

ORIGINAL ARTICLE

# Haem oxygenase-1 induction prevents glucocorticoid-induced osteoblast apoptosis through activation of extracellular signal–regulated kinase1/2 signalling pathway



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## KEYWORDS

Apoptosis;  
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**Abstract** *Background:* High-dose glucocorticoid (GC) therapy always causes osteoporosis partly by inducing osteoblast apoptosis. However, the underlying mechanisms of GC-induced apoptosis remain elusive. Haem oxygenase-1 (HO-1) is a cytoprotective protein that rescues cells from H<sub>2</sub>O<sub>2</sub> or high glucose–induced apoptosis. In bone metabolism, HO-1 also participates in osteoclast and osteoblast differentiation.

*Objective:* The present study aimed to investigate the protective role of HO-1 against GC-induced osteoblast apoptosis and to elucidate the underlying mechanism.

*Methods:* Mouse osteoblastic MC3T3-E1 cells were treated with dexamethasone (Dex) for 24 h in the presence or absence of cobalt (III) protoporphyrin IX chloride (CoPP, an inducer of HO-1). In some experiments, U0126 was added to the culture 1 h before CoPP treatment. The induction of apoptosis was determined by flow cytometry. Cell viability was evaluated using a cell counting kit-8 (CCK-8) assay. The expression levels of Bax and bcl-2 were measured by real-time polymerase chain reaction and Western blot. HO-1, extracellular signal–regulated kinase (ERK)-1/2 and pERK1/2 protein levels were measured by Western blot analysis.

*Results:* Dex promoted apoptosis and inhibited cell viability in MC3T3-E1 cells. In addition, Dex significantly increased Bax expression and reduced Bcl-2 expression. The expression of HO-1

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was also reduced after Dex treatment. HO-1 induction by CoPP significantly attenuated Dex-induced apoptosis as evidenced by Annexin V/PI staining. The mRNA expression level of anti-apoptotic gene Bcl-2 was also increased after CoPP treatment. Moreover, CoPP treatment increased the phosphorylation of ERK1/2. U0126, an inhibitor of ERK activation, significantly abrogated the protective effects of CoPP.

**Conclusion:** Our results demonstrate that HO-1 induction by CoPP can attenuate Dex-induced apoptosis of mouse osteoblastic MC3T3-E1 cells. The antiapoptotic effect of HO-1 induction may be correlated with the activation of ERK1/2 signalling pathway.

**The translational potential of this article:** HO-1 induction by CoPP can prevent GC-induced osteoblast apoptosis. Our findings will highlight the therapeutic potential of HO-1 induction in GC-induced osteoporosis.

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## Introduction

Glucocorticoids (GCs) are effective agents used in treating cancer, autoimmune diseases and inflammatory disorders [1,2]. However, high-dose or long-term use of GCs is the primary cause of secondary osteoporosis because GCs exert direct effects on the skeletal system. GCs suppress bone formation and increase bone resorption, which finally lead to GC-induced osteoporosis (GIOP) [3,4]. Osteoporotic fractures may occur in 30–50% of patients receiving long-term GC administration [4]. Thus, there is a great need to clarify the pathogenesis of GIOP and develop effective therapeutic strategies.

Coordinated cycles of bone formation and resorption require an orchestrated interplay among osteoblasts, osteocytes and osteoclasts [5]. GCs can increase osteoclast generation and induce osteocyte apoptosis [6–8]. Osteoblasts are responsible for bone formation. The inhibition of bone formation is critical to the pathogenesis of GIOP. GCs significantly decrease the number of osteoblasts. On the one hand, GCs inhibit the production of new osteoblast precursors. On the other hand, GCs induce the apoptosis of mature osteoblasts [9–11]. Previous studies have reported that GCs can induce osteoblast apoptosis through triggering endoplasmic reticulum (ER) stress, inducing proapoptotic proteins or suppressing tissue inhibitor of metalloproteinase-1 [12–14]. However, the underlying mechanism of GC-induced osteoblast apoptosis has not been fully elucidated.

Haem oxygenase (HO) is the rate-limiting enzyme that degrades haem to biliverdin, carbon monoxide and free iron [15]. Three isoforms of HO (HO-1, HO-2 and HO-3) have been identified. HO-1 is a protective protein possessing anti-apoptotic, antiinflammatory and antioxidant effects [16–18]. HO-1 was also found to play a critical role in bone and fat metabolism [19–21]. Cobalt (III) protoporphyrin IX chloride (CoPP) is a potent HO-1 inducer. HO-1 induction by CoPP can protect HepG2 cells from cadmium-induced apoptosis *in vitro* [22]. The antiapoptotic effects of HO-1 induction may attribute to activating the extracellular signal-regulated kinase (ERK)/nuclear factor erythroid 2-related factor 2 (Nrf2) signalling pathway, inhibiting ER stress and c-Jun N-terminal kinase activation [18]. Although the cytoprotective effect of HO-1 has been extensively studied, the mechanisms of HO-1 against GC-induced osteoblast apoptosis remain unclear.

