Calycosin induces apoptosis in osteosarcoma cell line via ERβ-mediated PI3K/Akt signaling pathways

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Abstract. Previous studies have shown that calycosin, a natural phytoestrogen which is structurally similar to estrogen, inhibits proliferation and induces apoptosis in estrogen-dependent cancer types via the estrogen receptor (ER)β-induced inhibition of PI3K/Akt. Therefore, the aims of the present study were to investigate the effects of calycosin on human osteosarcoma (OS), and to examine the molecular mechanisms associated with ERβ. Human OS MG-63 cells were treated with various concentrations of calycosin, and MTT and flow cytometry assays were used to assess the effects of calycosin on cellular proliferation and apoptosis. In addition, protein expression levels of ERβ, phosphorylated (p)-PI3K, p-Akt, cleaved poly (ADP-ribose) polymerase 1 (PARP) and cleaved caspase-3 were evaluated by western blot analysis. The present results suggested that calycosin inhibited proliferation and induced apoptosis in MG-63 cells. Furthermore, increased ERβ expression was detected in OS MG-63 cells treated with calycosin, and an ERβ inhibitor (PHTPP) reversed calycosin-induced cytotoxicity and apoptosis. Moreover, phosphorylation levels of PI3K and Akt were significantly downregulated after calycosin treatment, whereas PHTPP reversed their phosphorylation. ERβ-mediated PI3K/Akt downstream signaling pathways were found to influence the activity of poly (ADP-ribose) polymerase 1 and caspase-3. Thus, the present results indicated that calycosin inhibited proliferation and induced apoptosis in OS MG-63 cells, and that these effects were mediated by ERβ-dependent inhibition of the PI3K/Akt pathways.

Introduction

Osteosarcoma (OS) is the most common primary malignant bone tumor in pediatrics and young adolescents (1). Furthermore, the standard care for patients with OS, which includes surgical resection in combination with systemic chemotherapy, has a 5-year overall survival rate of 70% (2). Treatment of OS often fails due to the development of chemotherapy resistance and metastasis (3). It has been previously reported that sex hormones are involved in the occurrence and development of human OS, thus suggesting that novel endocrine therapy may be development for OS using clinically available estrogen inhibitors (4).

Plant-derived phytoestrogens and mammalian estrogens have similar structures and functions, and can cause anti-estrogen or estrogen-like effects (5). For this reason, plant-derived phytoestrogens are a current topic in research (6). Phytoestrogenic compounds are widely found in nature and can be divided into four categories: i) Isoflavones; ii), stilbene; iii), coumarins; and iv) lignans (7). Calycosin, a bioactive phytoestrogen isoflavone that is extracted from *Trifolium pratense* (red clover), has been shown to inhibit proliferation and induce apoptosis in cancer types (8,9). In addition, calycosin has been shown to induce apoptosis in human estrogen receptor (ER)-positive OS cells, but has no effect on ER-negative OS cells, suggesting that the inhibition of calycosin on ER-positive OS cells may be achieved by increasing ER expression (10).

ER belongs to the steroid hormone receptor family and consists of two subtypes, ERα and ERβ (11). A high ERα:ERβ ratio leads to increased cell proliferation, whereas a higher level of ERβ than ERα leads to decreased proliferation (12,13). Since the expression of ERβ has been shown to decrease during tumor progression, ERβ has been considered a potential tumor suppressor and therapeutic target in various types of cancer, including breast cancer and renal cell carcinoma (14,15). Moreover, ERβ agonists may be novel potential therapeutic candidates for OS endocrine therapy (16). Therefore, it was hypothesized that upregulation of ERβ may inhibit tumor development and progression.

The PI3K/Akt signaling pathway, an important regulator of cellular functions, has been found to be frequently hyperactivated in OS and contributes to tumorigenesis, proliferation, invasion, cell cycle progression and inhibition of apoptosis (17). Thus, suppressing this signaling pathway could inhibit disease initiation and development (18). Moreover, ERβ has an anti-tumor effect on OS cells by regulating the PI3K/Akt signaling pathway (16). In addition, ERβ can

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mediate inhibition of proliferation and activation of apoptosis in various types of cancer, including breast cancer and colorectal cancer following treatment with calycosin, which is shown to regulate the PI3K/Akt pathway (8,9). Therefore, the present study investigated whether calycosin had anti-tumor effects on OS MG-63 cells by mediating the ERβ-dependent PI3K/Akt signaling pathway.

Collectively, along with the anti-proliferative effect of calycosin on OS MG-63 cells, the present study examined the role of the ERβ-mediated PI3K/Akt pathway in OS MG-63 cells to help facilitate the current understanding of the molecular mechanism underlying calycosin functions.

