

# 4-Phenyl butyric acid prevents glucocorticoid-induced osteoblast apoptosis by attenuating endoplasmic reticulum stress

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**Abstract** Apoptosis of osteoblasts triggered by high-dose glucocorticoids (GCs) has been identified as a major cause of osteoporosis. However, the molecular mechanisms underlying GC-induced osteoporosis remain elusive. This study was conducted to make clear the mechanism of GC-induced osteoblast apoptosis and to examine whether reduction of ER stress by 4-PBA inhibited osteoblast apoptosis. After treatment with dexamethasone (Dex) or hydrocortisone, cell viability was assessed using an MTT assay. Flow cytometry was performed to assess the apoptosis of MC3T3-E1 cells. The expression levels of ER stress-related proteins (CHOP, GRP78, eIF2 $\alpha$ , and phospho-eIF2 $\alpha$ ) and apoptosis-related proteins (cleaved Caspase-3, Bcl-2, and Bax) in MC3T3-E1 cells were measured by Western blot analysis. We found that both Dex and hydrocortisone reduced cell proliferation and promoted apoptosis in MC3T3-E1 cells. In addition, the protein expression levels of cleaved Caspase-3 and Bax increased and the protein expression level of Bcl-2 decreased in MC3T3-E1 cells exposed to Dex. In addition, the Dex exposure also resulted in a release of cytochrome *c* (Cyt C) from mitochondria. The cellular ATP content was decreased following prolonged treatment with Dex. 4-PBA attenuated ER stress and mitochondrial dysfunction induced by Dex in MC3T3-E1 cells. Dex-mediated apoptosis of MC3T3-E1 cells is aggravated by ER stress. Moreover, Dex-induced apoptosis in MC3T3-E1 cells was inhibited by 4-PBA,

suggesting that ER stress involved in Dex-induced apoptosis. In conclusion, inhibition of ER stress by 4-PBA could reduce GC-induced apoptosis in MC3T3-E1 cells.

**Keywords** 4-Phenylbutyric acid · Glucocorticoid · Osteoblasts · Endoplasmic reticulum stress

## Introduction

Osteoporosis is a severe bone-wasting disease and a common complication of glucocorticoids (GCs) therapy for the treatment of diseases caused by an overactive immune system [1]. The main features of osteoporosis are bone mass loss and bone tissue degeneration, which lead to a reduction in bone strength and an increased risk of fracture [2]. A number of studies have shown that osteoporosis is caused by an imbalance between bone resorption and bone formation. Osteoblasts have a crucial role in the process of bone rebuilding. GCs promote bone loss by enhancing hypofunction and apoptosis of osteoblasts [3]. A previous study suggested that dexamethasone (Dex) may induce osteoblast apoptosis by upregulating pro-apoptotic proteins Bcl-XL, IAP-2, and XIAP [4]. Increasing numbers of studies have suggested that endoplasmic reticulum (ER) stress is implicated in osteoblast apoptosis. However, the exact molecular mechanisms involved in GC-induced osteoporosis are still not well known.

The endoplasmic reticulum (ER) is a crucial organelle with an important function in a variety of cellular functions, such as folding, assembly, and quality control of proteins, glycosylation, lipid biosynthesis, Ca<sup>2+</sup> storage, and localization of secreted and transmembrane proteins [5]. ER stress is a condition in which the homeostasis between protein folding load and capacity of ER is disrupted. When

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cells are subjected to ER stresses induced by agents such as brefeldin A or thapsigargin, the protein folding capacity of ER severely decreased, resulting in accumulation of misfolded or unfolded proteins in the ER lumen [6, 7]. Excess ER stress represents a fundamental threat to cell viability and activates multiple apoptotic signaling pathways in mammalian cells. Cells respond to ER stress by activating glucose-regulated protein 78 (GRP78), a ER molecular chaperone that regulates the levels of accumulation of unfolded or misfolded proteins [8]. Under normal physiological conditions, GRP78 is kept inactive through binding to protein kinase RNA-like ER kinase (PERK) and ATF6 proteins [9]. In conditions of ER stress, GRP78 is dissociated from these complexes by binding to unfolded or misfolded polypeptide chains or unassembled multisubunit proteins, thereby activating ER stress signal transducers [10]. Eukaryotic translation initiation factor 2 subunit- $\alpha$  (eIF2 $\alpha$ ) mediates the binding of amino acylated tRNA to ribosomes in a GTP-dependent manner. Phosphorylation of the  $\alpha$ -subunit of the eIF2 $\alpha$  attenuated the ability of protein synthesis and promoted apoptosis in mouse embryo fibroblasts treated with a proteasome inhibitor [11]. Another study showed that salubrinal, a selective chemical inhibitor of eIF2 $\alpha$  dephosphorylation, was proven to have a capability of protecting cells from ER stress [12]. Severe or prolonged ER stress initiated apoptotic processes through transcriptional induction of CCAAT/enhancer-binding protein homologous protein (CHOP), c-Jun NH2-terminal kinase (c-JNK), and caspase-12-dependent signaling pathways [13].