In the present study, we evaluate the effects of HO-1 on GC-induced osteoblast apoptosis and explored the underlying mechanisms. Dexamethasone (Dex), a synthetic GC, was used to induce osteoblast apoptosis. We present evidence that HO-1 induction by CoPP can prevent GC-induced osteoblast apoptosis. The antiapoptotic mechanism of HO-1 may involve the activation of ERK1/2 signalling pathway. Our findings indicate that HO-1 induction may be a useful therapeutic strategy for treating GIOP.

## Materials and methods

### Cell culture and treatment

Mouse osteoblastic MC3T3-E1 cells were grown in Dulbecco's Modified Eagle's Medium (high glucose; Gibco, Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% foetal bovine serum (Gibco, Thermo Fisher Scientific, Inc.), 100 U/ml penicillin (Sigma–Aldrich, St. Louis, MO, USA) and 100 µg/ml streptomycin (Sigma–Aldrich) in a humid incubator (Thermo Fisher Scientific, Inc.) with 5% CO<sub>2</sub> at a temperature of 37°C. To induce apoptosis, MC3T3-E1 cells were treated with different concentrations of Dex (Sigma–Aldrich) for 24 h.

To induce HO-1 expression, CoPP (3 µM; Frontier Scientific, Logan, USA) was added to the culture 12 h before Dex treatment. In some experiments, U0126 (an inhibitor of ERK activation, 10 µM; Sigma–Aldrich) or tin protoporphyrin IX (an inhibitor of HO-1, SnPP, 20 µM; Cayman Chemical, Ann Arbor, MI, USA) was added to the culture 1 h before CoPP treatment.

### Cell viability assay

Cell viability was assessed using a cell counting kit-8 (Dojindo Molecular Technologies, Inc., Kumamoto, Japan). Briefly, MC3T3-E1 cells ( $1 \times 10^4$ ) were seeded in 96-well plates, incubated overnight and treated with different concentrations of Dex for 24 h. Ten microlitres of cell counting kit-8 solution was added to the wells, and the cells were incubated for 2.5 h in the incubator. The absorbance values at 450 nm were measured using a Multi-Volume Spectrophotometer System (BioTek Epoch, Vermont, USA).

## Apoptosis assay

Cell apoptosis was assessed by flow cytometry using an Annexin V Apoptosis Detection Kit (BD Biosciences, San Jose, CA, USA) according to the manufacturer's instructions.

## Reactive oxygen species measurement

MC3T3-E1 cells were cultured with Dex for 24 h and then washed three times with phosphate buffer solution. After this, cells were incubated with 10  $\mu$ M dihydroethidine (Sigma–Aldrich) for 30 min and then harvested and washed three times with phosphate buffer solution. The fluorescence was determined by flow cytometry.

## HO-1 activity measurement

HO-1 activity was measured as previously described with minor modification [23]. Cell lysates were added to the reaction mixture containing 30  $\mu$ M haemin (Sigma–Aldrich), 2 mg/ml of rat liver cytosol, 1 mM MgCl<sub>2</sub>, 3 units of glucose-6-phosphatase dehydrogenase (Sigma–Aldrich), 1 mM glucose-6-phosphate (Solarbio Science & Technology, Beijing, China), 2 mM reduced nicotinamide adenine dinucleotide phosphate (Beyotime Institute of Biotechnology, Jiangsu, China) and 0.1 M potassium phosphate buffer. The reaction was conducted at 37°C in the dark for 30 min and terminated by the addition of 1 ml of chloroform. The concentration of bilirubin was determined as the difference between the concentrations measured at 464 nm and 530 nm. The results were expressed as picomoles of bilirubin per milligram of protein.

## Quantitative real-time polymerase chain reaction

Total RNA was extracted from MC3T3-E1 cells using TRIzol (Invitrogen Life Technologies, Paisley, UK) according to the manufacturer's instructions and reverse transcribed. Real-time polymerase chain reaction was performed using SsoFast EvaGreen Supermix (Bio-Rad, Hercules, CA) and conducted with the Bio-Rad CFX96 (Bio-Rad). The primers for Bax were 5'-TGAAGACAGGGCCTTTTGTG-3' and 5'-AATTCGCCGGAGACTCG-3'. The primers for Bcl-2 were 5'-GTCGCTACCGTCGTGACTTC-3' and 5'-CAGACATGCACCTACCAGC-3'. The primers for  $\beta$ -actin were 5'-GGCTGTATCCCTCCATCG-3' and 5'-CCAGTTGGTAACAATGCCATGT-3'.