Materials and methods

Calycosin. Calycosin (purity 98%; Tianjin JAHE Science and Technology Co. Ltd.) solution was diluted into a 250 μg/ml stock solution with DMSO (Sigma-Aldrich; Merck KGaA).

Cell culture. Human OS cells (MG-63) and human fetal osteoblast cells (hFOB1.19; Shanghai Institute of Biochemistry and Cell Biology) were incubated in DMEM (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.) and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin) in a humidified incubator containing 5% CO_2 at 37˚C. The medium was changed every 48 h.

MTT assay. Cell viability was determined by MTT (Sigma-Aldrich; Merck KGaA) assay. Cells were harvested using 0.25% trypsin and seeded into 96-well plates at a density of $3x10^4$ cells/well at 37˚C for 24 h. Then, cells were treated with calycosin (at concentrations of 0, 25, 50 or 100 μM) at 37˚C for 24, 48 and 72 h, or with 100 μM calycosin in the presence or absence of the ERβ inhibitor PHTPP (50 μM; MedChemExpress) at 37˚C for 48 h. In total, 20 μl MTT (5 mg/ml) was added to cells for 4 h at 37˚C. Following incubation, DMSO (100 μl) was added to dissolve the formazan crystals and shaken at room temperature for 10 min. Subsequently, cell viability was assessed by measuring the absorbance at 570 nm using a microplate reader (Thermo Fisher Scientific, Inc.). Proliferation rate (%) was calculated as follows: Optical density (OD) treatment group/OD control x100%.

Flow cytometry assay. Flow cytometry was used to study the effects of calycosin treatment on apoptosis of the OS cells using the FITC Annexin V Apoptosis Detection Kit I (BD Biosciences), according to the manufacturer's protocol. MG-63 cells were treated with calycosin (0, 25, 50 or 100 μM), or calycosin (100 μM) in the presence or absence of PHTPP at 37˚C for 48 h. Cells were harvested and washed with PBS. Apoptotic cells were identified by double staining with 5 μl FITC-conjugated Annexin V and 5 μl PI. Data were obtained and analyzed using a FACS-Canto flow cytometer (Beckman Coulter, Inc.) with Cell Quest software (version 5.1; BD Biosciences). Cells stained positive for Annexin V-FITC and negative for PI were considered early apoptotic, and cells stained positive for Annexin V-FITC and positive for PI were considered in late apoptosis.

Western blot analysis. After being treated with calycosin (0, 25, 50 and 100 μM), or calycosin (100 μM) in the presence or absence of PHTPP for 48 h, MG-63 cells were harvested with ice-cold PBS and lysed on ice in lysis buffer (Beyotime Institute of Biotechnology) for 30 min. The lysates were centrifuged at 4˚C at 700 x g for 10 min and collected. Then, the total protein was measured with a bicinchoninic protein assay kit (Tiangen Biotech Co., Ltd.). The samples (20 μg) were separated via 12% SDS-PAGE and then transferred to PVDF membranes (EMD Millipore). The membranes were blocked with 5% non-fat dried milk in TBST (0.2% Tween-20) buffer for 1 h at room temperature, and then incubated with the following primary antibodies overnight at 4˚C: ERβ (cat. no. sc8974; 1:500), PI3K (cat. no. 4255; 1:1,000), phosphorylated (p)-PI3K (cat. no. 17366; 1:1,000), Akt (cat. no. 9271; 1:1,000), p-Akt (cat. no. 9611; 1:2,000), cleaved caspase-3 (cat. no. 9661; 1:1,000), cleaved poly (ADP-ribose) polymerase 1 (cleaved PARP-1; cat. no. 9185; 1:1,000) and β-actin (cat. no. 7077; 1:1,000). After three washes with TBST, the membranes were subsequently incubated with a horseradish peroxidase-conjugated secondary antibody (cat. no. 7074; 1:5,000) for 1 h at room temperature. The protein signal was detected via electrochemiluminescence with an ECL-Plus kit (Beyotime Institute of Biotechnology) and analyzed using ImageJ software (National Institutes of Health). Anti-ERβ was purchased from Santa Cruz Biotechnology, Inc. The other antibodies were purchased from Cell Signaling Technology, Inc.

Statistical analysis. Data were obtained from ≥3 independent experiments and are presented as the mean ± SD relative to the control value. Statistical analysis for multiple comparisons was performed using one-way ANOVA followed by Tukey's post hoc test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Inhibition of proliferation and induction of apoptosis in OS MG-63 cells by calycosin. To evaluate the anti-proliferative effect of calycosin, MG-63 and hFOB1.19 cells were incubated with different concentrations of calycosin for 24, 48 and 72 h. It was found that calycosin caused a time- and concentration-dependent inhibition on the proliferation of MG-63 cells (Fig. 1A). However, the inhibitory effect of calycosin on the proliferation of hFOB1.19 cells was not significant (Fig. 1B), thus suggesting that the effect of calycosin was negligible on healthy osteoblasts.

