PINK1-mediated mitophagy contributes to glucocorticoid-induced cathepsin K production in osteocytes

Jun Yuan a,b,1, You-shui Gao c,1, De-lin Liu a,b,1, Andrew Chi Pang Tai a,b, Hong Zhou d, John M. Papadimitriou e, Chang-qing Zhang c,***, Ming-hao Zheng a,b,**, Jun-jie Gao c,***

a Centre for Orthopaedic Translational Research, Medical School, The University of Western Australia, Nedlands, Western Australia, 6009, Australia
b Perron Institute for Neurological and Translational Science, Nedlands, Western Australia, 6009, Australia
c Department of Orthopaedic Surgery, Shanghai Jiao Tong University Affiliated Sixth People’s Hospital, Shanghai, 200233, China
d Bone Research Program, ANZAC Research Institute, The University of Sydney, Sydney, NSW, Australia
e Pathwest Laboratories and Faculty of Health and Medical Sciences, The University of Western Australia, Nedlands, WA, 6009, Australia

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ABSTRACT

Background: Glucocorticoid (GC) is one of frequently used anti-inflammatory agents, but its administration is unfortunately accompanied with bone loss. Although sporadic studies indicated that osteocytes are subject to a series of pathological changes under GC stress, including overexpression of cathepsin K, the definite role of osteocytes in GC-induced bone loss remains largely unclear.

Methods: Gene expression of Ctsk and protein levels of cathepsin K were assessed in MLO-Y4 cell lines exposed to dexamethasone (Dex) of different time (0, 12, 24 hours) and dose (0, 10-8 and 10-6 M) courses by RT-qPCR and western blotting, respectively. Confocal imaging and immunostaining were then performed to evaluate the effects of osteocyte-derived cathepsin K on type I collagen in a primary osteocyte ex vivo culture system. MitoTracker Red was used to stain mitochondria for mitochondria morphology assessment and JC-1 assay was employed to evaluate the mitochondria membrane potential in MLO-Y4 cells following Dex treatment. Activation of PINK1-mediated mitophagy was evaluated by immunostaining of the PINK1 protein and CytoID assay. Mdivi-1 was used to inhibit mitophagy and siRNAs were used for the inhibition of Pink1 and Atg5.

Results: GC triggered osteocytes to produce excessive cathepsin K which in turn led to the degradation of type I collagen in the extracellular matrix in a primary osteocyte ex vivo culture system. Meanwhile, GC administration increased mitochondrial fission and membrane depolarization in osteocytes. Further, the activation of PINK1-mediated mitophagy was demonstrated to be responsible for the diminishment of dysfunctional mitochondria in osteocytes. Examination of relationship between mitophagy and cathepsin K production revealed that inhibition of mitophagy via knocking down Pink1 gene abolished the GC-triggered cathepsin K production. Interestingly, GC’s activation effect towards cathepsin K via mitophagy was found to be independent on the canonical autophagy as this effect was not impeded when inhibiting the canonical autophagy via Atg5 suppression.

Conclusion: GC-induced PINK1-mediated mitophagy substantially modulates the production of cathepsin K in osteocytes, which could be an underlying mechanism by which osteocytes contribute to the extracellular matrix degradation during bone loss.

Abbreviations: GC, glucocorticoid; CTSK, cathepsin K; TRAP, tartrate-resistant acid phosphatase; CTX, carboxyterminal telopeptide of type I collagen; Drp1, dynamin-related protein 1; Mfn2, mitofusin-2; PINK1, PTEN-induced putative kinase 1; ΔѰm, mitochondria membrane potential; CCCP, Carbonyl cyanide chloroophenylhydrazone; MTR, MitoTracker Red; TFEB, transcription factor EB.

* Corresponding author.
** Corresponding author. Centre for Orthopaedic Translational Research, Medical School, The University of Western Australia, Nedlands, Western Australia, 6009, Australia.
*** Corresponding author.

E-mail addresses: zhangcq@sjtu.edu.cn (C.-q. Zhang), minghao.zheng@uwa.edu.au (M.-h. Zheng), colingjj@163.com (J.-j. Gao).

1 These authors have contributed equally to this work and share first authorship.

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1. Introduction

Glucocorticoid (GC), as an anti-inflammatory pharmacological agent, has been widely used for the treatment of a variety of inflammatory diseases, allergic and autoimmune disorders [1]. Despite its therapeutic efficacy, GC administration is often accompanied with bone loss, which is the most common cause of medication-induced osteoporosis [2]. Previous evidence suggests that GC induces bone loss by means of enhancing osteoclastic bone resorption [3] and inhibiting osteoblastic bone formation [4,5]. Even though osteoclasts and osteoblasts are recognized to be responsible for the pathological mechanism of GC-induced bone loss, the role of osteocytes in this pathological change remains unknown.