## Western blot analysis

Protein extracts were resolved on sodium dodecyl sulfate–polyacrylamide gels and transferred to polyvinylidene difluoride membrane. The membrane was blocked with 5% freshly prepared milk-tris buffered saline Tween 20 for 2 h at room temperature and then incubated overnight at 4°C with antibodies specific for  $\beta$ -actin (Abcam, Cambridge, MA), HO-1, Bax, Bcl-2 (Beyotime), ERK1/2 and pERK1/2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Signals were detected with horseradish peroxidase–labelled secondary antibodies by chemiluminescence labelling.

## Statistical analysis

The results were presented as means  $\pm$  standard deviation. The significance between two groups was determined using Student *t* test. One-way analyses of variance were used for multiple comparisons. *p* Values less than 0.05 were considered statistically significant. All statistical analyses were performed using the SPSS 16.0 software (SPSS, Chicago, IL, USA).

## Results

### Dex induces the apoptosis of MC3T3-E1 cells

Previous studies have indicated that osteoblasts are one of the main targets for GCs. The effect of Dex on the viability of osteoblasts was determined in mouse osteoblastic MC3T3-E1 cells. Significant declines in cell viability were observed after 24 h of treatment with 1 or 5  $\mu$ M Dex as compared with the control (Fig. 1A). We then examined the cellular apoptosis using Annexin V-FITC/PI double staining method. The apoptotic rates of MC3T3-E1 cells were significantly increased after treatment with 1 or 5  $\mu$ M Dex as compared with the control (Fig. 1B). We also examined the expression of apoptosis-related molecules. Dex treatment reduced Bcl-2 expression but increased Bax expression (Fig. 1C, D). All data demonstrated that Dex can promote apoptosis of mouse osteoblastic MC3T3-E1 cells.

### CoPP induces HO-1 expression in MC3T3-E1 cells

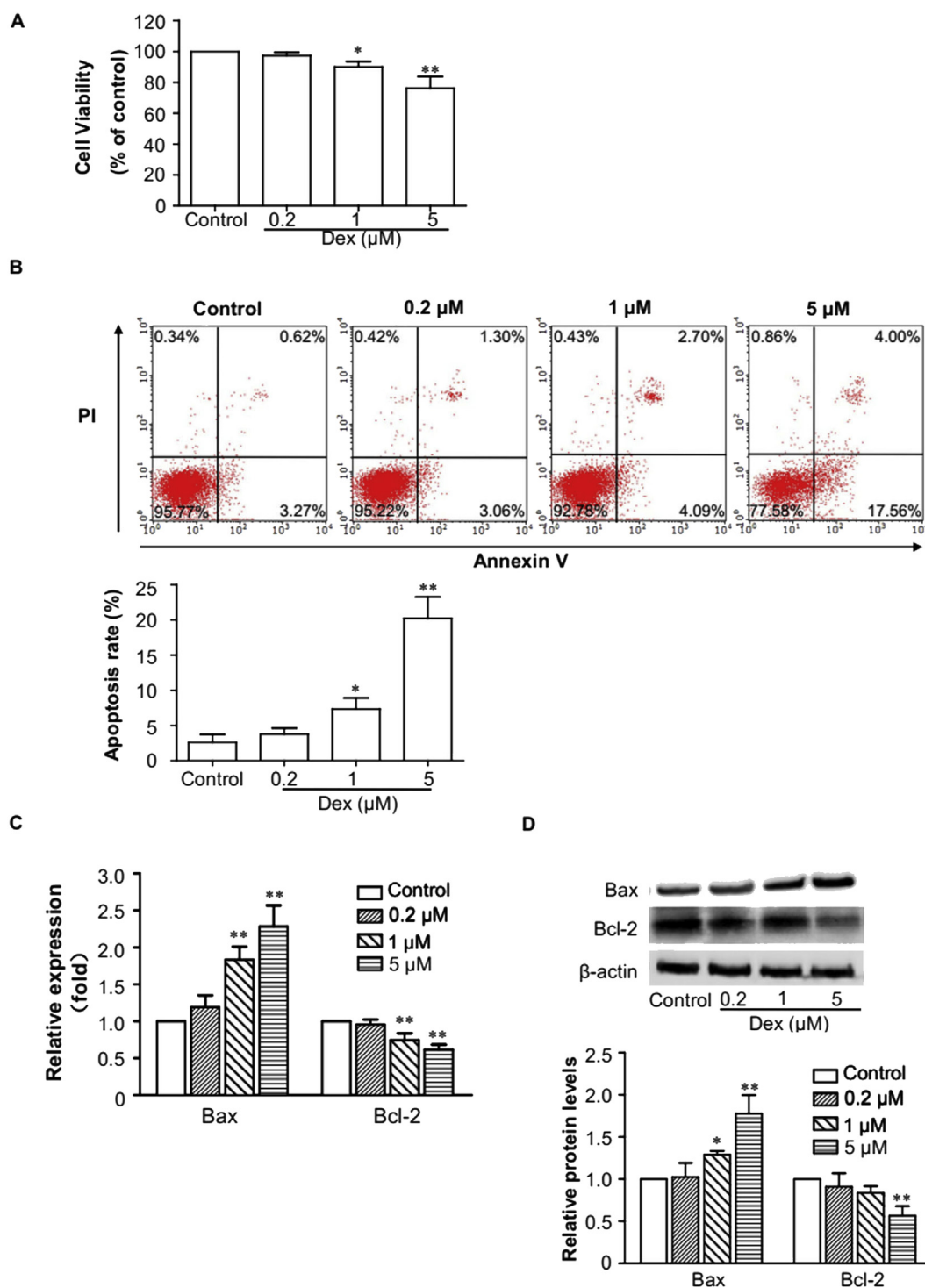
CoPP is a potent HO-1 inducer. To test whether CoPP could induce HO-1 expression in MC3T3-E1 cells, we performed Western blotting to determine the protein level of HO-1. MC3T3-E1 cells were cultured under different concentrations of CoPP for 36 h. CoPP increased the expression of HO-1 in MC3T3-E1 cells in a dose-dependent manner (Fig. 2A). Particularly, the incubation of MC3T3-E1 cells with 5  $\mu$ M CoPP caused a significant decrease in cell viability. CoPP at the concentration of 3  $\mu$ M did not affect the viability of MC3T3-E1 cells (Fig. 2B). Thus, we chose 3  $\mu$ M as the optimal concentration of CoPP to induce HO-1 in MC3T3-E1 cells.