Consistent with the aforementioned results, flow cytometry assay results demonstrated that calycosin induced MG-63 cell apoptosis in a concentration-dependent manner, and induced a significant increase in the percentage of early and late apoptotic cells (Fig. 1C and D).

Upregulation of ERβ in OS MG-63 cells by calycosin. ERβ is a traditional estrogen receptor, whose activity is inversely related to the occurrence and development of tumors (19). Therefore, the expression of ERβ was examined in MG-63 cells following treatment with calycosin. It was demonstrated that ERβ protein expression was increased significantly in a dose-dependent manner after calycosin treatment (Fig. 2A and B). Moreover,

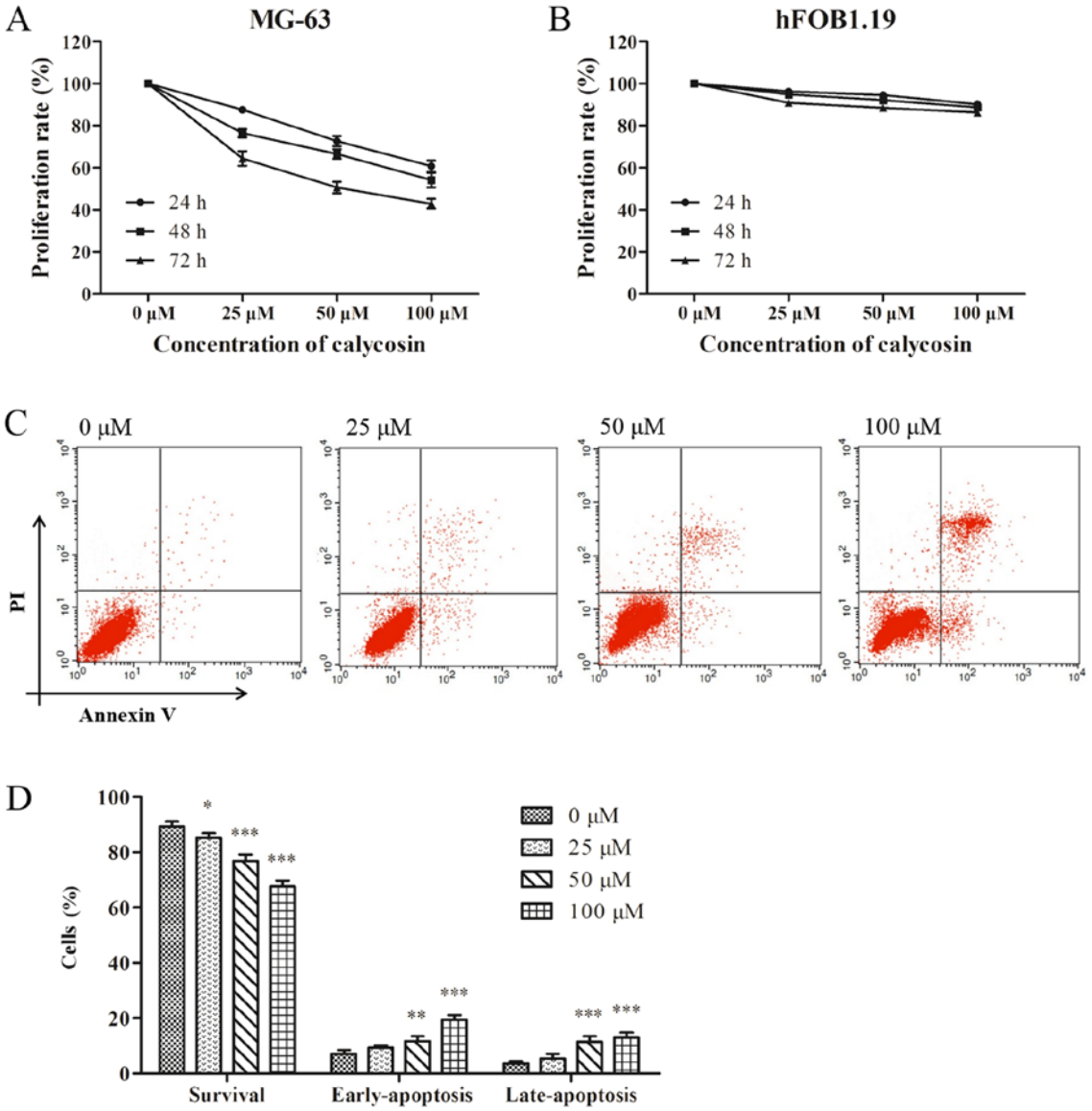

Figure 1. Inhibition of proliferation and apoptosis of osteosarcoma MG-63 cells by calycosin. Cells were incubated with varying concentrations of calycosin for 24, 48 and 72 h. Cell viability of (A) MG-63 cells and (B) hFOB1.19 cells were detected by MTT. Apoptosis of MG-63 cells was determined by (C) flow cytometry and (D) the results were quantified. These independent experiments were performed three times. *P<0.05, **P<0.01, ***P<0.001 vs. 0 μM calycosin. PI, propidium iodide.