As an inhibitor of ER stress, 4-phenyl butyric acid (4-PBA) eliminates intracellular unfolded protein response (UPR), which is a homeostatic signaling network. UPR is activated by unfolded proteins accumulated in the ER [14]. 4-PBA inhibits ER stress by regulation of several vital proteins, including GRP78, PERK, c-JNK, X-box-binding protein 1 (XBP-1), and CHOP [15]. Upregulation of some ER stress markers, especially GRP78 and CHOP, was observed in apoptotic osteoblasts, suggesting that ER stress was implicated in the pathogenesis of osteoporosis [16, 17]. During the process of osteoblast apoptosis induced by hyperhomocysteinemia, the expression levels of GRP78, inositol-requiring transmembrane kinase, endonuclease 1 $\alpha$ , and spliced XBP-1 significantly increased [18]. These findings suggested that inhibition of ER stress using 4-PBA may be a novel therapeutic strategy for osteoporosis.

In the present study, a GC analogue, Dex, was used to examine whether GCs promoted osteoblast apoptosis, and whether Dex induced ER stress in MC3T3-E1 cells. The protective effect of 4-PBA on Dex-induced ER stress and cell apoptosis was also examined in MC3T3-E1 osteoblast-like cells.

## Materials and methods

### Cell culture

MC3T3-E1 cells were cultured in minimum essential medium-alpha medium ( $\alpha$ -MEM; GIBCO, Carlsbad, CA, USA) with 10 % fetal bovine serum (GIBCO), 100 U/ml penicillin (Sigma-Aldrich, St. Louis, MO, USA), and 100  $\mu$ g/ml streptomycin (Sigma-Aldrich). Cells were maintained in a humid incubator (Thermo Scientific, Rockford, IL, USA) containing 95 % O<sub>2</sub> and 5 % CO<sub>2</sub> at a temperature of 37 °C.

### MTT assay

MC3T3-E1 cells ( $3 \times 10^3$  cells/well) were seeded in 96-well plates and incubated overnight at 37 °C. After 24 h incubation with 1  $\mu$ M Dex or 1  $\mu$ M hydrocortisone (Sigma-Aldrich), 10  $\mu$ l 5 mg/ml MTT reagent was added into each well and further incubated for 4 h. Optical densities (OD) of cell supernatants were detected at 570 nm with a DU800 spectrophotometer (Beckman Coulter, Fullerton, CA, USA).

### Cell death assay

The apoptosis of MC3T3-E1 cells was detected by Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) double-staining assay. After incubation with 1  $\mu$ M Dex, 1  $\mu$ M Dex + 0.5 mM 4-PBA, 1  $\mu$ M thapsigargin (TG; Sigma-Aldrich) or 1  $\mu$ M TG + 0.5 mM 4-PBA for 24 h, MC3T3-E1 cells in suspension and adherent cells were collected and then centrifuged at 200 g for 5 min, washed twice with phosphate-buffered saline (PBS), resuspended in 500  $\mu$ l Annexin V binding buffer, and incubated with 5  $\mu$ l FITC-labeled Annexin V and 5  $\mu$ l PI for 10 min at room temperature in the dark. The scatter parameters of MC3T3-E1 cells were acquired by a FAC Scan flow cytometer, and data were analyzed with FACS Diva software (BD Biosciences, San Jose, CA, USA).

### Western blot

The protein was extracted using RIPA lysis buffer (Beyotime Institute of Biotechnology, Jiangsu, China), denatured for 10 min at 95 °C, and cooled on ice for 2 min. Equal amounts of protein samples were separated on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels and transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, MA, USA). The membranes were blocked with 5 % nonfat milk in TBST buffer at room temperature for 1 h and then incubated

overnight at 4 °C with the primary antibodies including anti-GRP78 antibody, anti-CHOP antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-eIF2 $\alpha$  antibody (Cell Signaling Technology, Beverly, MA, USA), anti-phospho-eIF2 $\alpha$  antibody (Cell Signaling Technology), anti-Bcl-2 antibody (Santa Cruz), anti-Bax antibody (Santa Cruz), anti-active caspase-3 antibody (Abcam, Cambridge, MA, USA), and anti-GAPDH antibody (Santa Cruz). After overnight incubation with the primary antibody, the membranes were washed 3  $\times$  10 min with TBST buffer and incubated with horseradish peroxidase (HRP)-conjugated anti-mouse secondary antibody (Sigma-Aldrich) or anti-rabbit secondary antibody (Sigma-Aldrich) for 1 h at room temperature. The membranes were washed 3  $\times$  15 min with TBST buffer, and then blots were visualized with enhanced chemiluminescence reagent reagent (CWBI, Beijing, China) and exposed to an X-ray film. Quantification of Western blots was performed using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