Cathepsin K (CTSK) is a cysteine protease mainly produced by osteoclasts and has the unique capacity to degrade type I collagen in an acidic environment [6]. Type I collagen is the most abundant collagen species contributing to the mechanical scaffold of bone matrix, and degradation of type I collagen has been recognized as the essential event in bone resorption [7]. Measurement of the serum carboxyterminal telopeptide of type I collagen (CTX), which is released by cathepsin K cleavage of type I collagen, is employed to assess bone resorption in patients with metabolic bone disease. Studies have reported an increasing concentration of serum CTX in patients accepting short-term GC therapy [8,9], indicating a crucial role of cathepsin K in GC-induced bone loss. Although osteocytes have been demonstrated to utilize mechanisms similar to those in osteoclasts to remove mineralized matrix by overexpressing tartrate-resistant acid phosphatase (TRAP) and cathepsin K during stressful conditions including GC treatment by the mean of autophagy coupled events. Intriguingly, osteocytes are suggested to respond to dysregulation of autophagic activity mediates the onset and progression of multiple bone diseases [13–16]. The study from Jia et al. reported the existence of autophagy and increased production of cathepsin K in osteocytes after GC administration [17], raising the possibility that autophagy activation and osteocytic cathepsin K overproduction could be coupled events. Intriguingly, osteocytes are suggested to respond to stressful conditions including GC treatment by the mean of autophagy [17,18], suggesting that autophagy in osteocytes could be a new target for preventing GC-induced osteoporosis. However, later research reported that suppression of autophagy in osteocytes could not reverse the negative impact of GC on bone mass [19], implying that some other biological processes, besides autophagy, also participate in the pathogenesis of GC-related bone loss. Mitophagy is a type of non-canonical autophagy that selectively removes damaged mitochondria via an autophagic mechanism [20,21]. Mitochondria as the so-called “powerhouse” of cells provide essential energy for cell metabolism. GC as a stress factor has been reported to have the potential to interfere with mitochondrial homeostasis and the dysfunction of mitochondria has been associated with various diseases [22,23]. A previous study has shown that GC can influence neuronal function and survival by directly regulating mitochondrial functions [24]. Given the fact that inhibition of autophagy cannot stop the GC-induced bone loss [19], investigating the role of mitophagy in this pathological process and its relationship with cathepsin K will be of high significance.

Herein, we demonstrated a novel mechanism whereby mitophagy in osteocytes contributes to GC-induced cathepsin K production. Using an ex vivo cell culture system, we observed that GC-induced production of cathepsin K in osteocytes caused the degradation of extracellular type I collagen. Meanwhile, we revealed that GC stress led to increasing mitochondrial fission as well as impaired mitochondria membrane potential (∆Ψm), and subsequent activation of PINK1-mediated mitophagy in osteocytes [25,26]. Additionally, we showed that GC-induced mitophagy, rather than canonical autophagy, promoted the production of cathepsin K in osteocytes. Our results therefore indicate a possible role of osteocyte mitophagy in GC-induced bone loss.

2. Materials and methods

2.1. Animals

C57BL/6 mice were maintained in-house with a 12-h light/dark cycle and normal chow diet. The use of animals was approved by Animal Ethics Committee of Shanghai Jiao Tong University Affiliated Sixth People's Hospital (NO. 2019-0312). All animal operative procedures and animal housing were conducted under Permission to Use Animals (PUA).

2.2. MLO-Y4 cell culture

MLO-Y4 cells were maintained in α-MEM (Gibco) supplemented with 10% fetal bovine serum (FBS; Gibco), 1% penicillin/streptomycin (P/S; Sigma–Aldrich) and incubated in a 5% CO2 incubator at 37 °C. Cells were seeded in six-well plates (Nuncun Delta, Thermo Fisher) at a density of 10^5 cells/well or on 12 mm coverslips (ProSciTech) at a density of 2.5 × 10^4 cells/slice. Treatment using dexamethasone (Dex; D-2915, Sigma–Aldrich) or mdivi-1 (M0199, Sigma–Aldrich) were started at time point 0 or 12 hours after initial seeding.

2.3. Ex vivo calvaria culture

Primary osteocyte ex vivo culture was conducted according to our previous study [27]. In brief, fresh calvaria with intact pericranium were extracted from young mice (1-week old). Following triple washing with phosphate buffered solution (PBS), and three washes with α-MEM containing 1% P/S, the pericranium were then gently stripped off from both sides of calvaria through scraping and extensive washing with microsurgical instruments under the dissecting microscope. The calvaria were then cut into small pieces with a diameter of 5 mm and were directly cultured with complete α-MEM media and incubated in a 5% CO2 incubator at 37 °C. Calvaria pieces were treated with Dex for different time courses (0, 12 and 24 h) and concentration courses (0, 10–8 M and 10–6 M).

