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Parathyroid Hormone (PTH) Induces Autophagy to Protect Osteocyte Cell Survival from Dexamethasone Damage

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Statistical Analysis C
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Manuscript Preparation E
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Background: Glucocorticoids (GC) have direct adverse effects on osteocytes, the most abundant bone cell type, and play an important role in osteonecrosis of the femoral head (ONFH). Teriparatide has been reported to be an effective treatment for ONFH. However, the underlying mechanism is unclear.

Material/Methods: An osteocyte cell line, MLO-Y4, was used under various doses of dexamethasone (Dex) with or without rhPTH (1–34). Cell viability, autophagy, and apoptosis markers and osteocyte characteristic mRNAs were investigated to better understand this phenomenon.

Results: Induction of apoptosis by Dex was increased in a time- and dose-dependent manner in MLO-Y4 cells. Autophagy markers (LC3-II and Beclin-1) were increased at the low dose of Dex (10^{-7} or 10^{-6} M) and decreased at the high dose (10^{-5} M). In MOL-Y4 cells, rhPTH (1–34) was shown to be protective against Dex-induced apoptosis. The upregulation of LC3-II and Beclin-1 and decreased level of Caspase-3 was observed in the rhPTH (1–34)-treated group compared with the Dex-only-treated group. Furthermore, the changes induced by Dex in osteocytes, such as increased SOST, RANKL, and DMP-1 mRNA level and decreased Destrin mRNA level, were reversed by rhPTH (1–34). A similar result was found in osteocyte-specific proteins sclerostin expression encoded by SOST mRNA, which acted as a bone formation inhibitor.

Conclusions: The self-activation of autophagy may be a protective mechanism against apoptosis induced by Dex. The protection effect of rhPTH (1–34) for GC-induced ONFH thus results, at least in part, from enhanced autophagy.

MeSH Keywords: Dexamethasone Isonicotinate • Osteocytes • Osteonecrosis • Parathyroid Hormone

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Background

Non-traumatic osteonecrosis of the femoral head (ONFH) often leads to progressive collapse of the femoral head. Glucocorticoid (GC) administration was considered to be the most common risk factor for osteonecrosis [1]. Many etiologies that disrupt the blood circulation of the femoral head ultimately lead to an imbalance of self-repair and necrosis developed finally. However, the mechanism of ONFH is unclear. Many studies have shown that the damage to the bone structure and changes in macromechanical properties lead to collapse during the process of bone restoration [2]. Although it is possible to diagnose osteonecrosis before clinical symptoms appear, especially with magnetic resonance imaging (MRI), there is still no satisfactory treatment for early-stage ONFH.

In addition to bisphosphonates and statins, teriparatide (rhPTH[1–34]), which is a bone anabolic agent approved to treat osteoporosis [3], has been reported to benefit ONFH in an animal model. *In vitro* and *in vivo* studies have shown that rhPTH(1–34) protects osteoblasts and osteocytes from apoptosis and then stimulated new bone formation [4,5]. Osteocytes, the most abundant bone cell type, are a heterogenous population of terminally differentiated osteoblasts that direct bone remodeling in response to applied mechanical loading of bone. GCs have direct adverse effects on osteoblasts, osteoclasts, and osteocytes, decreasing lacunar canalicular fluid, bone vascularity, and bone strength via their effects on osteocytes [6]. Furthermore, GCs are known to induce osteocyte apoptosis [7,8] and to increase circulating SOST mRNA or sclerostin levels [9,10]. Therefore, osteocytes have a vital role in GC-induced ONFH. Autophagy is a lysosomal degradation pathway that is essential for cell growth, survival, differentiation, development, and homeostasis [11]. As autophagy is a regulated process that helps to maintain a balance, it can be a self-repair process that leads to cell death. Autophagy is involved in the effect of GCs on osteocytes, and the cell fate is determined by GCs dose [12].