To test whether HO-1 is involved in Dex-induced apoptosis of MC3T3-E1 cells, we then examined the effect of Dex on HO-1 expression. The results showed that Dex treatment caused a significant decrease in HO-1 expression as compared with the control (Fig. 2C). However, CoPP preconditioning significantly increased HO-1 expression as compared with Dex-treated group (Fig. 2C).

In addition, flow cytometry results showed that Dex significantly increased reactive oxygen species (ROS) generation as compared with the control. CoPP preconditioning reduced ROS levels as compared with the Dex-treated group (Fig. 2D).

### HO-1 induction prevents Dex-induced apoptosis of MC3T3-E1 cells

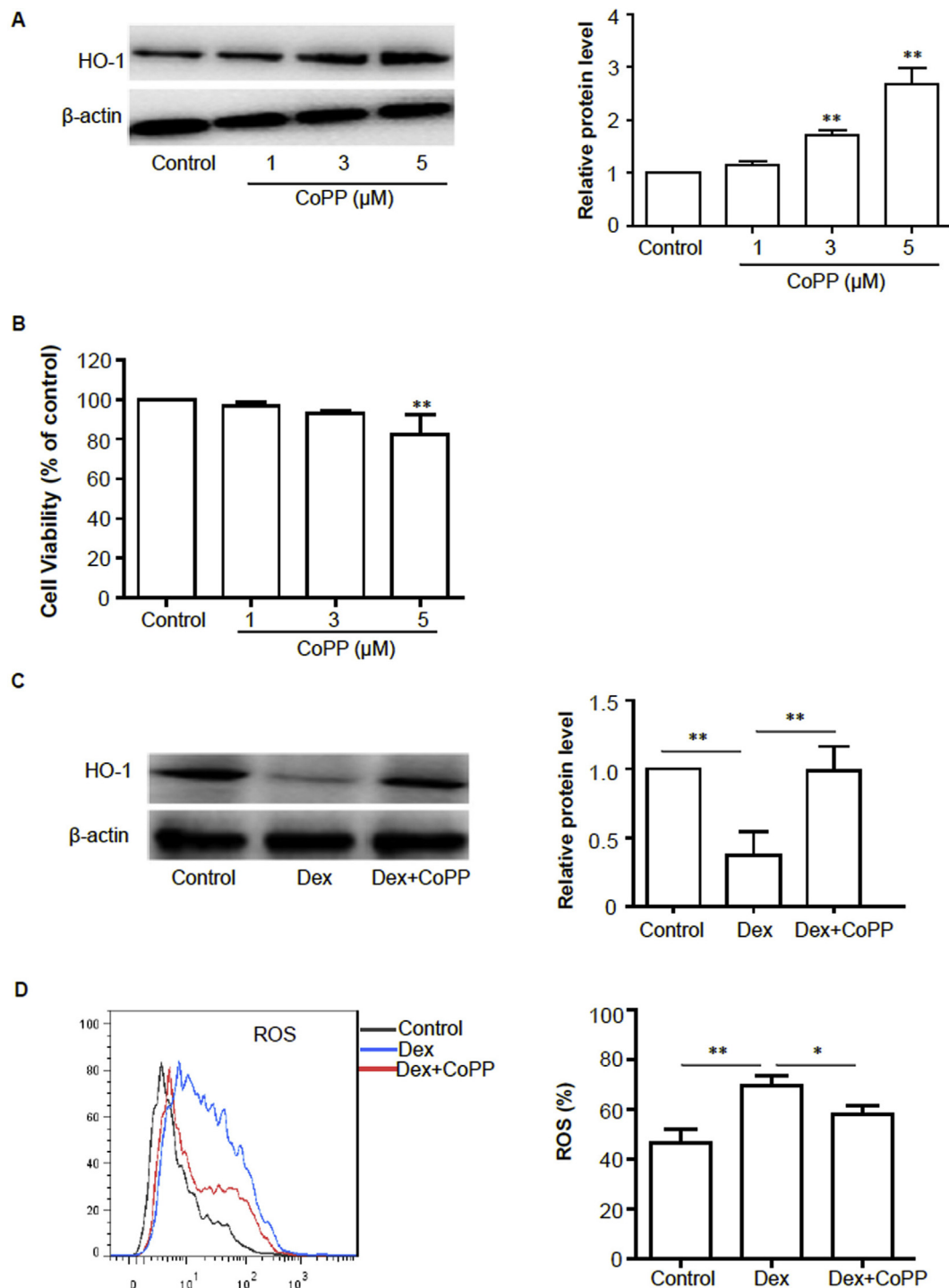
As expected, in the presence of Dex, cells pretreated with CoPP showed a significant decrease in the rate of apoptosis (Fig. 3A). However, the beneficial effects of CoPP were reversed by additional pretreatment with SnPP, a HO-1