2.4. Cell transfections

For siRNA transfection, cells were transfected with PINK1 siRNA (s206144, ThermoFisher), Atg5 siRNA (MSS247019, ThermoFisher) or negative control siRNA (AM4611, ThermoFisher) using Lipofectamine 3000 transfection reagents (L3000008, Invitrogen) following the manufacturer’s protocol. In brief, when cells reached an 80% confluence at transfection, Lipofectamine 3000 reagent was diluted in Opti-MEM and mixed well. Master mix of siRNA was prepared by diluting siRNA in Opti-MEM medium (111,058,021, Gibco) and mixed thoroughly, which was then added to each tube of diluted Lipofectamine 3000 reagent (1:1 ratio) and incubated at room temperature for 15 min. Subsequently, siRNA transfection was performed for 48 h followed with further analysis. Gene knock down efficiency was evaluated by analyzing corresponding gene and protein levels through real-time PCR and western blotting, respectively.
2.5. Western blotting

Cellular proteins were extracted by incubating in RIPA lysis buffer containing protease inhibitor cocktail (Roche) and phosphatase inhibitor cocktails (Sigma–Aldrich) for 20 min at 4 °C. Clarified cell lysate (centrifuge at 12,000 g for 20 min at 4 °C) were diluted and boiled at 95 °C with 4 × SDS sampling buffer for 5 min. Proteins were loaded in each lane and fractionated on 10%–17.5% SDS-PAGE gel and resolved protein transferred to nitrocellulose membrane (Millipore). Membranes were then blocked and incubated with primary antibodies including β-actin (JLA20 (1:5000, Developmental Studies Hybridoma Bank), VDAC1 (1:1000, ab15595, Abcam), TOM20 (1:500, sc-1774, Santa Cruz Biotechnology), Cox IV (1:1000, #4844, Cell Signaling Technology), cathepsin K (1:500, sc-48353, Santa Cruz Biotechnology), PINK1 (1:500, ab23707, Abcam), LC3A/B (1:1000, #4108, Cell Signaling) and the corresponding HRP-conjugated secondary antibodies including HRP-conjugated goat anti-mouse IgG (1:5000, A9197, Sigma–Aldrich), HRP-conjugated goat anti-rabbit IgG (1:5000, A0545, Sigma–Aldrich). Proteins were ultimately visualized by enhanced chemiluminescence reagent (Perkin Elmer) and autoradiography (C hemiDoc MP Imaging Systems, Bio-Rad). Immunoblotting images presented are representatives of at least three independent experiments.

2.6. Immunofluorescence

MLO-Y4 cells seeded on 5 mm or 8 mm coverslips (ProSciTech) were fixed in 4% PFA at room temperature for 20 min, and ex vivo cultured mice calvaria were fixed for 2 h, washed 3 times in PBS and permeabilized with using 0.1% Triton X-100 in PBS for 5 min at room temperature. Cells or mice calvaria were again washed in PBS for 3 times and non-specific antibody binding was blocked by 3% BSA-PBS for 30 min at room temperature. Cells were incubated with PINK1 antibody (1:250, ab23707, Abcam), LC3B antibody (1:1000, #3868, Cell Signaling) in 0.2% BSA-PBS overnight at 4 °C. Calvaria were incubated with collagen I antibody (1:100, ab34710, Abcam), cathepsin K antibody (1:100, abcam19207, Abcam), Alexa Fluor 647 Phallolidin (1:500, A22287, Thermo Fisher), Alexa Fluor 488 Phalloidin (1:500, A12379, Thermo Fisher) for 45 min, or Hoechst dye (1:5000; 33342, Perkin Elmer) for 15 min at room temperature. Cells and calvaria were then washed 3 times in PBS and incubated with Goat anti-Rabbit IgG (H + L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 647 (1:1000; A-21244, Thermo Fisher), Goat anti-Rabbit IgG (H + L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 568 (1:1000; A-11011, Thermo Fisher), Goat anti-Mouse IgG (H + L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 488 (1:1000; A-11001, Thermo Fisher). After 3 times 0.2% BSA-PBS wash and 3 times PBS wash, cells were incubated with Hoechst dye (1:5000; 33342, Perkin Elmer) for 15 min at room temperature. The cells were then rinsed three times in 0.2% PBS and mounted in ProLong Diamond Anti-fade medium (Invitrogen).