MG-63 cells were treated with 100 μM calycosin in the presence or absence of PHTPP, and then the expression of ERβ was assessed. It was found that calycosin in combination with PHTPP significantly decreased ERβ expression (P<0.01; Fig. 2C and D). Therefore, the present results suggested that the inhibition of calycosin on MG-63 cells occurred via the regulation of ERβ.

ERβ-mediated inhibition of proliferation and activity of apoptosis in MG-63 cells by calycosin. Previous studies have shown that ERβ is a key negative mediator of cell proliferation, and positive regulator of apoptosis in cancer (8,9,16). Therefore, to study the effect of ERβ on proliferation and apoptosis, MG-63 cells were pretreated with 100 μM calycosin in the presence or absence of PHTPP for 48 h. It was identified that calycosin + PHTPP reversed the calycosin-mediated inhibition of cell proliferation (P<0.001; Fig. 3A). In addition, calycosin + PHTPP abolished calycosin-induced apoptosis (P<0.001, Fig. 3B and C). Consistent with the findings of previous studies, calycosin-induced cytotoxicity and apoptosis were found to be mediated by the ERβ-signaling pathway.

Regulation of ERβ-mediated PI3K/Akt signaling in OS MG-63 cells by calycosin. It has been previously shown that inhibiting the PI3K/Akt signaling pathway may be a novel treatment strategy for OS (17). Therefore, the PI3K/Akt signaling pathway was examined in the present study. After a 48 h exposure to calycosin (0, 25, 50 or 100 μM) in MG-63 cells, PI3K phosphorylation and Akt expression were found to be downregulated in a concentration-dependent manner (Fig. 4A and B). Thus, the present results indicated that calycosin inactivated the PI3K/Akt signaling pathway in MG-63 cells. In addition, to assess the relationship between

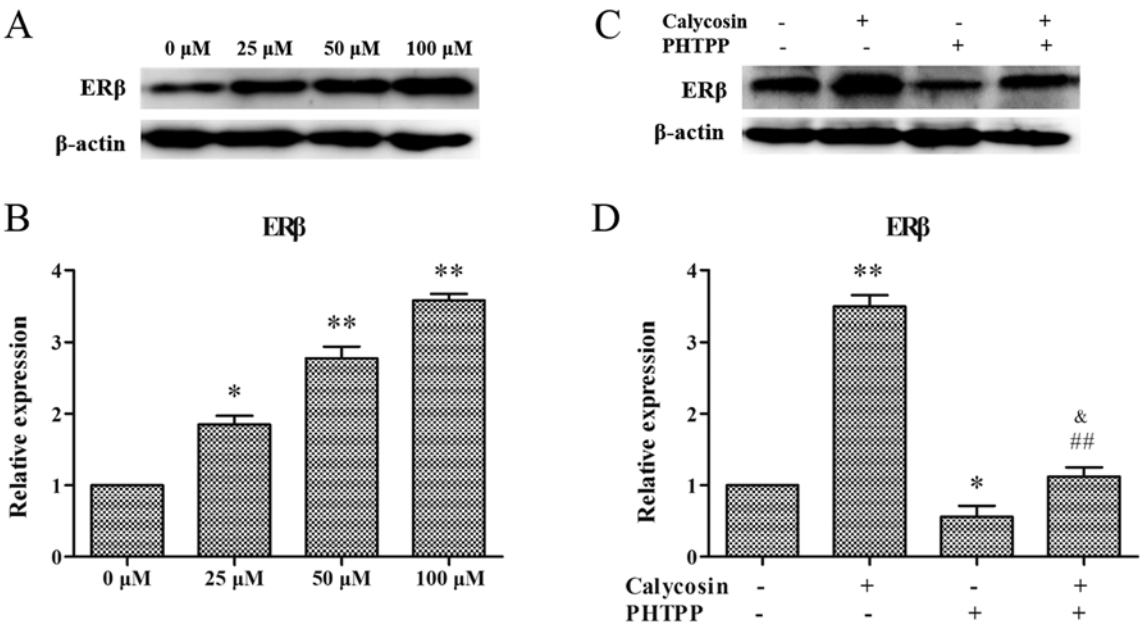

Figure 2. Calycosin upregulates ERβ expression in osteosarcoma MG-63 cells. (A) Western blotting results of (B) protein expression of ERβ after treatment with different concentrations of calycosin. (C) Western blotting results of (D) protein expression of ERβ after 100 μM calycosin treatment in the presence or absence of PHTPP for 48 h. These independent experiments were performed three times. *P<0.05, **P<0.01 vs. 0 μM calycosin. $^{\#\#}$P<0.01 vs. calycosin alone. $^{\&}$P<0.05 vs. PHTPP alone. ERβ, estrogen receptor β.