**Figure 1** The effects of Dex on the apoptosis of MC3T3-E1 cells. Mouse osteoblast MC3T3-E1 cells were cultured with indicated concentrations of Dex for 24 h. (A) Cell viability was measured using a CCK-8 kit. (B) Cell apoptosis was measured by flow cytometry. (C) The expressions of Bax and Bcl-2 were analyzed by real-time PCR. (D) The expressions of Bax and Bcl-2 were analyzed by Western blot. Data were represented as means  $\pm$  SD of three individual experiments. \* $p < 0.05$ , \*\* $p < 0.01$ . Dex = dexamethasone; PCR = polymerase chain reaction; SD = standard deviation; CCK-8 = cell counting kit-8.

inhibitor (Fig. 3A). SnPP can suppress HO-1 activity. We then analyzed the activity of HO-1 in MC3T3-E1 cells. As shown in Fig. 3B, Dex showed a tendency to reduce HO-1 activity in MC3T3-E1 cells. CoPP treatment increased HO-

1 activity significantly as compared with the Dex-treated group. The effects of CoPP were reversed by additional pretreatment with SnPP. We further assessed the mRNA expression levels of Bax and Bcl-2. Dex increased Bax

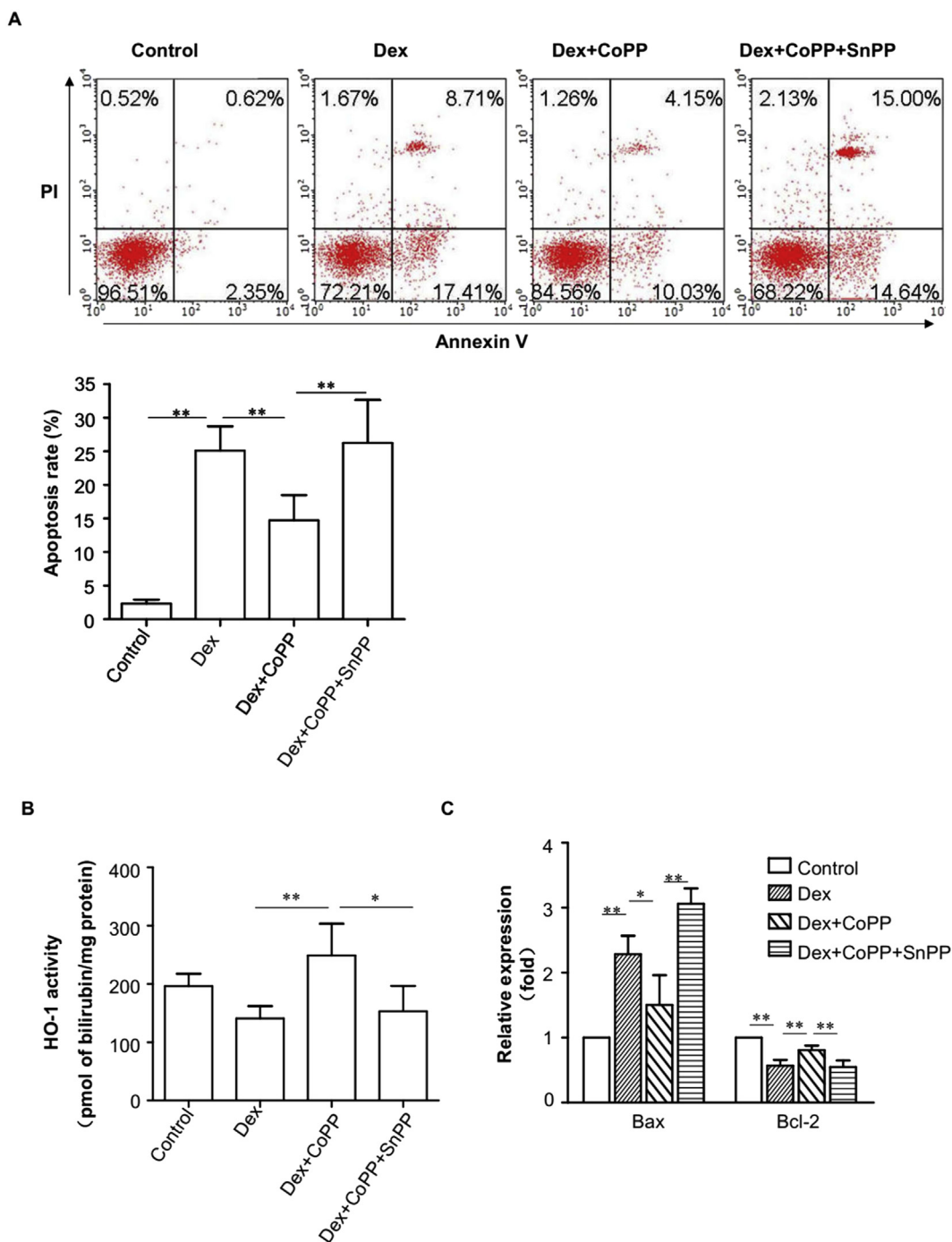


**Figure 2** The effects of CoPP on HO-1 expression in MC3T3-E1 cells. (A) Mouse osteoblast MC3T3-E1 cells were cultured with indicated concentrations of CoPP for 36 h. Western blot and quantitative analysis were performed to detect the expression of HO-1. (B) Cell viability was measured using a CCK-8 kit. (C) CoPP was added 12 h before Dex treatment. The expression of HO-1 was measured by Western blot 24 h after Dex treatment. (D) The expression of ROS was measured by flow cytometry. Data were represented as means  $\pm$  SD of three individual experiments. \* $p < 0.05$ , \*\* $p < 0.01$ .

CoPP = cobalt (III) protoporphyrin IX chloride; Dex = dexamethasone; HO-1 = haem oxygenase-1; ROS = reactive oxygen species; SD = standard deviation; CCK-8 = cell counting kit-8.

expression and inhibited Bcl-2 expression in MC3T3-E1 cells. CoPP treatment negated the effects of Dex on Bax and Bcl-2 expression. The effects of CoPP were also reversed by

additional pretreatment with SnPP (Fig. 3C). These results demonstrate that HO-1 induction by CoPP protects MC3T3-E1 cells against Dex-induced apoptosis.



