as means±SD. ($n=3$). *$P<0.05$. **d** The content of LDH release induced by baicalein was attenuates by GSH, NAC, or SOD treatment. Results were presented as fold change compared to control cells. Data are expressed as means±SD. ($n=3$). *$P<0.05$. **e** Analysis of MG-63 cell apoptosis of baicalein alone or in the presence of ROS scavenger GSH, NAC, and SOD by flow cytometry. **f** The percentage of cell apoptosis were shown (baicalein, 21.8 %; baicalein+GSH, 13.1 %; baicalein+NAC, 12.3 %; baicalein+SOD, 15.3 %). Data are expressed as means±SD. ($n=3$). *$P<0.05$. These results showed that baicalein induces ROS-triggered apoptosis in human osteosarcoma cells. **g** ROS scavenger GSH, NAC, and SOD attenuated baicalein-mediated apoptosis in human osteosarcoma cell. The expression of apoptosis-related protein was detected in response to baicalein alone or in the presence of ROS scavenger GSH, NAC, and SOD

Next, we want to known whether ROS scavenger attenuated the toxicity of baicalein in MG-63 cells. The apoptosis cells with baicalein with or without ROS scavenger were detected using either annexin V and propidium iodide staining; ROS scavenger can attenuate the toxicity of baicalein in MG-63 cells, and the percentage of cell apoptosis is 5.9±0.8 % (control), 44.7±5.3 % (baicalein), 16.2±2.3 % (baicalein+GSH), 18.2±2.7 % (baicalein+NAC), and 22.0±2.3 % (baicalein+SOD), respectively (Fig. 3e, f). To further determine the involvement of apoptosis in baicalein-induced cytotoxicity with ROS scavenger (GSH, NAC, and SOD) treatment, various related apoptotic proteins were examined. Compared to baicalein treatment alone, baicalein combined with ROS scavenger treatment significantly decreased the expression of active caspase-3, while increased the expression of Bcl-2 (Fig. 3g). These results showed that baicalein induced ROS production and ROS-triggered apoptosis in MG-63 cells.

Baicalein-induced ROS production and cell apoptosis involves BNIP3

Next, we note that baicalein also induces the mitochondrial fragmentation which precedes the cell apoptosis, and it is concluded that baicalein-induced mitochondrial change is necessary for subsequent cell apoptosis. Mitochondria are dynamic organelles which undergo continual fusion and fission to maintain their normal morphology and functions; it is also reported that mitochondrial oxidative stress-induced ROS production can induce mitochondrial fragmentation and cell death [13–15]. Therefore, mitochondrial fission and fusion-related gene expressions were tested by real-time PCR, including Parkin, PINK, Mfn1, Mfn2, Drp1, Mul1, March5, and


A
MitoSOX Red
Dapi
Merge
Control
Beicalein 25μM
Beicalein+GSH
Beicalein+NAC
Beicalein+SOD
B
Fold Change in Fluorescence
Control
25μM Bai
Bai+10mM GSH
Bai+10mM NAC
Bai+2500U SOD
C
DCF Fluorescence intensity
D
Fold Change of LDH Release
E
control
Bai
Bai+GSH
Bai+NAC
Bai+SOD
Propidium Iodide (PI)
Annexin v FITC
F
Death cells(%)
G
Bai 25μM
GSH 10mM
NAC10mM
SOD 2500U
DMSO
Active caspase3
caspase3
Bcl-2
Actin

BNIP3. As Fig. 4a showed, baicalein induced some mitochondrial fission-related gene expression, like Drp1, Mul1, and BNIP3. Among the three proteins, BNIP3 belongs to the pro-apoptotic Bcl2 family member which has a single BH3 domain and a C-terminal transmembrane (TM) domain. Although it belongs to the Bcl2 family, its pro-cell death activity is distinct from those of other family members. BNIP3 directly interacts with Bcl2 and Bcl-XL, suggesting that BNIP3 could activate Bax through an indirect mechanism. Moreover, it is also reported that the increase of ROS production can induce BNIP3 expression [16]. Therefore, we conclude that baicalein induces ROS production and BNIP3 expression with the subsequent activation of Bax, which in turn causes cell apoptosis. First, we used Western blotting to measure levels of BNIP3 protein after baicalein exposure. The results (Fig. 4b) show that baicalein induced BNIP3 in a dose-dependent manner. Next, we tested whether ROS scavenger treatment can reduce BNIP3 expression in baicalein-treated cells. GSH, NAC, and SOD treatment attenuated BNIP3 expression in baicalein-treated cells, which showed that the induced expression of BNIP3 might be caused by ROS production (Fig. 4c). At last, two siRNAs were used to knockdown BNIP3, and the expression of apoptotic-related protein with baicalein treatment was tested. As the results showed, compared with control cells, knockdown of BNIP3 significantly reduced cleavaged caspase-3 and the decrease of Bcl-2 (Fig. 4d). FACS showed that knockdown of BNIP3 significantly reduced apoptosis cells induced by baicalein treatment (Fig. 4e). The percentage of cell apoptosis is 5.6±1.3 % (control siRNA), 46.5±4.7 % (baicalein+control siRNA), 20.0±2.1 % (baicalein+BNIP3 siRNA-1), and 20.01±2.054 % (baicalein+BNIP3 siRNA-2), respectively. In conclusion, these results demonstrated that baicalein-induced ROS production and cell apoptosis involves BNIP3.