For fluorescent dye staining, cells were placed on 35 mm glass-bottom petri dishes (P35g-1.5-14-C, MatTek), and were transfected with Mitotracker Red (MTR) CMXRos (M7512, Thermo Fisher), CYTO-ID Autophagy detection kit (ENZ-5131-K200) or OsteoSense (1:100, 6 M Dex for 24 h. Following washes, cells were stained with 33 μM MTR, and incubated for 30 min at 37 °C. Cells displayed sign(s) of phototoxicity such as blebbing or vacuolization were excluded from analysis. The Nikon A1 microscope was employed to acquire the confocal images using the 60X/1.4 immersion objective and digital images were acquired using NIS Elements Software. All images were analyzed and counted by ImageJ (NIH).

2.7. RNA isolation and quantitative real-time PCR

Total RNA was isolated from cells using TRIzol reagent (Invitrogen, 15596-026) and PureLink RNA Mini Kit (Invitrogen, Thermo Fisher) following the manufacturer’s instructions. RNA (1 mg) was reverse-transcribed into cDNA using M-MLV Reverse Transcriptase (Promega). Quantitative real-time PCR was performed using 2 × SYBR Green Master Mix (Bio-Rad) in a CFX Connect Real-Time PCR detection system (Bio-Rad). Each sample was run in triplicate and gene expression levels were normalized to housekeeping gene Gapdh. All measurements were analyzed using the 2−ΔΔCt method. The primers used for real-time PCR are listed as follows (5′–3′): TGGCCTTCCGTTGCTCTAC (forward), GAGTTGGTGTAGGTCGCA (reverse) for Gapdh; AGCAACAGCATGGCTCTATAAGTCGTC (reverse) for Pink1; GGAGAAGAACCTGAAAG (forward), ATTCTGGAGACTCGAGG (reverse) for Catk; GTGATCCCCGCCAGACAGAAC (forward), CAACAAAGCACCGG (reverse) for Actg5.
following high dose Dex administration (Fig. 1B and C). To further investigate whether osteocyte-derived cathepsin K has the ability to degrade type I collagen, we immunostained cathepsin K and type I collagen in ex vivo cultured osteocytes following GC treatment of different concentrations. Interestingly, we observed that osteocytes exposed to high dose of Dex contained type I collagen fragments that were found to colocalize with cathepsin K in their cytoplasm. Conversely, such damaged fragments were absent after vehicle or low dose Dex (10⁻⁶ M) administration (Fig. 1D and E), implying a digestion process of cathepsin K towards to type I collagen induced by GC stress. Quantitative analysis showed that there were increased numbers of type I collagen puncta-like staining in the edge of lacunae when exposed to high dose Dex (Fig. 1G) indicating an increasing degradation of type I collagen in bone matrix following GC treatment. Collectively, these data suggested that GC induced cathepsin K production in osteocytes, which could be responsible for the degradation of type I collagen within lacunae.

3.2. GC causes mitochondria fission and membrane potential impairment in osteocytes

The balance between mitochondria fission and fusion is known to be

Figure 1. GC-enhanced overproduction of cathepsin K in osteocytes promotes type I collagen degradation (A) Evaluation of Ctsk gene expression in MLO-Y4 treated with vehicle or Dex for 24 h by RT-qPCR. Relative expression was normalized to Gapdh. Data are presented as mean ± SD; n = 3, n.s., not significant, ***p < 0.001 (B, C) Evaluation of cathepsin K protein levels in MLO-Y4 cells treated with vehicle or Dex for 24 h by western blotting. Quantification of protein levels were normalized by β-actin. Data are represented as mean ± SD relative to vehicle group, n = 3, n.s., not significant, *p < 0.05 (D, E) Representative confocal images of ex vivo cultured primary osteocytes following 24 h vehicle or Dex treatment, cathepsin K (red) and type I collagen (green) are stained with corresponding antibodies (n = 3). Intensity profiles were obtained using ImageJ software, along the dashed line. Triangles and dashed lines refer to the typical colocalization points. Scale bar = 10 μm (F) Representative confocal images of type I collagen puncta (marked by white triangles) detected in ex vivo cultured primary osteocytes following 24 h vehicle or Dex treatment, bone mineral was labelled with OsteoSense (turquoise) (n = 3). Scale bar = 5 μm (G) Quantitative analysis of type I collagen puncta number in primary osteocytes with ImageJ (n = 3). Data are presented as mean ± SD; n.s., not significant, ***p < 0.001. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)
the key for maintaining healthy mitochondrial dynamics and normal mitochondrial function [28]. Increasing fission rate is indicated as a universal stress response and is able to induce the depolarization of the mitochondrial membrane potential (ΔΨm), which drives mitochondrial ATP synthesis [29]. In addition, mitochondrial membrane potential is claimed as one of the key mediators for clearance of damaged mitochondrial through mitophagy [32]. To explore the effects of GC on osteocyte mitochondria, we first examined mitochondrial morphology and membrane potential using an osteocyte-like cell line, MLO-Y4 [33]. Observations on confocal imaging revealed that the majority of mitochondria in the control group are tubular-like. However, increased mitochondrial fragmentations were observed in Dex treated group, in a dose-dependent manner, which is similar to the oxidative phosphorylation uncoupler (Carbonyl cyanide chlorophenylhydrazone, CCCP) [34]. Quantitative analysis showed that MLO-Y4 cells had a significantly larger population of fragmented mitochondria after the addition of high dose Dex and a lower proportion of tubular-like mitochondria (Fig. 2A). Further, we assessed the protein level of Drp1 and Mfn2 in osteocytes following GC treatment, which are demonstrated to play crucial roles in promoting fission and fusion, respectively [35, 36]. The immunoblotting results revealed an increased expression of dynamin-related protein 1 (Drp1) and mitofusin-2 (Mfn2) in high dose Dex administration group compared to that in control cells (Fig. 2C–E), which further suggested that mitochondrial fission was induced by Dex.