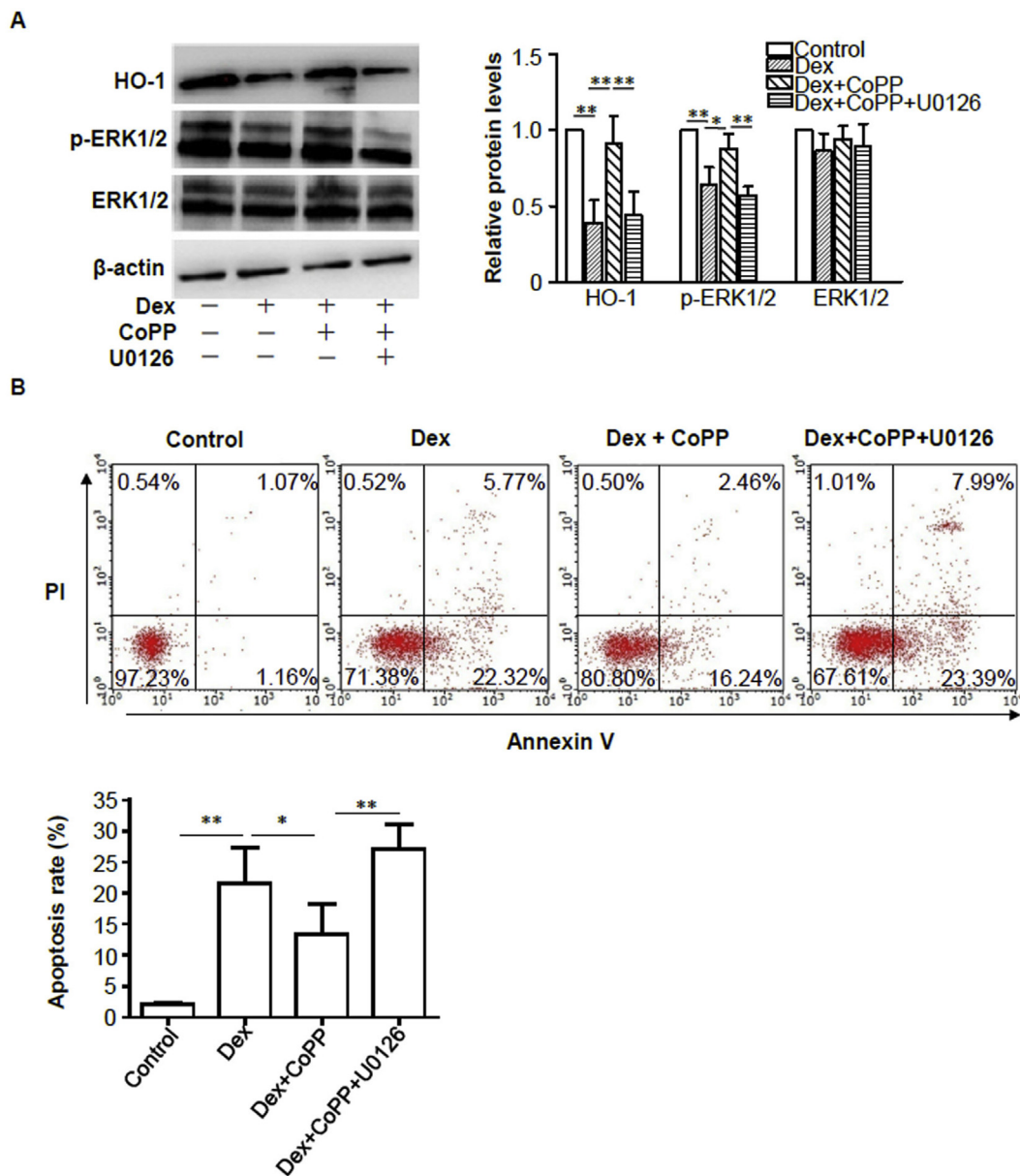
**Figure 3** The effects of HO-1 induction on Dex-induced apoptosis of MC3T3-E1 cells. Mouse osteoblast MC3T3-E1 cells were preconditioned with CoPP or SnPP 12 h before Dex treatment. (A) Cell apoptosis was measured by flow cytometry. (B) HO-1 activity was analyzed based on bilirubin formation. (C) The expressions of Bax and Bcl-2 were analyzed by real-time PCR. The data were represented as means  $\pm$  SD of three individual experiments. \* $p < 0.05$ , \*\* $p < 0.01$ .

CoPP = cobalt (III) protoporphyrin IX chloride; Dex = dexamethasone; HO-1 = haem oxygenase-1; PCR = polymerase chain reaction; SnPP = tin protoporphyrin IX; SD = standard deviation.

### HO-1 induction prevents Dex-induced apoptosis through ERK1/2 signalling

ERK pathway has been reported to participate in cell survival. To further elucidate the mechanism involved in the antiapoptotic effect of HO-1, we examined the activation

of ERK pathway in MC3T3-E1 cells. As shown in Fig. 4A, Dex inhibited the phosphorylation of ERK1/2 but did not affect total ERK1/2 expression. CoPP treatment restored the phosphorylation of ERK1/2. U0126, an inhibitor of ERK activation, significantly inhibited the phosphorylation of ERK1/2. Moreover, CoPP-induced HO-1 expression was also