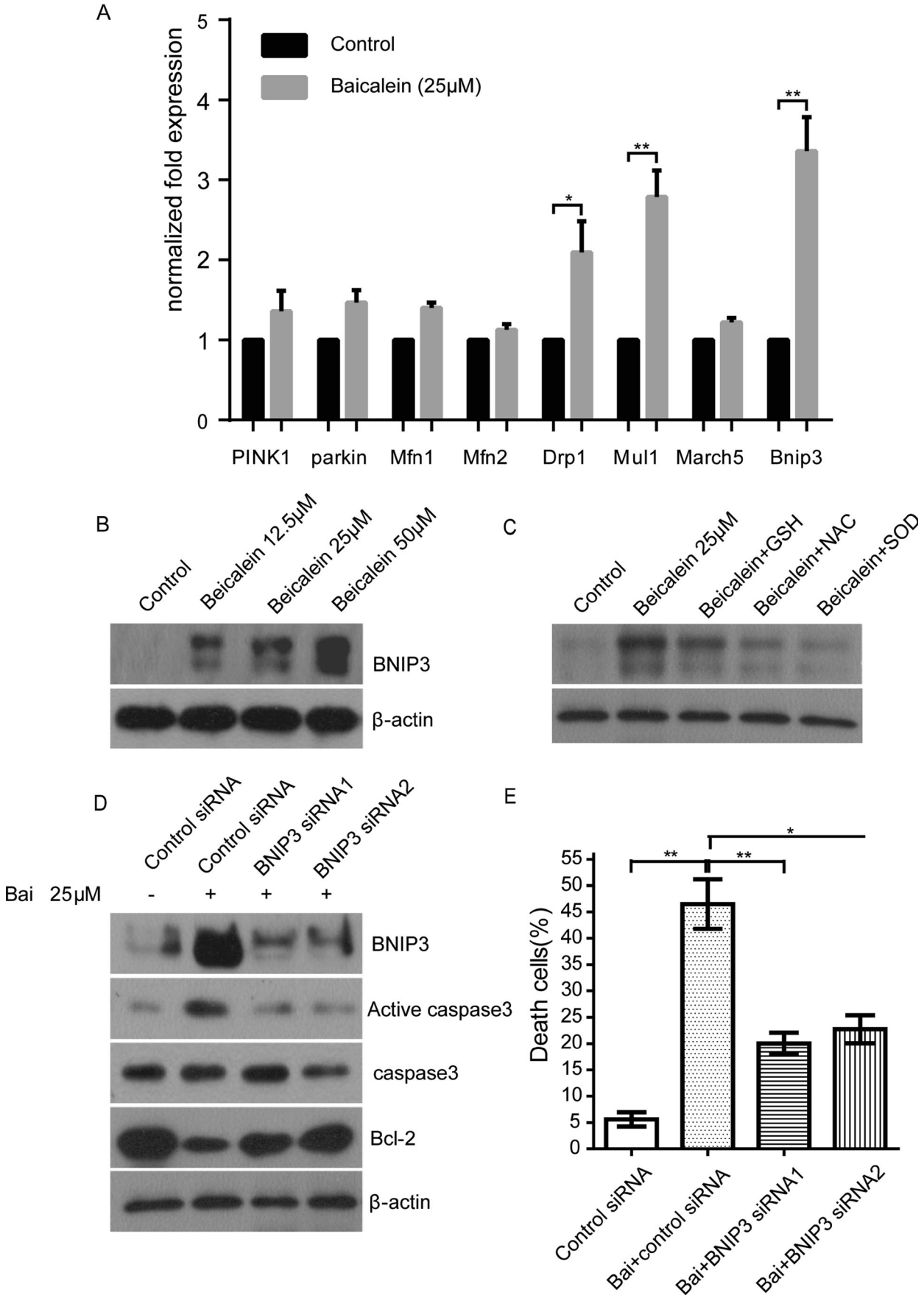
Fig. 4 Baicalein-induced ROS production and cell apoptosis involves BNIP3. **a** MG-63 cells were treated with or without baicalein for 12 h and the expression of Parkin, PINK, Mfn1, Mfn2, Drp1, Mul1, March5, and BNIP3 were determined by real-time PCR. **b** Dose-dependent increases of BNIP3 in MG-63 cells following baicalein treatment for 24 h. Cells were treated with various concentrations of baicalein for 24 h, and BNIP3 protein level were analysed by Western blotting. **c** Expression of BNIP3 protein was detected in response to baicalein alone or in the presence of ROS scavenger GSH, NAC, and SOD. **d** BNIP3 was knockdown with two siRNAs, and the expression of apoptotic related protein with baicalein treatment were tested. **e** The apoptosis cells in control or BNIP3 knockdown groups induced by baicalein treatment were tested by FACS