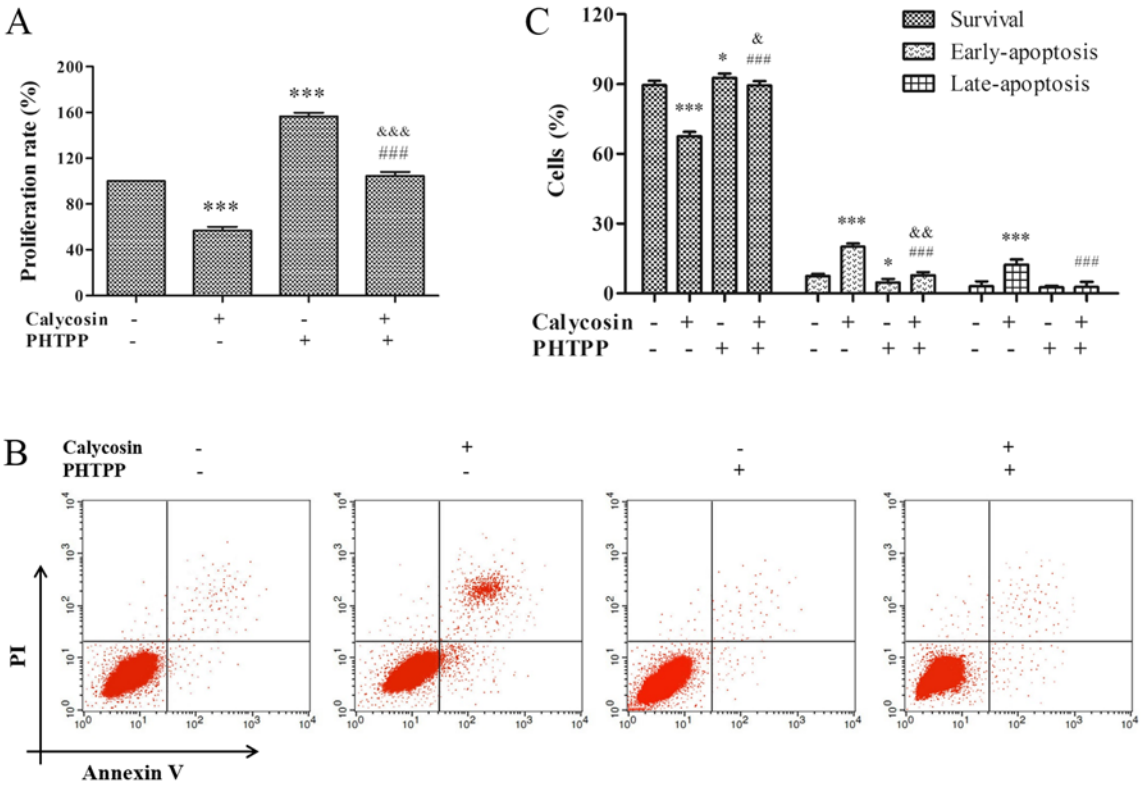

Figure 3. Estrogen receptor β-mediated inhibition of proliferation and apoptosis osteosarcoma MG-63 cells by calycosin. Cells were incubated with 100 μM calycosin in the presence or absence of PHTPP for 48 h. (A) Cell viability was detected by MTT. (B) Apoptosis was determined by flow cytometry and (C) the results were quantified. These independent experiments were performed three times. *P<0.05, ***P<0.001 vs. 0 μM calycosin. $^{\#\#\#}$P<0.001 vs. calycosin. $^{\&}$P<0.05, $^{\&\&}$P<0.01, $^{\&\&\&}$P<0.001 vs. PHTPP. PI, propidium iodide.