### ATP assay

MC3T3-E1 cells were seeded in a 96-well plate and then cultured in the presence of 1  $\mu$ M Dex for 6, 12, and 24 h, respectively. The ATP levels in cells were detected with a commercial CellTiter-Glo Luminescent ATP Assay kit (Promega, Madison, WI, USA) according to the protocol provided by the manufacturer. In brief, the assay buffer was gently mixed with the substrate at room temperature. The mixed reagent (100  $\mu$ l) was added into each well and then incubated with shaking for 15 min at room temperature to induce cell lysis. After incubation, luminescence was measured using a microplate reader (Beckman Coulter).

### Statistical analysis

The unpaired Student's *t* test was used to analyze differences between two groups. Analysis of variance (ANOVA) was used to compare the means of three or more groups. All data were expressed as mean  $\pm$  SD, and a value of *P* < 0.05 was considered to be statistically significant. All statistical analyses were performed using the SPSS 16.0 software (SPSS, Chicago, IL, USA).

## Results

### Dex promotes osteoblast apoptosis

Various studies have indicated that osteoblast apoptosis is a cause of osteoporosis. To investigate the mechanism underlying GC-induced osteoblast apoptosis, the MC3T3-E1 cell line was used as a cellular model to examine proapoptotic

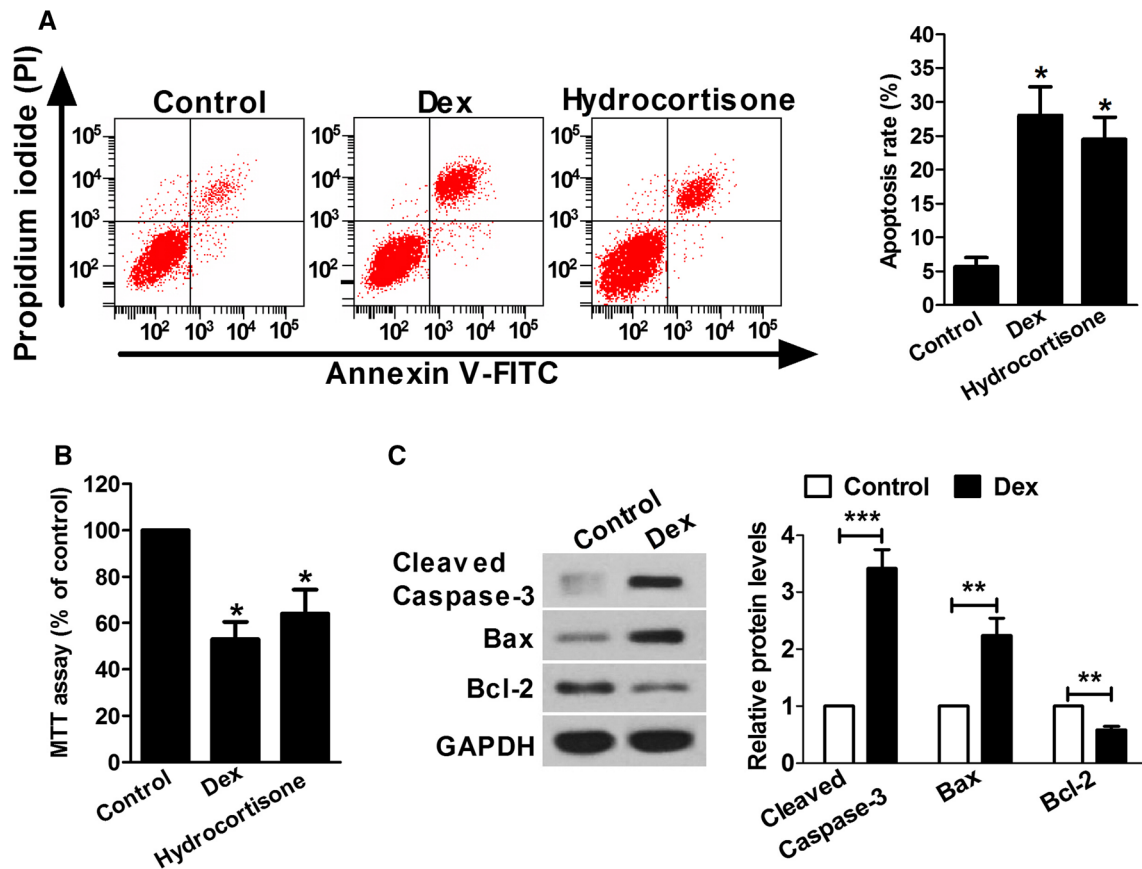
effects of Dex and hydrocortisone on osteoblasts. We first examined the apoptosis levels in cells treated with Dex or hydrocortisone using the Annexin V-FITC/PI double-staining method. The apoptosis rates of MC3T3-E1 cells in both Dex and hydrocortisone group remarkably increased compared with that in the control group (Fig. 1a), indicating that Dex and hydrocortisone were inducers of osteoblast apoptosis. The effects of Dex and hydrocortisone on cell survival in MC3T3-E1 cells were assessed using an MTT assay. We found that the cell proliferation ability of MC3T3-E1 cells exposed to 1  $\mu$ M Dex or hydrocortisone was significantly decreased when compared with a non-treated group (Fig. 1b). In addition, we detected the expression levels of apoptosis-related proteins Bax and Bcl-2. The data showed that cleaved Caspase-3 and Bax expression were increased and Bcl-2 expression was decreased in cells exposed to Dex (Fig. 1c). These results revealed that Dex at the concentration of 1  $\mu$ M promoted osteoblast apoptosis.