**Figure 4** The effects of HO-1 induction on ERK1/2 activation in MC3T3-E1 cells. Mouse osteoblast MC3T3-E1 cells were pre-conditioned with CoPP 12 h before Dex treatment. U0126 was added 1 h before CoPP treatment. (A) Western blot and quantitative analysis were performed to detect the expression of HO-1, ERK1/2 and pERK1/2. (B) Cell apoptosis was measured by flow cytometry. \* $p < 0.05$ , \*\* $p < 0.01$ .

CoPP = cobalt (III) protoporphyrin IX chloride; Dex = dexamethasone; ERK = extracellular signal-regulated kinase; HO-1 = haem oxygenase-1.

reduced by U0126 (Fig. 4A). Flow cytometry analysis showed that U0126 abrogated the antiapoptotic effect of CoPP (Fig. 4B). These results indicate that HO-1 induction may alleviate Dex-induced apoptosis through the activation of ERK1/2 signalling pathway.

## Discussion

GIOP is the most prevalent drug-induced osteoporosis, characterized by reduced bone formation and increased bone resorption. Increased osteoblast apoptosis is thought

to be one of the main mechanisms that account for the disorder of bone metabolism. In the present study, we investigated the role of HO-1 in Dex-induced osteoblast apoptosis. We provide evidence that HO-1 induction by CoPP protects mouse osteoblasts against Dex-induced apoptosis through the activation of ERK1/2 signalling pathways.