ERβ and the PI3K/Akt signaling pathway, MG-63 cells were treated with calycosin in the presence or absence of PHTPP. It was demonstrated that the combination of calycosin + PHTPP reversed the decrease in the phosphorylation levels of PI3K and Akt (P<0.01; Fig. 4C and D), while no changes were observed in the expression levels of total Akt and total PI3K. Therefore,

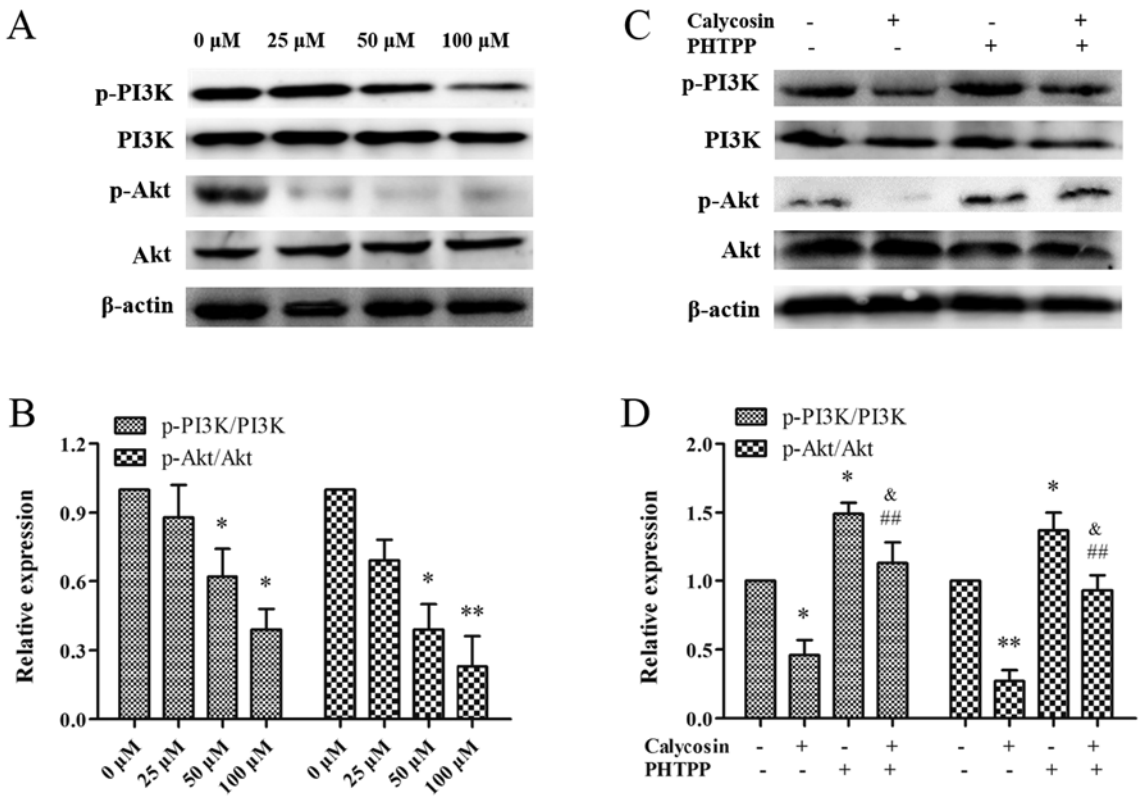

Figure 4. Regulation of estrogen receptor β-mediated PI3K/Akt signaling in osteosarcoma MG-63 cells by calycosin. (A) Western blotting results of (B) protein expression levels of p-PI3K/PIK3 and p-Akt/Akt after treatment with different concentrations of calycosin. (C) Western blotting results of (D) protein expression levels of p-PI3K/PIK3 and p-Akt/Akt after treatment with 100 μM calycosin, in the presence or absence of PHTPP for 48 h. These independent experiments were performed three times. *P<0.05, **P<0.01 vs. 0 μM calycosin. $^{\#\#}$P<0.01 vs. calycosin. $^{\&}$P<0.05 vs. PHTPP. p, phosphorylated.

the present results suggested that the ERβ-mediated decrease in PI3K/Akt activity was involved in calycosin-induced cell death.

ERβ-mediated increase in the expression levels of apoptotic-associated protein in OS MG-63 cells treated with calycosin. Apoptosis is often associated with the cleavage of specific substrates, such as cleaved PARP-1 and caspase-3, whose excessive activation can promote apoptosis (20). When MG-63 cells were treated with 0, 25, 50 and 100 μM calycosin for 48 h, it was found that the protein expression levels of PARP-1 and cleaved caspase-3 increased in a time- and dose-dependent manner (Fig. 5A and B). This suggested that activation of PARP-1 and cleavage of caspase-3 may be involved in calycosin-mediated cell apoptosis. In addition, calycosin + PHTPP treatment reduced PARP-1 and caspase-3 cleavage protein expression levels (P<0.05; Fig. 5C and D). Collectively, the present results indicated that PARP-1 and caspase-3 may be downstream targets of the ERβ-mediated PI3K/Akt pathway.