Discussion

Osteosarcoma is one of the most common malignant bone tumors. Currently, the main treatment for the disease is radical surgery, assisted with chemotherapy and other comprehensive therapy. However, drug resistance of osteosarcoma has been a major obstacle of chemotherapy. Therefore, the exploring for safe and effective chemopreventers is booming at present. Tumor cell is characterized by an uncontrollable increase in cell proliferation and/or a reduction in cell apoptosis. Disrupted apoptosis may result in chemotherapy resistant of tumor cell, which hinders the treatment of malignant tumors, including osteosarcoma [17]. Baicalein has been found to be active against a wide variety of cancer cells by inducing apoptosis [18–20]. The mechanism of action of baicalein has not been extensively studied. It is assumed to be of increasing importance due to its involvement in the suppression of many tumor-related processes including oxidative stress, apoptosis, proliferation, and metastasis. Baicalein has multiple targets that interacts with a variety of proteins and meanwhile modifies the target protein activities and expression [21]. Thus, it is a good candidate for either cancer preventive agents or therapeutic agents. The molecular mechanism underlying baicalein-induced apoptosis in human osteosarcoma cell line MG-63 is currently elucidated. Herein, our results indicate that baicalein induced inhibition of cell proliferation and apoptosis in MG-63 cells. Baicalein induces the activation of caspase-3, modulation of Bax/Bcl2, and release of cytochrome c. It is reported that baicalein induced ROS production and LDH release in HL-60 cells [12], which are replicable in our study with MG-63 cells. Furthermore, inhibition of ROS generation by the antioxidant GSH, NAC, or SOD effectively inhibited apoptosis induced by baicalein. These results show that baicalein induces ROS-triggered apoptosis in human osteosarcoma cells.

ROS is mostly produced by NAD(P)H oxidases family. Other possible sources include mitochondrial electron transport enzymes, lipoxygenase, and nitric oxide synthase [22]. ROS induces free radicals such as superoxide (O_2), hydroxy radical (OH−), and derivatives of oxygen like H_2O_2. Free radical generation has been shown to induce cellular apoptosis by damaging cellular components, including DNA, proteins, and lipid membranes. Also, they have been suggested to regulate the process involved in the initiation of mitochondrial apoptotic signaling [23]. Mitochondrial apoptosis pathways are critically regulated by anti-apoptotic protein Bcl-2 and pro-apoptotic multidomain (Bax). The Bcl-2 family regulates apoptosis by controlling mitochondrial permeability, while BAX promotes apoptosis by antagonizing the Bcl-2 protein and induce the release of cytochrome c [24]. However, the exact mechanism of ROS production in apoptosis pathways is not clearly understood; our study shed new light on ROS-triggered cell death. We provide the evidence that ROS may contribute to apoptosis through the regulation of BNIP3; it is possible that BNIP3 mediates baicalein-induced apoptosis through the regulation of Bcl2 and the activation of Bax [25].

Our results also show that Baicalein induces the mitochondrial fragmentation which precedes the cell apoptosis, which


A
normalized fold expression
Control
Baicalein (25μM)
PINK1
parkin
Mfn1
Mfn2
Drp1
Mul1
March5
Bnip3
B
Control
Beicalein 12.5μM
Beicalein 25μM
Beicalein 50μM
BNIP3
β-actin
C
Control
Beicalein 25μM
Beicalein+GSH
Beicalein+NAC
Beicalein+SOD
D
Control siRNA
Control siRNA
BNIP3 siRNA1
BNIP3 siRNA2
Bai 25μM
BNIP3
Active caspase3
caspase3
Bcl-2
β-actin
E
Death cells(%)
Control siRNA
Bai+control siRNA
Bai+BNIP3 siRNA1
Bai+BNIP3 siRNA2

indicates that cell apoptosis is the result of mitochondrial dysfunction. This morphological alteration is accompanied by the increase of mitochondrial protein BNIP3 as well as Mul1 and Drp1, which is consistent with the previous studies [15, 26]. Mitochondrial fragmentation and dysfunction are always related to cell apoptosis, but the mechanism underlying this event remains vague. Based on the current research results, we conclude that BNIP3 can interact with mitochondrial dynamic-related proteins, which causes mitochondrial fragmentation and damage and then BNIP3 mediates cell apoptosis through the regulation of Bcl2, Bax, and cytochrome c. However, the relationship among baicalein, BNIP3, mitochondrial dynamic-related proteins, and Bcl-2 needs further investigation.

In summary, our results demonstrated that baicalein induces human osteosarcoma cell line MG-63 apoptosis via ROS-induced BNIP3 expression. The current study may facilitate a clearer understanding of the mechanism of baicalein-induced cell apoptosis and provide the theoretical basis for the antitumor effects of baicalein.

Conflicts of interest None

Authors' contributions Fangfan Ye drafted the article. Honghan Wang, Lusi Zhang, Yongyi Zou, and HaiLong Han were in charge in acquisition of data, analysis and interpretation of data, and revision of the article. Jia Huang took control in the conception and design.

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