### Dex induces ER stress response and mitochondrial dysfunction in osteoblast cells

A large number of studies have reported that the protein expression levels of GRP78 and CHOP are increased as well as the phosphorylation level of eIF2 $\alpha$  when severe ER stress occurs [19, 20]. In this study, ER stress response was identified by detecting the protein expression levels of ER stress markers GRP78, CHOP, and phospho-eIF2 $\alpha$ . The expression of GRP78, CHOP, and phosphorylated eIF2 $\alpha$  were increased in the Dex group compared with the control group (Fig. 2a), indicating that ER stress was involved in GC-induced osteoblast apoptosis. The Dex exposure also resulted in a release of cytochrome *c* (Cyt C) from mitochondria, as seen by an increase in the cytoplasmic level of Cyt C (Fig. 2b). Because decreased production of ATP is a consequence of mitochondrial depolarization, therefore we further detected the cellular levels of ATP following Dex treatment for 6, 12, or 24 h. The results showed that cellular ATP content was decreased following prolonged treatment of Dex (Fig. 2c).

### 4-PBA reduces ER stress and mitochondrial dysfunction induced by Dex in osteoblast cells

4-PBA, one member of the small molecular chaperone class, facilitates protein folding and subsequent trafficking through the secretory pathway, thereby relieving ER stress and UPR in cells [21]. To test whether 4-PBA could alter the ER stress and mitochondrial dysfunction in osteoblasts exposed to GC, MC3T3-E1 cells were co-incubated with 1  $\mu$ M Dex and 0.5 mM 4-PBA. After 24 h, the expression levels of ER stress proteins were measured using Western blot. The quantitative analysis of Western blot showed that



**Fig. 1** Effect of dexamethasone (Dex) on apoptosis in MC3T3-E1 cells. MC3T3-E1 cells were treated with 1  $\mu$ M Dex or hydrocortisone for 24 h. **a** Cell apoptosis rate was analyzed by flow cytometry. **b** Cell proliferation was determined by MTT assay. **c** Cell lysates

were subjected to Western blot analysis with anti-cleaved Caspase-3, anti-Bcl-2, and anti-Bax antibodies. GAPDH was used as a loading control. Data were expressed as mean  $\pm$  SD ( $n = 3$ ). \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$