Discussion

OS usually occurs in adolescence when the synthesis of sex hormones, such as estrogen or androgen, peaks, thus indicating that sex steroids and their receptors may be involved in the development of OS (21). Phytoestrogens have recently received increased attention due to their ability to bind to ERs. Previous studies have suggested that certain phytoestrogens exhibit antiestrogenic activity via ER-mediated signaling pathways (22,23). Moreover, calycosin is a phytoestrogen isoflavone that exhibits estrogenic activity and anti-tumor effects on several cancer types, by inducing apoptosis of tumor cells *in vitro* and *in vivo* (8,9). Furthermore, *in vitro* and *in vivo* studies have also shown that calycosin has antiapoptotic and antimetastatic activities against OS (10,24). Consistent with these previous studies, the present results suggested that calycosin effectively inhibited cell proliferation and induced apoptosis in a time- and dose-dependent manner in OS MG-63 cells. In addition, calycosin had a low cytotoxicity in osteoblast hFOB1.19 cells.

Several previous studies have shown that calycosin inhibits tumorigenesis and tumor progression by regulating ERβ expression (8,9,25). Moreover, the downregulation of ERβ has been observed in various types of cancer (8,9). In the present study, ERβ protein expression in OS cells was found to be decreased, while its expression increased significantly in a dose-dependent manner after calycosin treatment. A previous study showed that estrogen extracted from *Astragalus membranaceus* could inhibit etoposide-induced apoptosis of human OS cells by activating ERβ (26). Furthermore, despite the reduction in ERβ expression, which may be related to different subtypes of OS cells, the estrogen inhibitor fulvestrant exhibits significant anti-OS activity in OS 143B cells (27). The present results suggested that the ERβ inhibitor PHTPP reversed the increase in the protein expression of ERβ, and significantly reversed the cytotoxicity and apoptosis detected following calycosin treatment in MG-63 cells. Therefore, the present