However, the mechanisms underlying the beneficial effect of rhPTH(1–34) in treatment of ONFH in animal models are unclear. This is the first study to show that autophagy enhanced by rhPTH(1–34) protects osteocyte cell survival from Dex damage and reversed Dex-induced changes in MLO-Y4 cells.

Material and Methods

Cell culture and treatment

MLO-Y4 osteocyte cells were provided by Prof. Bonewald (University of Missouri). Cells were cultured in phenol red-free α-MEM supplemented with 10% FBS and antibiotics (100 U/mL penicillin G and 100 mg/mL streptomycin). Medium was added to the wells at the same time as the cells were seeded, and was changed every 48 h thereafter. Treatment of cells for all assays consisted of incubation with fresh medium containing different concentrations of Dexamethasone (Dex) (Sigma, St. Louis, MO, USA) with or without rhPTH (1–34) (Sangon Biological Engineering Technology Company, Shanghai, China). The current study was approved by the Ethical Committee of Fudan university, China.

Cell viability assay and TUNEL staining

Cell proliferation was assessed using a CCK8 assay. Cells were resuspended in a 200-µl cell culture medium and seeded at a density of 1×10^3 cells/ml in 96-well microtiter plates, then incubated overnight for cell attachment. Viable cells were measured at 24, 48, and 72 h by CCK-8 (Dojindo Laboratory, Kumamoto, Japan) after Dex treatment. The result of cell viability measurement is expressed as the absorbance at OD_{450} and the value of absorbance was defined as 100% survival. Cells seeded on coverslips and grown in medium containing Dex with or without rhPTH (1–34) for 72 h, then we used the One Step TUNEL Apoptosis Assay Kit (Beyotime Institute of Biotechnology) according to the manufacturer's instructions. The results were examined by a fluorescence microscope (ZEISS, AXIO)

Indirect immunofluorescence analysis

Cells were seeded on coverslips and grown in medium contain Dex for 72 h. Cells were then fixed and the epitope was blocked. Rabbit anti-mouse Caspase 3, Sclerostin, or F-actin antibody was added to the cells and incubated at 4°C overnight. Then, goat anti-rabbit fluorescent antibody and DAPI were added and incubated at room temperature for 1 h. Samples were observed by a microscope (ZEISS, AXIO).

Quantitative real-time PCR

Expression of osteocyte genes was analyzed with quantitative real-time PCR according to the manufacturer's instructions. In brief, total RNA was extracted from osteocytes under Dex treatment with or without rhPTH(1–34) using TRIzol reagent (Invitrogen, Life Technologies; Carlsbad, CA, USA). Total RNA (2 µg) was used to generate single-stranded cDNA. Then, SYBR Green PCR Master Mix was used for PCR on an ABI 7500 Real-Time PCR System (Applied Biosystems 7500 System, Foster City, CA, USA). The sense and antisense primers used were as follows: mouse SOST sense, 5'-ACAACCAGACCATGAACCG-3' and antisense, 5'-CAGGAAGCGGGTGTAGTG-3'; mouse Destrin sense, 5'-CTTTGTATGACGCCAGCTTTG-3' and antisense, 5'-ATCCTTCGAGCTTGCATAGATC-3'; mouse RANKL sense, 5'-ACTTTCGAGCGCAGATGGAT-3' and antisense, 5'-CCAGAGTCGAGTCCTGCAAA-3'; dentin matrix protein 1 (Dmp1) sense, 5'-CTGAAGAGAGGACGGGTGATT-3' and antisense, 5'-CGTGTGGTCACTATTTGCCTG-3';mouse