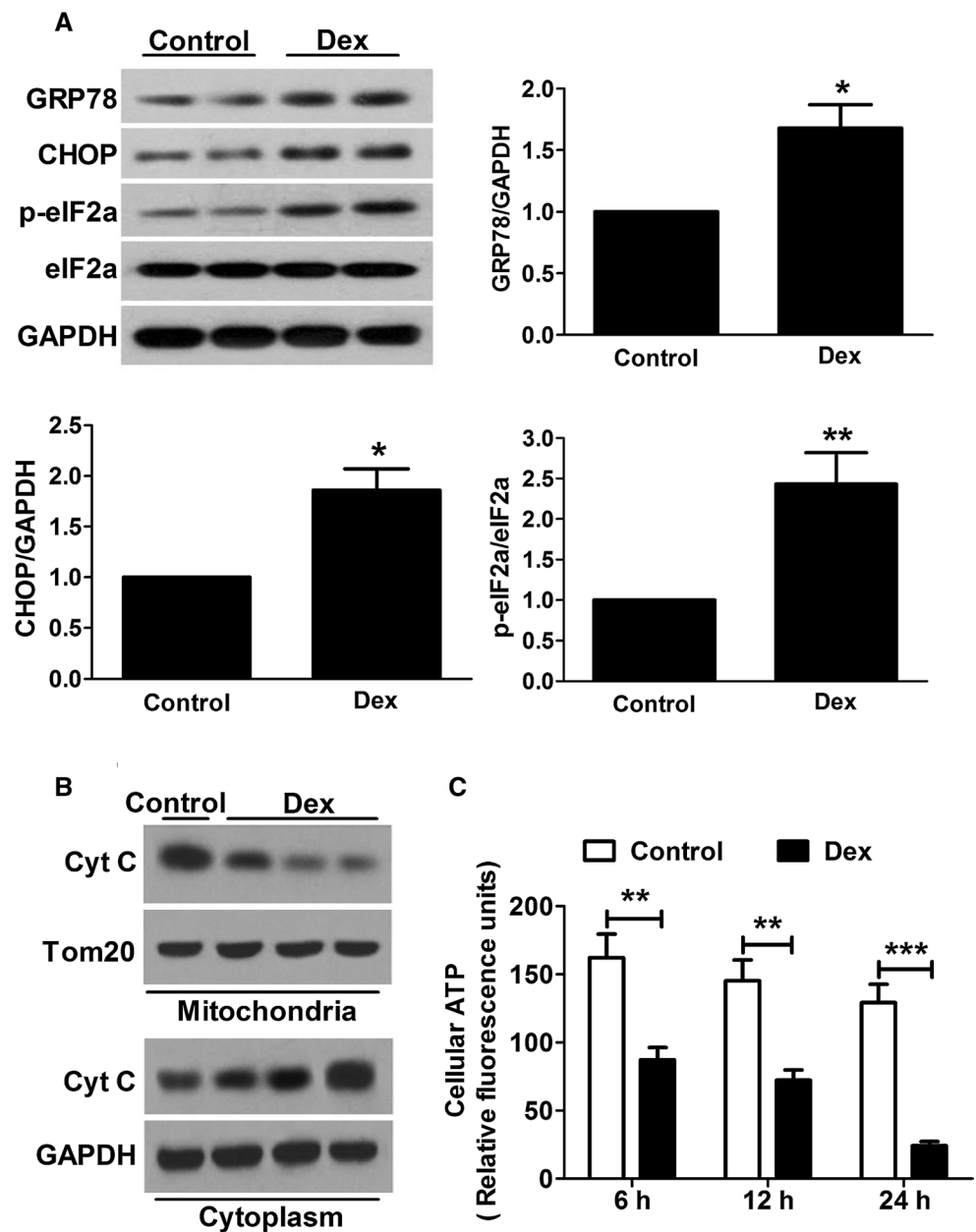
the level of phospho-eIF2 $\alpha$ /eIF2 $\alpha$  and the expression levels of GRP78 and CHOP were significantly decreased in the Dex + 4-PBA group in comparison with the Dex group (Fig. 3a). In addition, Dex-induced Cyt C release was suppressed by 4-PBA treatment (Fig. 3b). The cellular ATP content in the 4-PBA + Dex group was increased compared with the Dex group (Fig. 3c). These findings suggested that 4-PBA treatment at a concentration of 0.5 mM effectively rescued Dex-induced ER stress response and mitochondrial dysfunction in MC3T3-E1 cells.

**4-PBA reduces apoptosis induced by Dex and ER stressors in osteoblast cells**

Low-grade ER stress is modulated by the activation of the UPR to facilitate the recovery of ER function, but prolonged or severe ER stress will cause cellular apoptosis [22]. TG is known as a ER stress inducer that triggers ER stress by disturbing intracellular calcium homeostasis [23]. To examine whether 4-PBA ameliorates Dex-induced

osteoblast apoptosis, MC3T3-E1 cells were treated with 1  $\mu$ M Dex or 1  $\mu$ M TG for 24 h in the presence of 0.5 mM 4-PBA, and the apoptosis rates were then assessed by flow cytometry measurement. The apoptosis rate of cells co-cubated with TG and 4-PBA was decreased when compared with cells only in presence of TG (Fig. 4a). Moreover, the apoptosis rate of cells in the Dex + 4-PBA group was significantly lower than that in the Dex group. We further assessed the expression levels of cleaved Caspase-3, Bax, and Bcl-2 by Western blot. The data indicated that Dex promoted the expression of cleaved Caspase-3 and Bax and inhibited Bcl-2 expression in MC3T3-E1 cells. However, treatment with 4-PBA significantly reversed the upregulation of cleaved Caspase-3 and Bax and downregulation of Bcl-2 induced by Dex or TG in MC3T3E1 cells (Fig. 4b–e). The CHOP expression level was increased in the Dex or TG group, but decreased after addition of 4-PBA (Fig. 4f, g). Taken together, these results demonstrated that 4-PBA significantly ameliorated Dex-induced apoptosis by inhibiting ER stress in MC3T3-E1 cells.