To explore whether GC can cause mitochondrial membrane depolarization, we performed the JC-1 assay to evaluate ΔΨm after Dex treatment (10−6 M). Confocal imaging showed that, compared to the control group, accumulated in mitochondria indicated by a fluorescence emission shift from red to green in GC-treated MLO-Y4 cells (Fig. 2F). Further, quantification by spectrophotometer verified a significant

Figure 2. GC causes increased mitochondria fission and membrane potential impairment in osteocytes (A) Representative images of mitochondria morphology in MLO-Y4 cells stained with MTR (50 nM, 30 min) and visualized by fluorescence microscopy. Cells were exposed to vehicle or Dex (10−8 M and 10−6 M) for 24 h, CCCP (20 nM, 24 h) was used as positive control (n = 3). Scale bar = 5 μm (B) Quantitative scoring of mitochondrial morphology graded as tubular, intermediate and fragmented, with >250 cells counted from three independent experiments in each group (C–E) Western blotting analysis for protein level of Mfn2 and Drp1 in MLO-Y4 cells treated with vehicle or 10−6 M Dex for 24 h. Quantification of protein levels were normalized by β-actin. Data are represented as mean ± SD relative to vehicle group, n = 3, p < 0.05 (F, G) Evaluation of mitochondria membrane potential in MLO-Y4 treated vehicle or 10−6 M Dex for 24 h by JC-1 assay. Data are presented as a ratio of 590 nm (red):520 nm (green) fluorescence values. Scale bar = 10 μm. Data are represented as mean ± SD, n = 5, ***p < 0.001. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)
decreased ratio of red/green fluorescence intensity in GC-treated group, compared to those untreated cells (Fig. 2G). These data suggested that mitochondria membrane potential in MLO-Y4 was impaired after GC administration. Together these findings indicate that GC treatment induced mitochondrial fission and mitochondrial membrane depolarization, which both strengthen the possibility of mitophagy induction.

3.3. GC causes degradation of dysfunctional mitochondria in osteocytes

Given that previous studies have demonstrated the induction of autophagy [17,18], together with our finding of increased mitochondria fission in GC-treated osteocytes, we then explored whether autophagic degradation was also activated for the removal of accumulated stressed mitochondria. As the engulfment of dysfunctional mitochondria by autophagosome is a central process of mitophagy, we first labelled autophagosomes and mitochondria in MLO-Y4 with CytoID probe and MTR, respectively. CytoID probe is a cationic amphiphilic trace dye that can incorporate into pre-autophagosomes, autophagosomes, and autolysosomes while minimally stain the lysosomes. Following the stimulation of Dex (10⁻⁶ M), confocal imaging showed colocalizations between CytoID positive autophagosomes and the MTR positive mitochondria which are fragmented, while such kind of colocalization was absent in control group (Fig. 3A and B). This indicated that dysfunctional mitochondria are likely to be removed by autophagic process under GC stress. To further confirm mitochondria degradation, we analyzed the level of mitochondrial proteins including TOM20, VDAC1 and COX IV by western blot assay. Quantification of the protein band intensity showed a decrease of the mitochondria proteins level in a GC dose-dependent manner (Fig. 3C–F).