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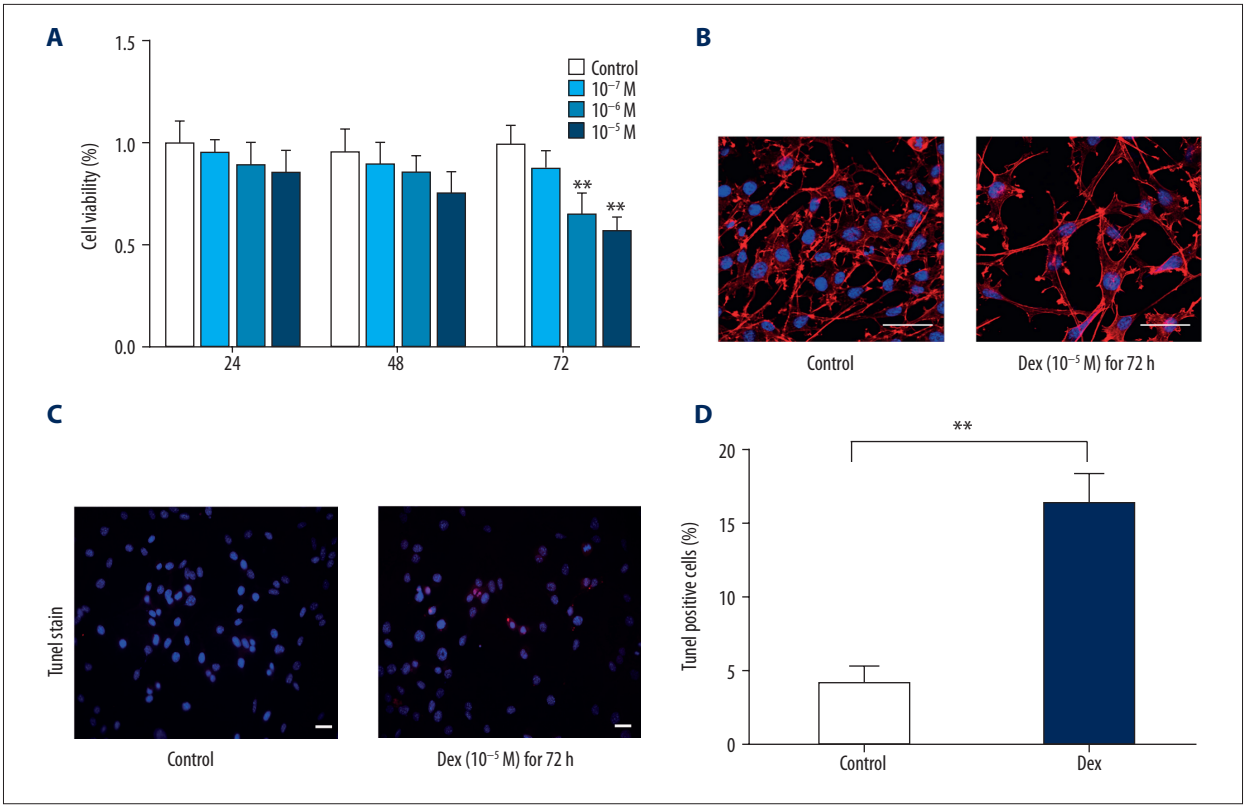

Figure 1. Effect of Dex in MLO-Y4 osteocyte cells. (**A**) Proliferation of MLO-Y4 cells was measured by CCK-8 after cells were treated at different concentrations of Dex from day 1 to day 3 (time interval 24 h). ** p<0.01. (**B**) Confocal image of the cells with 10^{-5} M Dex for 72 h. Scale bars are 20 μm. (**C**) The TUNEL stain of the MLO-Y4 cells with 10^{-5} M Dex for 72 h. Scale bars are 30 μm. (**D**) Five different fields were examined in each experiment. Statistical analysis of TUNEL-positive cells with or without 10^{-5} M Dex for 72 h. ** p<0.01.

β-actin sense: 5'-CTGTCCCTGTATGCCTCTG-3' and antisense, 5'-ATGTCACGCACGATTTCC-3'.