**Fig. 2** Dex triggers endoplasmic reticulum (ER) stress response in MC3T3-E1 cells. **a** MC3T3-E1 cells were collected at indicated times and lysed. Western blots and quantitative analysis were conducted to assess the expression levels of GRP78, CHOP, and phospho-eIF2a in MC3T3-E1 cells treated with or without Dex. GAPDH was used as an internal control. **b** MC3T3-E1 cells were treated with 1  $\mu$ M Dex for 6, 12, or 24 h. The expression levels of cytochrome *c* (Cyt *C*) in the cytoplasm and mitochondria were determined by Western blot. **c** Total cellular levels of ATP were analyzed as described in Materials and methods. Data were expressed as mean  $\pm$  SD ( $n = 3$ ). \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$



## Discussion

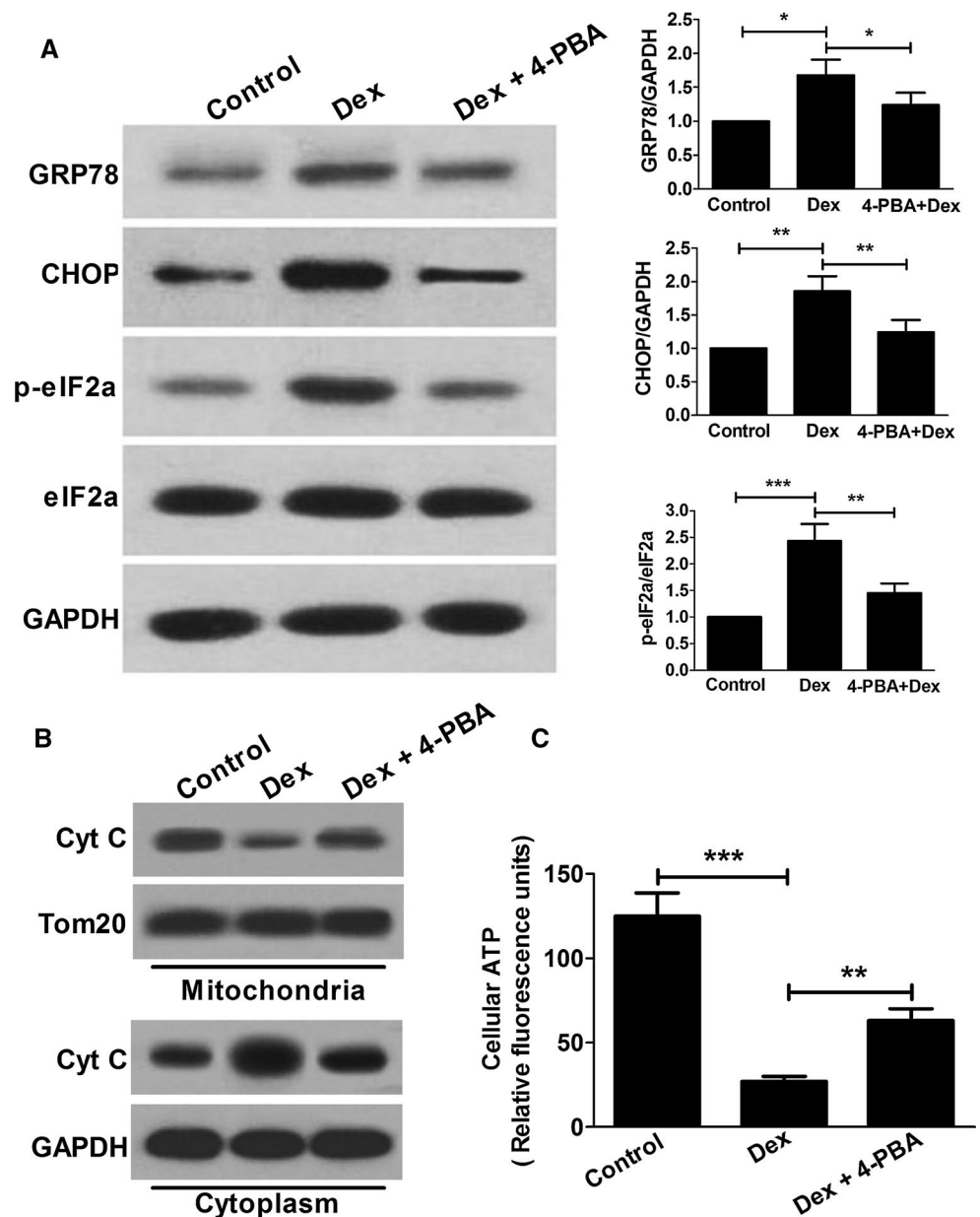
Osteoporosis is the most common bone disease all over the world and severely impairs the life quality of patients, especially those in old age. GCs are unsurpassed immunomodulatory and antiinflammatory agents and are widely used in the clinical setting [24]. However, long-term therapeutic use of GC is thought to be an important cause of secondary osteoporosis, which is partially the result of osteoblast apoptosis [25]. To investigate the mechanism by which GCs induce apoptosis in osteoblasts and to determine whether ER stress is involved in this process, we detected the expression levels of ER stress indicators and assessed effects of 4-PBA on Dex-induced apoptosis of MC3T3-E1

osteoblast-like cells. We found that a chemical chaperone, 4-PBA, reversed Dex-induced apoptosis in mouse osteoblast-like cells by inhibiting ER stress. This new discovery is of great significance for molecular intervention against GC-induced osteoporosis.