3.4. PINK1-mediated mitophagy is responsible for mitochondria degradation in osteocytes

Currently, the recognized mechanism of mitophagy is based on the initiation by PTEN-induced putative kinase 1 (PINK1). Under naive conditions, PINK1 is imported into mitochondria and rapidly degraded by proteolysis [37]. Nevertheless, in the stressed scenario, stabilized PINK1 proteins are accumulated at the mitochondrial outer membrane [38,39]. To examine whether PINK1 is involved in this GC-induced cleavage towards damaged mitochondria, we first stained PINK1

Figure 3. GC causes degradation of dysfunctional mitochondria in osteocytes

(A, B) Representative confocal images showed colocalization between autophagosomes and mitochondria labelled with CytoID (green) and MTR (red), respectively. MLO-Y4 cells were treated with vehicle or 10⁻⁶ M Dex for 24 h (n = 3). Intensity profiles were obtained using ImageJ software, along the white dashed line. White triangles refer to the typical colocalization points. Scale bar = 5 μm (C, D) Evaluation of mitochondrial proteins including TOM20, VDAC1 and COX IV expression levels in MLO-Y4 cells by western blotting. Cells were exposed to vehicle or Dex (10⁻⁶ M and 10⁻⁸ M) for 24 h, CCCP (20 nM, 24 h) was used as positive control (n = 3). Quantification of protein levels were normalized by β-actin. Data are represented as mean ± SD relative to vehicle group, n = 3, n.s., not significant, *p < 0.05, **p < 0.01, ***p < 0.001. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)
protein with anti-PINK1 antibody and labelled mitochondria with MTR probe. Notably, we observed that more PINK1 puncta accumulated around mitochondria and an increasing fluorescence intensity of PINK1 staining following Dex (10^{-6} M) treatment compared with cells in vehicle-treated group (Fig. 4A and B). We then analyzed the protein level of PINK1 under high GC stress by western blot, the immunoblotting result showed an increased production of PINK1 protein (Fig. 4C and D). To further investigate whether PINK1 positive mitochondria are delivered into autophagosome for degradation, we analyzed the position association between PINK1 stained protein and Cyto ID positive autophagosome. As expected, confocal imaging together with fluorescence intensity profile demonstrated an obvious co-localization between PINK1 and Cyto ID positive autophagosomes during GC administration (Fig. 4E and F), indicating that the increased autophagic degradation towards dysfunctional mitochondria is associated with PINK1. Together, our findings implied that GC induces PINK1-mediated mitophagy in osteocytes.

3.5. PINK1-mediated mitophagy induces cathepsin K production in osteocytes

Mitophagy, as a selective autophagic degradation, and its activation is accompanied with an increased demand for autophagic and lysosomal proteins, of which the gene expressions are regulated by transcription factor EB (TFEB) [40,41]. A study from Nezich et al. demonstrated that the tendency of translocation into the nucleus and transcriptional activity of TFEB are dependent on PINK1 and Parkin [42]. Notably, previous study has identified that cathepsin K is a transcriptional target of the MiT/TFE family (including TFE3, TFEB and TFEC) in osteoclast [43]. These lead us to hypothesize that PINK1-mediated mitophagy has the potential to mediate cathepsin K production through promoting TFEB translocate into nucleus. To verify the translocation of TFEB, we analyzed the protein level of TFEB in both cytoplasmic and nuclei lysates. The immunoblotting results showed an increasing TFEB in cell nuclei
following high dose GC administration, indicating that GC induced more TFEB transfer into nucleus in osteocytes (Fig. S1). To determine whether PINK1-mediated mitophagy is involved in the effects of GC on osteocyte-derived cathepsin K, we first knocked down the expression of Pink1 via transfecting siRNA (Figs. S2A and B). In the siRNA scramble group, the induction of cathepsin K occurred at both gene and protein level after GC treatment. On the contrary, a significant decrease of cathepsin K production was observed in the Pink1 knockdown group (Fig. 5A-C). A mitophagy inhibitor, mdivi-1 [44, 45] was then used to suppress mitophagy activation (Fig. 5D and E). Consistently, the GC-induced cathepsin K production was compromised by the inhibition of mitophagy with mdivi-1 addition (Fig. 5F-H). Thus, together the reverse effects observed after knockdown Pink1 which is a major mitophagy regulator and directly inhibiting mitophagy with mdivi-1, we conclude that PINK1-mediated mitophagy contributes to cathepsin K production in osteocytes under GC administration.

Owing to the fact that GC also induces autophagy in osteocytes, we next verified if mitophagy triggered upregulation of cathepsin K is associated with autophagy. As Atg5 has been characterized as an indispensable gene for canonical autophagy [47,48], we suppressed canonical autophagy by knocking down the expression of Atg5 (Fig. 5I, Figs. S2C and D). Immunoblotting showed the inhibition of canonical autophagy had no influence on cathepsin K production in osteocytes (Fig. 5J and K). Therefore, it appears that it is GC-induced PINK1-mediated mitophagy that enhances the production of ostecytic cathepsin K rather than canonical autophagy.