Western blot analysis

The protein samples were extracted from osteocytes under Dex treatment with or without rhPTH(1–34) for 72 h. Protein samples were fractionated by SDS-PAGE and electrophoretically transferred onto a nitrocellulose membrane. After blocking, the membrane was incubated overnight in 1 of the following primary antibodies: cleaved caspase 3 (1: 1000), Cleaved-PARP (1: 1000), Beclin-1 (1: 1000), or LC3 (1: 1000), and β-actin (1: 1000) was used as an internal control (Cell signaling technology, Beverly, MA, USA). After 2-h incubation with horseradish peroxidase-conjugated secondary antibody (1: 5000), the images were detected using the Enhanced Chemiluminescence Western Blot System (Amersham Biosciences).

Statistical analysis

Experiments were repeated at least 3 times and results are expressed as the means ±SD. Differences between treated and untreated groups were assessed by the *t* test. One-way ANOVA models were used to compare multiple comparisons with Tukey's Studentized range test as post hoc analysis. The significance level was set at 0.05.

Results

GC treatment reduced the viability of osteocytes.

A previous study reported that after exposure for 6 to 24 h, MLO-Y4 cells treated with 10^{-6} M Dex had dendritic shortening [13]. Therefore, in our experiment, the longer-term effect of Dex stimulation on the osteocyte was determined using the CCK-8 assay. As shown in Figure 1A, Dex reduced the numbers of MLO-Y4 cells in a time- and dose-dependent manner compared with the control group. During the short-term treatment (24–48 h) in all groups, the difference was not significant. However, following 72-h treatment, the viability of cells in the 10^{-6} M and 10^{-5} M groups was significantly decreased (p<0.01). To further observe the character of osteocytes, the confocal image of osteocytes showed that the number of dendrites and

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cells decreased with Dex (10^{-5} M) treatment compared with controls (Figure 1B). Furthermore, as shown in TUNEL staining (Figure 1C, 1D), after 72-h exposure of Dex, cells exhibited an increased ratio of TUNEL-positive (bright red nuclei indicative of apoptosis) cells compared with controls ($p<0.01$). In brief, results indicated that higher level and longer treatment with Dex resulted in significant levels of apoptosis-associated cell death.

GC treatment induced apoptosis and autophagy in osteocytes

To investigate the apoptosis and autophagy activity after Dex exposure, Western blot analysis was used. As shown in Figure 2A, after 72-h exposure, the increase in LC3-II levels starting from the lowest dose (10^{-7} M), and the maximal response was seen at 10^{-6} M Dex. The level of Beclin-1 expression was increased most at 10^{-6} M Dex. Interestingly, when the MLO-Y4 cells were treated with 10^{-5} M Dex, the expression of LC3-II and Beclin-1 were significantly reduced compared to the 10^{-6} M Dex group, which was almost back to normal level (Figure 2B, 2C). However, the apoptosis marker Cleaved-PARP and Cleaved Caspase 3 in the 10^{-5} M Dex group were still significantly increased compared with controls ($p<0.01$) (Figure 2D–2F). These observations suggest that GCs treatment induces autophagy and apoptosis in osteocytes, and the autophagy can be suppressed at high level of GCs.

Activation of autophagy by rhPTH(1–34) reversed Dex-induced osteocytes apoptosis and functions

rhPTH(1–34) has been reported to induce autophagy [14] and benefit the GC-induced ONFH [3]. To further confirm the protective effect of rhPTH(1–34), the cells were treated under 10 nM rhPTH(1–34) with or without Dex 10^{-5} M for 72 h. Compared with Dex-only treatment, co-culturing with rhPTH(1–34) enhanced the activity of autophagy (LC3-II and Beclin-1) and reduced the apoptotic rate (Figure 3).

To further investigate the function of osteocytes, quantitative PCR for several typical mRNAs were used. As shown in Figure 4, the level of SOST mRNA in the Dex group was increased ($p<0.01$), as was the RANKL and DMP-1 mRNA level, compared to the Dex+PTH and control groups. In contrast, the Destrin mRNA level was decreased ($p<0.01$). Furthermore, consistent with the SOST mRNA result, the expression of sclerostin, the protein product of SOST, suggests that rhPTH(1–34) reversed Dex-induced changes in osteocytes (Figure 4A).