GCs promote osteoclastogenesis by inhibiting osteoprotegerin and stimulating the synthesis of receptor activator for nuclear factor  $\kappa$ B ligand, thereby promoting bone resorption [26, 27]. A recent study showed that GCs increased the rate of apoptosis in osteoblasts by promoting E4BP4 expression through upregulation of Bim [28]. The *in vitro* evidence suggested that high concentrations of Dex inhibited the proliferation but not the differentiation or maturation of human osteoblast precursors. Therefore, a



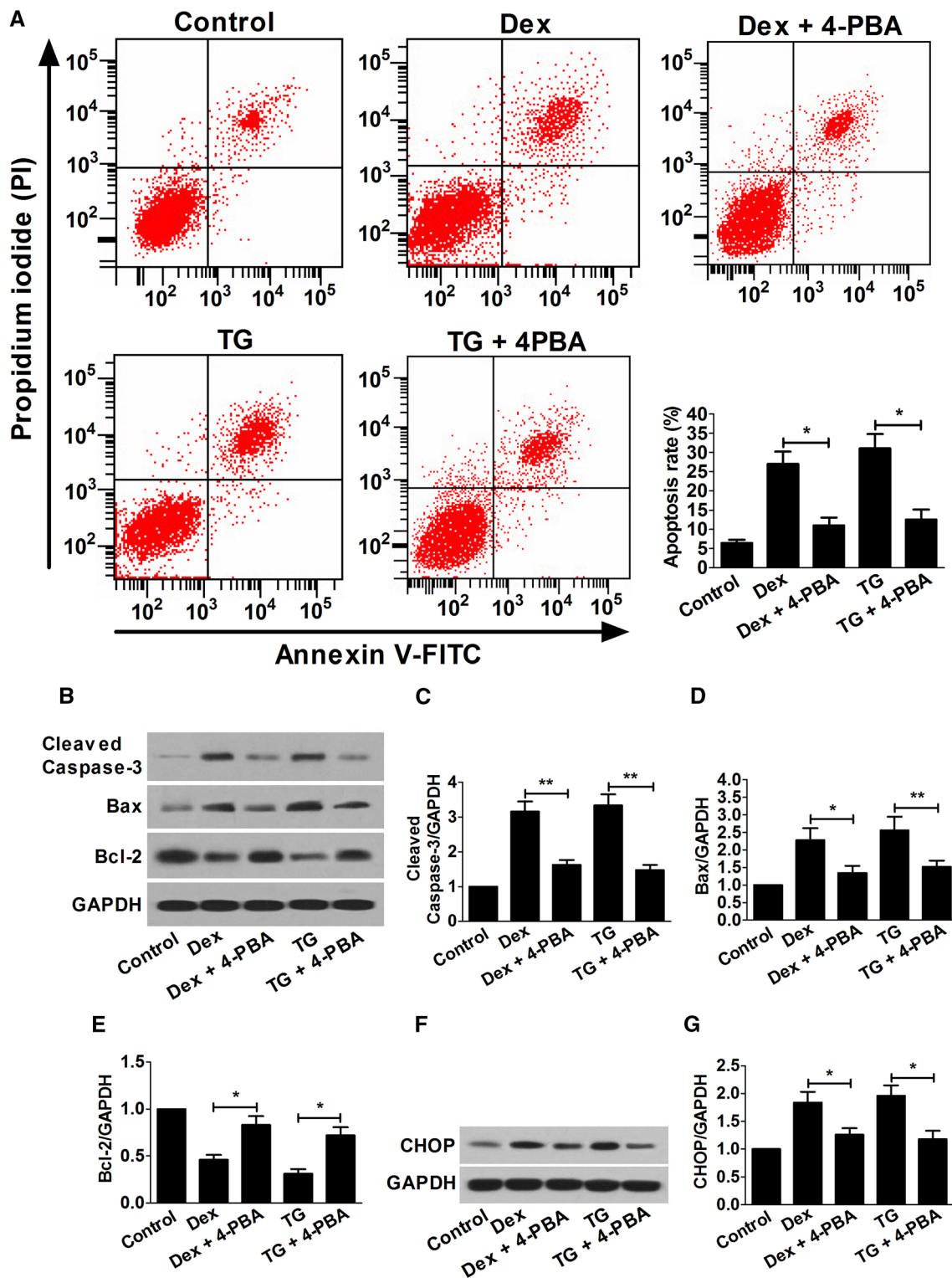
**Fig. 3** Effects of 4-phenyl butyric acid (4-PBA) treatment on ER stress response and mitochondrial dysfunction. **a** MC3T3-E1 cells were exposed to 1  $\mu$ M Dex in the presence or absence of 0.5 mM 4-PBA for 24 h. Western blot was performed to detect the expression levels of GRP78, CHOP, and phospho-eIF2a in MC3T3-E1 cells. **b** Release of Cyt C from mitochondria was assessed by Western blot. **c** Cellular ATP content was determined following treatment with Dex or Dex + 4-PBA for 24 h. Data are represented as mean  $\pm$  SD ( $n = 3$ ). \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$