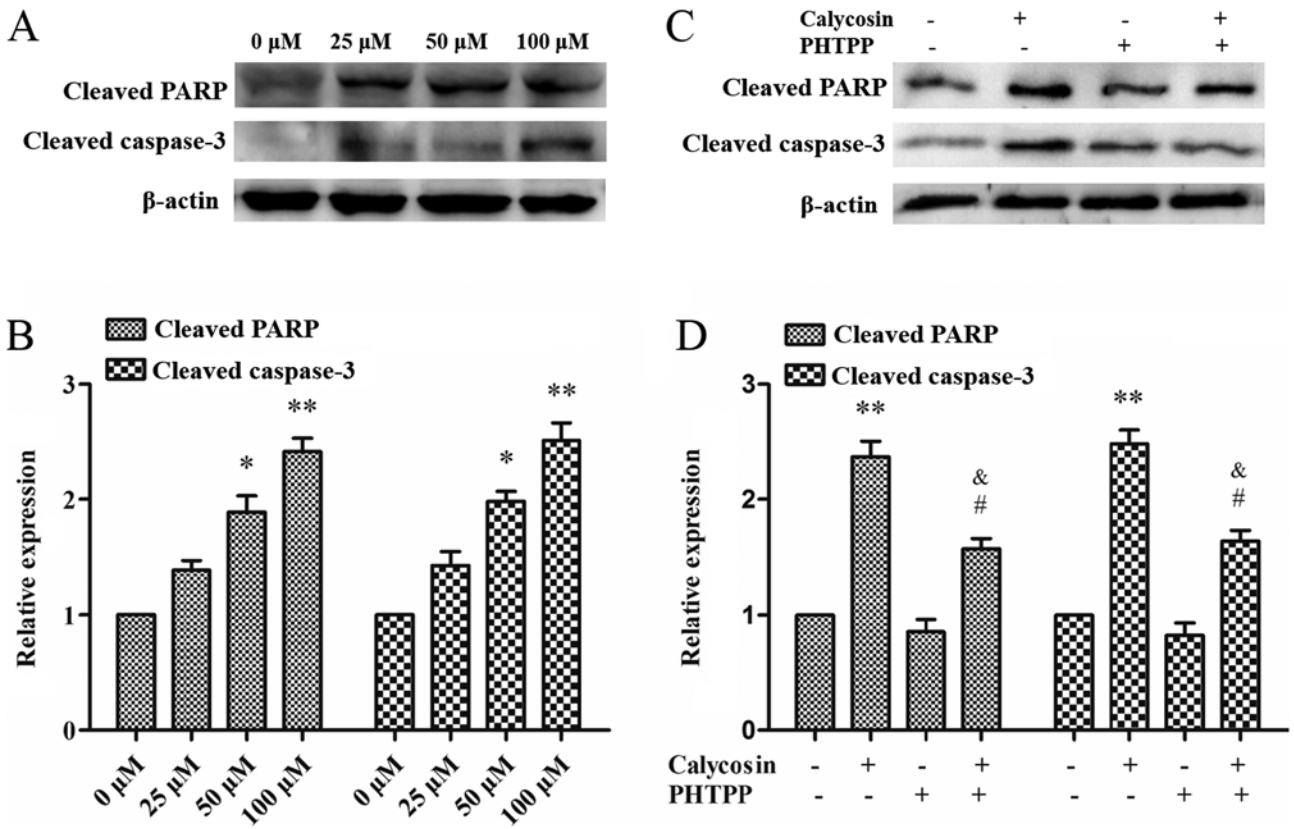

Figure 5. Induction of ERβ-mediated expression of apoptotic-associated proteins in ER-positive cells by calycosin. (A) Western blotting results of (B) protein expression levels of cleaved PARP and cleaved caspase-3 after treatment with different concentrations of calycosin. (C) Western blotting results of (D) protein expression levels cleaved PARP and cleaved caspase-3 after treatment with 100 μM calycosin, in the presence or absence of PHTPP for 48 h. These independent experiments were performed three times. *P<0.05, **P<0.01 vs. calycosin (0 μM or 0 h). $^{\#}$P<0.05 vs. calycosin. $^{\&}$P<0.05 vs. PHTPP. ER, estrogen receptor; PARP, poly (ADP-ribose) polymerase 1.

results indicated that the anti-tumor effects of calycosin were mediated by the ERβ signaling pathway.

In addition, the mechanism underlying the anti-tumor effect of ERβ was examined in the present study. The association between calycosin and ERβ was investigated, and calycosin was found to reduce OS cell proliferation by inhibiting the ERβ signaling pathway, in particular a potential downstream effector. The PI3K/Akt signaling pathway is frequently hyperactivated in OS and has been shown to be involved in tumorigenesis, proliferation, invasion, cell cycle progression and inhibition of apoptosis (17). Furthermore, ERβ can independently predict the prognosis of triple-negative breast cancer by interacting with the PI3K/Akt pathway (19). Estrogen can activate the PI3K/Akt pathway via ERβ in breast cancer (28). Moreover, ERβ has a significant anti-tumor effect on OS U2-OS cells by regulating the PI3K/Akt signal pathway (16). It has been shown that the inhibitory effects of calycosin on OS MG-63 cells are mediated by the PI3K/Akt pathway (10). In the present study, it was demonstrated that calycosin inactivated the PI3K/Akt signaling pathway in OS MG-63 cells, whereas the ERβ inhibitor PHTPP enhanced the phosphorylation of PI3K and Akt. Thus, the present results suggested that the anti-tumor effects of ERβ were associated with the PI3K/Akt signaling pathway.

Defects in apoptosis play an important role in tumor pathogenesis, and the cytotoxic effects of many antineoplastic drugs are usually accompanied by an increase in apoptosis (29). Experimental studies of OS cells and 143B-harbored nude mice have shown that calycosin possesses an anti-osteosarcoma effect, and the underlying mechanism is associated with the activation of apoptosis (30). In the present study, calycosin was found to induce apoptosis in MG-63 cells, as indicated by morphological changes, and the activation of caspase-3 and PARP-1. Caspases are known for their role as initiators and executors of apoptosis (31). Activation of different caspase cascades plays an important role in apoptosis by cleaving key factors involved in cellular function and viability (32). Moreover, the apoptotic executor factor caspase-3 can cleave the caspase substrate PARP-1 into two specific fragments, thus contributing to cell death (33). Therefore, cleaved PARP-1 and caspase-3 are considered as apoptotic markers. The present results indicated that there were increased expression levels of cleaved PARP-1 and caspase-3 following treatment with calycosin in a concentration-dependent manner, thus indicating the involvement of PARP-1 and caspase-3 in the effects of calycosin. Moreover, the PHTPP inactivated PARP-1 and caspase-3 cleavage, indicating that ERβ mediated PARP-1 and caspase-3 activity in calycosin-induced apoptosis.

Collectively, the present study provided further evidence for the interaction between calycosin and ERβ. In addition, the antiproliferative effects of calycosin were found to be mediated by the ERβ-dependent regulation of the PI3K/Akt pathways. Therefore, the present study provides a theoretical basis for the potential use of calycosin as a therapeutic to treat OS.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

WT performed the majority of the experiments and drafted the manuscript. ZWW helped perform the experiments. BMY analyzed the data and drafted the manuscript. YGB conceived the study, supervised the experiments and edited the manuscript. designed the study. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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