Discussion

GCs are effective anti-inflammatory and autoimmune modulating agents. However, adverse effects, such as bone loss and even ONFH, can negate the benefits of GC treatment. GC-induced osteoporosis (GIO) has been widely investigated, and most researchers accept that GIO is caused primarily through direct effects of GCs in bone cells [15]. However, the mechanisms of GC-induced ONFH are still unclear. Given the close relationship between the GIO and GCs-induced ONFH, many GC-induced cellular reactions may be similar. Imbalance of bone formation and resorption, regional endothelium dysfunction, and cell apoptosis are considered to be potential pathological changes in GC-induced ONFH [16,17]. Many studies have shown that GC treatment decreased the number of cells and induced apoptosis and autophagy [18–20]. Weinstein suggested that GC-induced osteocyte apoptosis, a cumulative and irreparable defect, could uniquely disrupt the mechanosensory function of the osteocyte network and thus start the inexorable sequence of events leading to collapse of the femoral head [7]. A recent study reported that teriparatide (rhPTH[1–34]) benefitted ONFH in an animal model [3]. In the present study, we observed that rhPTH(1–34) induced autophagy to protect osteocyte cell survival from Dex damage.

The anti-apoptotic effect of PTH(1–34) was observed in various cells, including osteoblastic and osteocytic cell lines [21,22]. Furthermore, the dual effect of PTH, which was antiapoptotic in preconfluent cells and proapoptotic in more differentiated postconfluent cells, was reported in a study by Chen, showing that both antiapoptotic and proapoptotic process were dependent on cAMP activation, while cAMP-signaling played a critical role in controlling autophagy [23]. Autophagy is a well-conserved mechanism among species and is involved in various biological events [11]. In our study, the increase in LC3-II and Beclin-1 levels both starting from the lowest dose (10^{-7} M), and the maximal response was seen at 10^{-6} M Dex. However, when exposed to 10^{-5} M Dex, the expression of LC3-II and Beclin-1 were significantly reduced. Beclin-1 and LC3 are major regulators and markers of the autophagy pathway [24]. Jia reported that the activation of osteocyte autophagy was significantly increased when the cells were treated with a low dose of GC; higher doses of GC activated the gene pathway for osteocyte apoptosis, which was then significantly increased [12]. During the initial autophagy process, cells may be able to remain viable during periods of metabolic stress. However, higher and persistent stress may generate a large accumulation of autophagosomes, leading to cell death. Therefore, this process works both ways. The protective effect of rhPTH(1–34)- increased autophagy is likely a self-protective mechanism used by osteocytes to attenuate the effect of Dex on osteocytes.

rhPTH(1–34) reversed Dex-induced changes in osteocytes, not only in cell apoptosis, but also in the function of osteocytes. The Dex-induced upregulation of SOST, RANKL, and DMP-1 mRNA were reversed by hPTH(1–34). The osteocyte-specific proteins sclerostin encoded by SOST mRNA, which acted as a bone formation inhibitor, was increased under Dex stimulation.