Dex has been shown to induce apoptosis in osteoblastic cell lines (MC3T3-E1, UAMS-32 cell) [24,25]. Consistent with previous reports, we find high-dose Dex (1 or 5  $\mu$ M) can induce apoptosis of MC3T3-E1 cells within 24 h as evidenced by Annexin V/PI staining. This was also confirmed by the

expression of apoptosis-related molecules. Dex treatment significantly increased bax expression while reduced bcl-2 expression. The proliferation of MC3T3-E1 cells was also reduced after Dex treatment. Although osteoblast apoptosis is commonly accepted as a key player in the pathogenesis of GIOP, the underlying mechanism remains poorly understood. Previous studies have shown that Dex induces osteoblast apoptosis by increasing E4BP4 expression or ER stress [26–28]. Autophagy activation also participates in Dex-induced apoptosis [28]. In the present study, we find that Dex significantly reduced HO-1 expression after incubation for 24 h with MC3T3-E1 cells. HO-1 is a stress-responsive enzyme that exerts potent cytoprotective effects in a wide range of cells and disease models. HO-1 deficiency results in increased apoptotic cells in mouse placentas [29]. Carbon monoxide, one of the by-products of HO-1, attenuates tumor necrosis factor- $\alpha$ -induced apoptosis of osteoblasts [30]. Based on previous studies, our findings suggest that HO-1 may participate in Dex-induced apoptosis of osteoblasts. Consistent with our results, Singh and Haldar [31] also observed that Dex represses HO-1 expression in peripheral blood mononuclear cells. However, Han et al [32] have reported that Dex increases HO-1 expression after incubation for 6 h with MC3T3-E1 cells. The difference may be caused by different culture conditions. HO-1 is responsible for ROS scavenging [33]. HO-1 deficiency results in increased ROS levels in visceral adipose precursor cells [34]. ROS induction leads to oxidative stress, a process that alters bone metabolism and contributes to the pathogenesis of osteoporosis [35]. As expected, we find a concomitant increase of ROS levels in MC3T3-E1 cells treated with Dex. Previous studies have reported that ROS is responsible for Dex- or homocysteine-induced apoptosis of osteoblasts [24,36]. This result provides further evidence that HO-1 is involved in Dex-induced apoptosis of MC3T3-E1 cells.

CoPP is a metalloprotoporphyrin used as a powerful inducer of HO-1. HO-1 induction by CoPP can protect astroglia against manganese-induced apoptosis or attenuate hepatocyte apoptosis. In the skeletal system, CoPP-induced HO-1 can protect osteoarthritic osteoblasts from senescence. In the present study, we find that 3  $\mu$ M CoPP significantly induced HO-1 expression in MC3T3-E1 cells without affecting their apoptosis. Most importantly, preconditioning cells with CoPP for 12 h significantly inhibited Dex-induced apoptosis. The protective effects of CoPP can be reversed by SnPP, an inhibitor of HO-1. Moreover, HO-1 induction by CoPP significantly inhibited Bax expression and increased Bcl-2 expression, which further confirmed that HO-1 induction attenuates Dex-induced apoptosis. Our data indicate a therapeutic potential for HO-1 against Dex-induced apoptosis of osteoblasts.

We also find that the antiapoptotic effects of HO-1 induction were associated with the activation of ERK1/2. The ERK1/2 pathway regulates cell apoptosis, proliferation, autophagy and differentiation. Previous studies have reported that ERK activation can attenuate osteoblast apoptosis induced by Dex or 17 $\beta$ -estradiol [37,38]. In the present study, using U0126, an inhibitor of ERK activation, we find that inhibition of ERK activation significantly abrogated the protective effect of CoPP against Dex-induced apoptosis. Moreover, inhibition of ERK activation

also reduced the expression of HO-1 in MC3T3-E1 cells. These results suggest that CoPP triggers the activation of ERK1/2 pathways, which induces an antiapoptotic effect in osteoblasts. Previous studies have shown that ERK kinase can phosphorylate Bad or Nrf2 [39,40]. Bad is a Bcl-2 family protein, and its phosphorylation can prevent apoptosis. Nrf2 is a key transcription factor that mediates antioxidant and antiinflammatory responses. ERK/Nrf2 signalling pathway plays an important role in CoPP-induced antiapoptotic response. However, identifying the downstream target of ERK needs more investigation.

## Conclusion

In summary, we find that preconditioning of MC3T3-E1 cells with CoPP alleviates Dex-induced apoptosis through activation of ERK1/2 signalling. These results suggest that HO-1 participates in Dex-induced osteoblast apoptosis. HO-1 induction may be a promising therapeutic strategy against GC-induced osteoporosis.

## Conflict of interest

The authors declare no conflict of interest.

## Acknowledgement

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