4. Discussion

GCs are hormones released by the adrenal glands and play a central role in stress response and survival. However, prolonged exposure to high GC levels has been found to lead to deleterious consequences, of which, GCs-induced osteoporosis is the notorious one. Although osteocytes are deeply located within mineralized bone matrix within lacunae, the dendritic processes of osteocytes are able to extend through canaliculi to form highly interconnected communication networks between neighboring osteocytes and bone surface cells to orchestrate the bone homeostasis [49-51]. In the present study, we observed type I collagen degradation as a result of GC-induced cathepsin K overproduction. Meanwhile, we uncovered that GC caused increased mitochondrial fission and mitochondrial membrane depolarization in osteocytes. As a result, mitophagy was activated as a cleavage method to remove the dysfunctional mitochondria which are destined to autophagosomes for degradation. PINK1, as one of the key mitophagy regulators of note, was found to be responsible for the induction of mitophagy in this situation. Importantly, our study has further forged a novel mechanistic association that the lysosome protein cathepsin K, as a downstream target of PINK1, is overproduced and participates in the GC-induced bone loss pathophysiology (Fig. 6). This is the first demonstration that mitophagy rather than canonical autophagy triggers GC-induced osteocytic cathepsin K. This could be a possible explanation of why autophagic inhibition in osteocytes could not attenuate GC induced bone loss [19].

In fact, various paths have been reported for osteocyte to regulate bone mass. One major regulation path is related to the mechanotransduction property of osteocytes [52]. It has been demonstrated that changes in mechanical environment that are sensed by osteocyte processes, will alter the expression of bone remodeling genes including SOST, RANKL, OPG. More recently, several proteins involved in regulating osteocyte mechanotransduction were demonstrated to participate in control bone homeostasis. It was reported that loss of the focal adhesion protein β1 integrin expression in osteocytes would cause severe decreasing of bone mass and impaired mechanotransduction [53]. In addition, Kindlin-2, which belongs to Kindlin family proteins that interact with integrins, participant in controlling bone homeostasis by mediating the differentiation of osteoprogenitor into osteocyte [54]. Here, we showed that osteocyte was involved in bone remodeling under GC stress via directly contributing to collagen degradation by producing cathepsin K, and this process was regulated by PINK1-mediated mitophagy.

PINK1-mediated mitophagy is the most common type of mitophagy. Knock-down of Pink1 abolished GC induced Csk expression suggests its major role in regulating cathepsin K production. However, there are several other types of mitophagy reported. One is the ubiquitin-independent mitophagy, in which various mitochondrial proteins including NIX/Bnip3, FUNDC1, Bcl2-L-13 and FBK8P act as receptors while the ubiquitin is not involved [55-58]. Another one is the Ivemectin-induced mitophagy, for which the initiation relies on mitochondrial fragmentation and ubiquitylation via TRAP2/COAP1/CIA2 [59]. The third uncommon type of mitophagy is one that does not rely on LC3 lipidation but requires mitochondrial fission, ULK1 and Rab9-positive membranes [60]. Whether these three types of mitophagy play the same role in GC-related bone loss have yet to be explored.

It was reported that osteocyte perilacunar remodeling by cathepsin K majorly occurs during development periods, which was not associated with pathological bone loss [61]. However, previous study has shown an overexpression of CTSK in osteocytes after GC stress [17], which is consistent to our findings here, suggesting the potential of osteocyte-derived CTSK in bone remodeling in both physiological and pathological situation. Given this mechanistic model for GC-induced cathepsin K production, a remaining question is whether GC can lead to acidification of the microenvironment surrounding osteocytes which is the prerequisite for the activation of cathepsin K to degrade type I collagen. The cleavage event of cathepsin K requires an acidified microenvironment during osteoclastic osteolysis [62], and V-ATPase complex is a versatile proton pump that is responsible for acidifying the intracellular compartments in eukaryotic cells as well as the extracellular space in some cases [63,64]. Studies from Jahn et al. revealed that osteocytes can remove calcium in the perilacunar matrix by acidifying their microenvironment through v-ATPase during lactation [65]. However, in the situation of GC stress, whether osteocytes or PINK1-mediated mitophagy is able to generate an acidified compartment remains to be determined. Another question is how PINK1-mediated mitophagy regulates cathepsin K secretion. One possibility is that osteocytes secrete cathepsin K via a mechanism similar to the secretory autophagy. Secretory autophagy is an unconventional protein secretion form that differs from the classical endoplasmic reticulum (ER)-Golgi pathway [66]. Increasing evidence reported that secretory autophagy is relevant to the secretion of a various of cytosolic proteins. In addition, different types of non-canonical autophagy are demonstrated to be involved in pathogen release from infected cells [67] and the protein trafficking into the plasma membrane [68]. In this sense, mitophagy as a form of the non-canonical autophagy, is likely to take part in the secretion of the lysosome proteins as the secretory autophagy.