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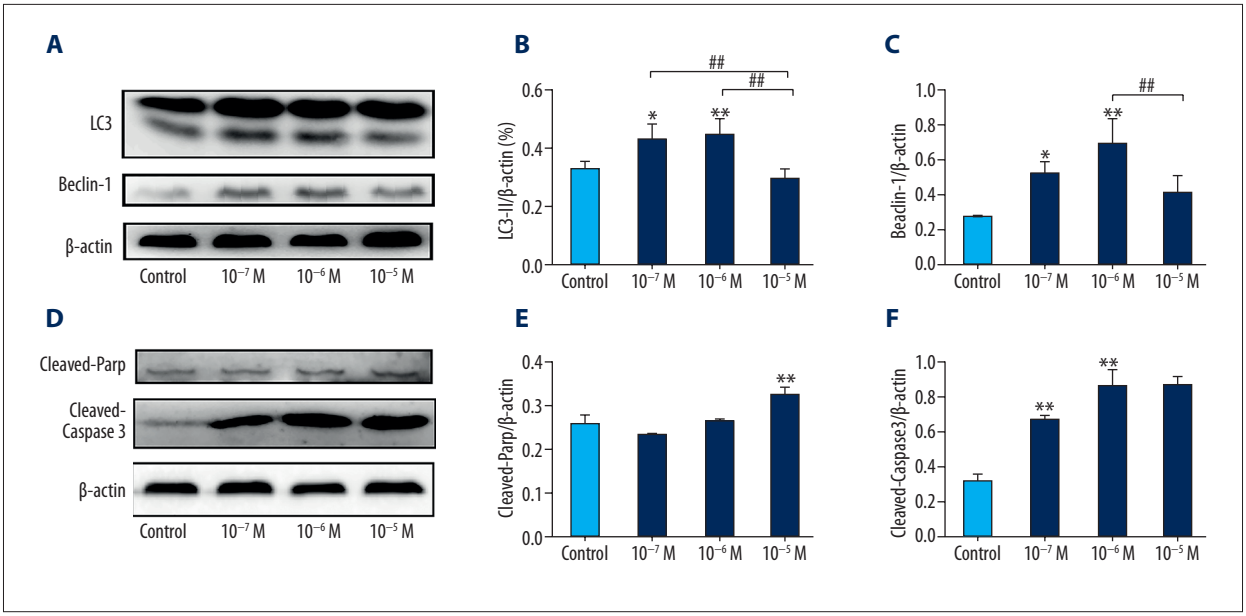

Figure 2. GC treatment induced apoptosis and autophagy in osteocytes. (**A**) Representative Western blot analysis of LC 3, Beclin-1, and β-actin in Dex (10^{-7}, 10^{-6}, 10^{-5} M)-treated MLO-Y4 cells (72 h). (**B**) Densitometric analysis was used to quantify the levels of LC 3-II and (**C**) Beclin-1. Values were normalized against β-actin. * p<0.05, ** p<0.01, *vs.* Control group; ## p<0.01. (**D**) Representative Western blot analysis of cleaved caspase-3, PARP, and β-actin in Dex (10^{-7}, 10^{-6}, 10^{-5} M)-treated MLO-Y4 cells (72 h). (**E**) Densitometric analysis was used to quantify the levels of cleaved caspase-PARP and (**F**) cleaved caspase-3. Values were normalized against β-actin. * p<0.05, ** p<0.01, *vs.* Control group.