decrease in the proliferation and an increase in the apoptosis of osteogenic precursors is more likely to be the key factor in GC-induced osteoporosis [29]. In addition, GCs suppress bone formation by attenuating osteoblast differentiation via the monomeric GC receptor [24]. In this study, we found that Dex led to an increased rate of apoptosis and a decline of proliferation in MC3T3-E1 osteoblast-like cells. Consistent with the effects of Dex, hydrocortisone also reduced cell proliferation and promoted apoptosis in MC3T3-E1 cells. The protein expression level of Bcl-2 was decreased in MC3T3-E1 cells treated with 1  $\mu$ M Dex for 24 h, but Bax and cleaved Caspase-3 were increased. Furthermore, we measured the expression levels of GRP78, CHOP, and phospho-eIF2a in Dex-treated osteoblast cells.

These data suggested that GC may induce osteoporosis by facilitating osteoblast apoptosis via enhancing ER stress.

The ER is the main site for protein synthesis, folding, and trafficking in cells. Various stressful conditions bring about the abnormal or excessive accumulation of mutant, unfolded, or misfolded proteins in the ER, thereby resulting in ER stress [30]. GRP78 is a critical regulator for ER integrity because of its antiapoptotic effects and its capability to control the activation of transmembrane ER stress sensors such as IRE1, PERK, and ATF6 [31]. Multiple studies have demonstrated that IRE1 $\alpha$  binds directly to misfolded or unfolded proteins in the ER. GRP78 desensitizes IRE1 $\alpha$  to attenuate ER stress and acts as a timer to modulate ER response time by contributing to IRE1 $\alpha$



**Fig. 4** 4-PBA protects MC3T3-E1 cells against apoptosis induced by Dex or thapsigargin (TG). Cells were treated with 1  $\mu$ M Dex, 1  $\mu$ M Dex + 0.5 mM 4-PBA, 1  $\mu$ M TG, or 1  $\mu$ M TG + 0.5 mM 4-PBA for 24 h. **a** Apoptotic cells were detected by flow cytometry. **b–g**

Western blot assay was conducted to measure the expression levels of apoptosis-related proteins cleaved by caspase-3, Bax, Bcl-2, and CHOP. Data are represented as the mean  $\pm$  SD ( $n = 3$ ). \* $P < 0.05$ , \*\* $P < 0.01$

deactivation once ER homeostasis is reestablished [32]. ER stress has been proved to be implicated in neurodegenerative diseases, inflammation, viral infections, metabolic diseases, and cancer, characterized by a serious accumulation of abnormal proteins in ER [33]. TG, an ER stress activator, selectively inhibits  $\text{Ca}^{2+}$  ATPases and is widely used to induce ER stress in various cell types [34]. In this study, we found that ER stress was implicated in GC-induced osteoblast apoptosis. Furthermore, we demonstrated that 4-PBA exhibited beneficial effects on inhibiting ER stress and ER stress-associated cell apoptosis and might be used in clinical treatment for osteoporosis in future. These findings may provide a new strategy to rescue osteoporosis by reducing osteoblast apoptosis through ER stress inhibition.

Release of Cyt C from mitochondria triggers activation of caspase proteases and death of cells by apoptosis [35]. A previous study showed that ER stress is involved in GC-induced apoptosis of osteoblasts and osteocytes [36], but it is not yet clear whether mitochondrial function also is affected by GCs. Here, we found that Cyt C was released from the mitochondria into cytoplasm when MC3T3-E1 cells were treated with Dex. After treatment with Dex, the cellular ATP content was decreased in a time-dependent manner. These results suggested that mitochondrial dysfunction was also involved in GC-induced osteoblast apoptosis. Furthermore, we examined whether 4-PBA could rescue Dex-induced mitochondrial dysfunction. We found that 4-PBA treatment attenuated Dex-induced mitochondrial dysfunction in MC3T3-E1 cells.

In summary, this study demonstrated that suppression of ER stress with 4-PBA inhibits GC-induced apoptosis by attenuating ER stress and mitochondrial dysfunction in MC3T3-E1 osteoblast-like cells. Further studies should be done to confirm the effects of 4-PBA on GC-induced osteoblast apoptosis and osteoporosis in vivo. This study may provide a novel strategy for molecular intervention against Dex-induced osteoporosis by inhibiting ER stress.

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#### Compliance with ethical standards

**Conflict of interest** The authors declare that there are no conflicts of interest.

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