Previous study has demonstrated positive outcomes for osteoporosis by modulating osteocyte autophagy level in animal models. For example, activating osteocyte autophagy by administration of rapamycin intra-peritoneally in 24-month-old male rats can reduce the severity of age-related bone changes in trabecular bone [69]. Similarly, mitophagy-regulating drugs targeting osteocyte may also exert protecting effects on bone following GC treatment. Therefore, our study provides a potential therapeutic approach to alleviate GC-induced osteoporosis by targeting PINK1-mediated osteocytic mitophagy. Further studies based on osteocyte PINK1-deficient animal models are required to reveal the role of osteocyte PINK1-mediated mitophagy axis in GC induced bone loss.

In conclusion, our study provides novel evidence that GC-induced bone loss can be mediated by mitophagy, which challenges the current view of GC-bone loss mediated by autophagy. Specifically, GC induces the activation PINK1-mediated mitophagy to eliminate damaged mitochondria in osteocytes, which then enhances the production and secretion of cathepsin K, and subsequent degradation of extracellular type I collagen. Therefore, PINK1-mediated osteocytic mitophagy could be a
promising therapeutic target in alleviating GC induced osteoporosis. In vivo studies using genetically modified animal models in which mitophagic activity are modulated will be valuable to provide further evidence to reveal the role of PNK1-mediated mitophagy in osteocyte.

**Author contributions**

J.J. Gao, M.H. Zheng and C.Q. Zhang designed the research; J. Yuan and Y.S. Gao wrote the manuscript; J. Yuan and D.L. Liu performed the research; J. Yuan and J.J. Gao analyzed the results and data; Y.S. Gao, H. Zhou and M.H Zheng contributed research materials; A. Tai and J. Papadimitriou provided precious advice for the writing and helped with proofreading for this article.

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**Declaration of competing interest**

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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**Appendix A. Supplementary data**

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jot.2022.11.003.

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Figure 5. **PNK1-mediated mitophagy induces cathepsin K production in osteocytes** (A) Evaluation of Ctsk gene expression in Pink1 knock-down MLO-Y4 treated with vehicle or 10^{-6} M Dex for 24 h by RT-qPCR. Relative expression was normalized to Gapdh. Data are presented as mean ± SD; n = 3, **p < 0.01, ***p < 0.001 (B, C) Evaluation of cathepsin K protein levels in Pink1 knock-down MLO-Y4 cells following vehicle or 10^{-6} M Dex treatment for 24 h by western blotting. Quantification of protein levels were normalized by β-actin. Data are represented as mean ± SD relative to vehicle group, n = 3, **p < 0.01 (D, E) Confocal images showing the inhibition of mitophagy by mdivi-1 in MLO-Y4 cells. MLO-Y4 were pre-treated with vehicle or mdivi-1 (50 μM) for 1 h followed with treatment of vehicle or 10^{-6} M Dex for 24 h. Intensity profiles were obtained using ImageJ software, along the white dashed line. White triangles and dashed lines refer to the typical colocalization points between LC3B (green) and mitochondria stained with MTR (red) (n = 3). Scale bar = 10 μm (F) Evaluation of Ctsk gene expression in MLO-Y4 pre-treated with vehicle or mdivi-1 (50 μM) for 1 h followed with treatment of vehicle or 10^{-6} M Dex for 24 h by RT-qPCR. Relative expression was normalized to Gapdh. Data are presented as mean ± SD; n = 3, *p < 0.05 (G, H) Evaluation of cathepsin K protein levels in MLO-Y4 cells by western blotting. Quantification of protein levels were normalized by total protein stained with ponceau red. Data are represented as mean ± SD relative to vehicle group, n = 3, *p < 0.05 (I) Evaluation of canonical autophagy in Atg5-knockdown MLO-Y4 cells following 24 h 10^{-6} M Dex treatment by western blotting assay toward Atg5 and LC3 protein (J, K) Evaluation of cathepsin K level in Atg5-knockdown MLO-Y4 cells following 10^{-6} M Dex treatment. Quantification of protein levels were normalized by β-actin. Data are represented as mean ± SD relative to vehicle group, n = 3, n.s., not significant. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Figure 6. **Diagram for PNK1-mediated mitophagy contributes to GC-induced cathepsin K production in osteocytes.** During GC administration, PNK1 accumulates on dysfunctional mitochondria membrane and initiates mitophagy to remove dysfunctional mitochondria. At the same time, the lysosome protein cathepsin K is over-produced and causes type I collagen degradation. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)
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