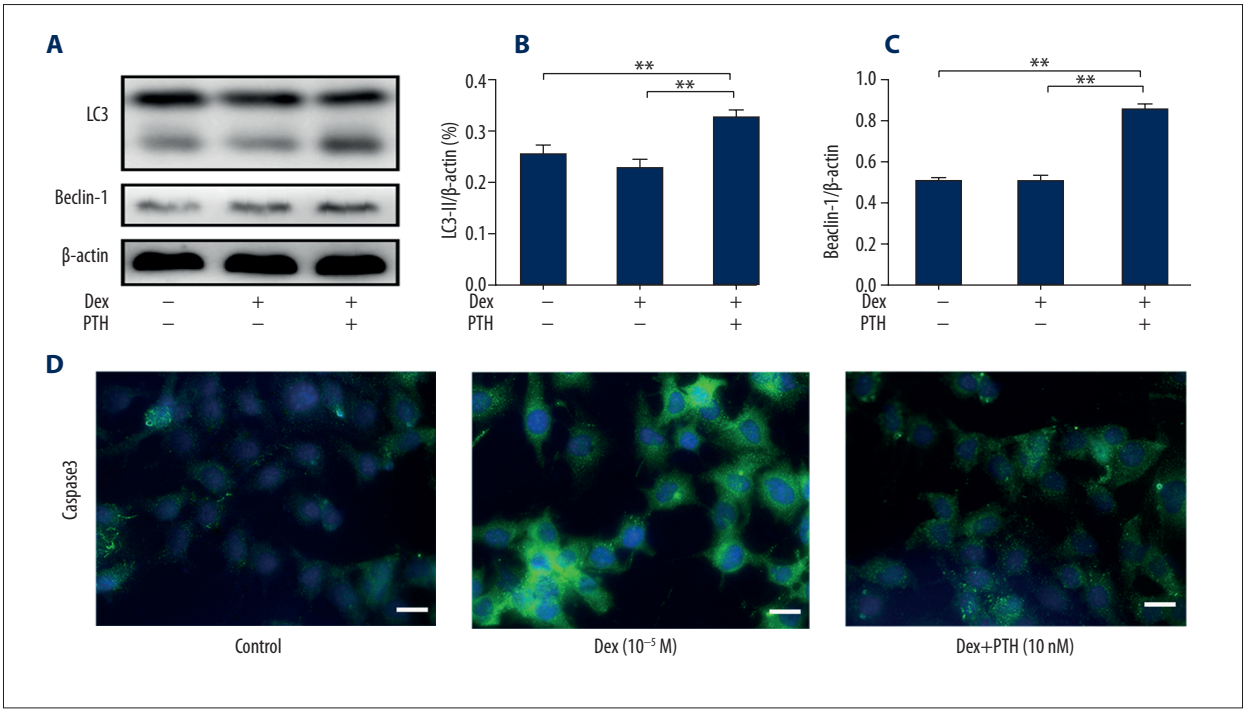

Figure 3. Effect of activation of autophagy by rhPTH(1–34) on apoptosis of osteocytes. (**A**) Representative Western blot analysis of LC 3, Beclin-1 and β-actin in MLO-Y4 cells treated as indicated for 72 h. (**B**) Densitometric analysis was used to quantify the levels of LC 3-II and (**C**) Beclin-1. Values were normalized against β-actin. * p<0.05, ** p<0.01; (**D**) The immunofluorescence image of Caspase-3 in the cells with 10^{-5} M Dex with or without 10 nM hPTH (1–34) for 72 h. Scale bars are 20 μm.

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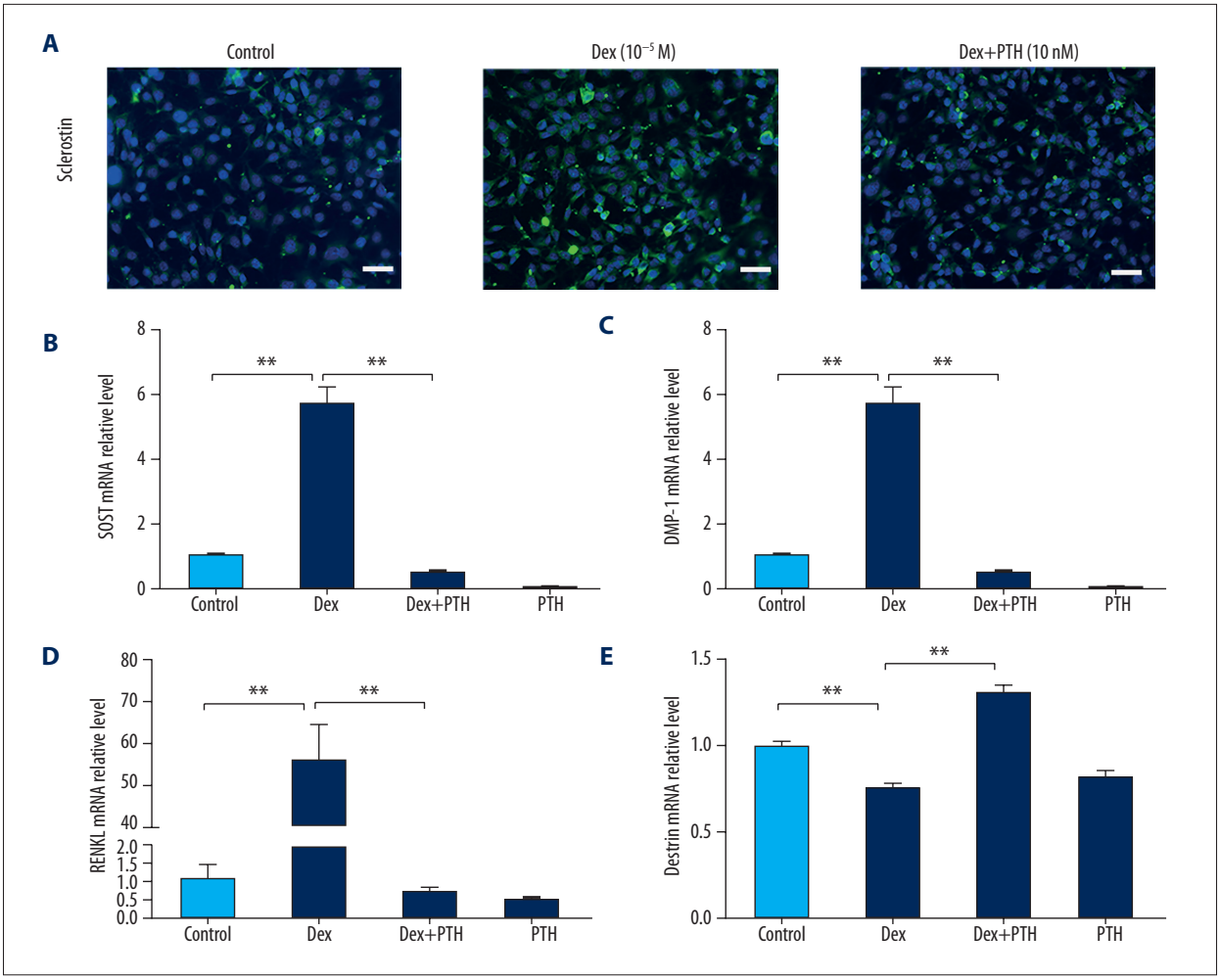

Figure 4. Effect of activation of autophagy by rhPTH(1–34) on function of osteocytes. (**A**) The immunofluorescence image of sclerostin in the cells with 10^{-5} M Dex with or without 10nM hPTH (1–34) for 72 h. Scale bars are 50 μm. (**B**) The relative expression of osteocytes SOST mRNA, (**C**) DMP-1 mRNA, (**D**) RANKL mRNA, and (**E**) Destrin mRNA in cells with 10^{-5} M Dex with or without 10nM hPTH (1–34) for 72 h. * P<0.05, ** P<0.01.

Increased RANKL mRNA showed the enhanced osteoclast activity induced by Dex. These may be reasons for GC-induced bone loss. DMP-1, a key molecule in controlling osteocyte formation and phosphate homeostasis, may be associated with cell apoptosis [25]. This result was consistent with the Western blot analysis, which indicated that pathological apoptosis markers caspase 3 and PARP were upregulated by Dex.

Osteocytes express type-1 PTH/PTHrP receptors (PTH1Rs), which are fully activated by aminoterminal PTH fragments and couple to multiple signal transducers, including adenylyl cyclase and phospholipase C. Intermittent administration of PTH is known to enhance bone mass, while continuous infusion of PTH decreases bone mineral density by increasing bone resorption [26]. Therefore, the regulation of PTH is a very delicate process that depends on the dose and frequency. In this study, Dex-induced apoptosis of osteocytes caused higher expression of SOST, RANKL, and DMP-1 mRNA and increased sclerostin secretion. These changes could be reversed by rhPTH (1–34).

Conclusions

We found that the self-activation of autophagy may be a protective mechanism against apoptosis induced by Dex. However, persistent exposure and high levels of GCs may result in osteocyte apoptosis and dysfunction. Furthermore, the protective effect of rhPTH (1–34) for GC-induced ONFH results at least in part from enhanced autophagy. Further study is required to elucidate the dose and frequency of PTH before application.

Conflict of interests

None.

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