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Suppression of Autophagy in Osteocytes Does Not Modify the Adverse Effects of Glucocorticoids on Cortical Bone

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Abstract

Glucocorticoid excess decreases bone mass and strength in part by acting directly on osteoblasts and osteocytes, but the mechanisms remain unclear. Macroautophagy (herein referred to as autophagy) is a lysosome-based recycling pathway that promotes the turnover of intracellular components and can promote cell function and survival under stressful conditions. Recent studies have shown that glucocorticoids stimulate autophagy in osteocytes, suggesting that autophagy may oppose the negative actions of glucocorticoids on this cell type. To address this possibility, we compared the impact of prednisolone administration on the skeletons of adult mice in which autophagy was suppressed in osteocytes, via deletion of Atg7 with a Dmp1-Cre transgene, to their control littermates. In control mice, prednisolone increased autophagic flux in osteocyte-enriched bone as measured by LC3 conversion, but this change did not occur in the mice lacking Atg7 in osteocytes. Nonetheless, prednisolone reduced femoral cortical thickness, increased cortical porosity, and reduced bone strength to similar extents in mice with and without autophagy in osteocytes. Prednisolone also suppressed osteoblast number and bone formation in the cancellous bone of control mice. As shown previously, Atg7 deletion in osteocytes reduced osteoblast number and bone formation in cancellous bone, but these parameters were not further reduced by prednisolone administration. In cortical bone, prednisolone elevated osteoclast number to a similar extent in both genotypes. Taken together, these results demonstrate that although glucocorticoids stimulate autophagy in osteocytes, suppression of autophagy in this cell type does not worsen the negative impact of glucocorticoids on the skeleton.

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Keywords

autophagy; osteocytes; glucocorticoids

INTRODUCTION

The therapeutic use of glucocorticoids is associated with loss of bone mass and strength, leading to at least one traumatic fracture in 30–50% of patients [1]. Glucocorticoids cause a profound reduction in osteoblast number and bone formation rate [2]. This is due in part to the action of glucocorticoids directly on cells of the osteoblast lineage [3, 4]. High glucocorticoid levels also cause an increase in bone resorption and prolong osteoclast lifespan by acting directly on this cell type [5]. In addition, we have shown earlier that protection of osteocytes from the direct actions of glucocorticoids prevents the increase in osteocyte death and the decrease in bone strength caused by administration of prednisolone [4].

Autophagy is a lysosome-based recycling pathway that degrades intracellular components in order to promote cell survival and function, especially under stressful conditions [6]. During this process, old organelles or protein aggregates become engulfed by a double membrane vesicle called an autophagosome that fuses with lysosomes allowing degradation of its contents [7]. In this way autophagy provides additional sources of energy and helps cells to eliminate damaged organelles such as mitochondria, both of which promote cell survival and function. Autophagy appears to be particularly important in long-lived cells and a decline in autophagy has been proposed as an explanation for the changes that occur in degenerative diseases [8]. Importantly, deletion of autophagy-related genes such as Atg7 completely suppresses the process of autophagy allowing one to examine the significance of this pathway in various cell types [9].

We have recently shown that deletion of Atg7 in mature osteoblast and osteocytes suppresses autophagy and causes skeletal changes similar to those that occur with age in wild type mice [10–13], suggesting that a decrease in autophagy in osteocytes may contribute to skeletal aging. In addition, glucocorticoids stimulate autophagy in the MLOY4 osteocytic cell line, and inhibition of autophagy in these cells aggravates the effect of glucocorticoids on cell viability [14, 15]. Together these observations suggest that autophagy may oppose the negative actions of glucocorticoids on osteocytes and therefore, in the absence of autophagy, the impact of glucocorticoids on osteocytes and the skeleton might be increased. The goal of the present study was to determine whether suppression of autophagy in osteocytes alters the negative effects of exogenous glucocorticoids on the skeleton. Our results demonstrate that although exogenous glucocorticoids stimulate autophagy in osteocytes, suppression of autophagy in this cell type does not worsen the negative impact of glucocorticoids.

MATERIALS AND METHODS

Animal studies

Experimental mice were generated by crossing mice harboring a conditional Atg7 allele [9] with mice harboring a transgene consisting of 9.6 kb of the Dmp1 gene 5'-flanking region inserted upstream from the Cre coding sequence [16], as previously described [13]. Both parental strains were backcrossed into the C57BL/6 genetic background for more than 12 generations. Offspring were genotyped by PCR using the following primer sequences: Cre-for, 5'-GCGGTCTGGCAGTAAAACTATC-3', Cre-rev, 5'-GTGAAACAGCATTGCTGTCACTT-3', product size 102 bp; Hind-Fw, 5'-TGGCTGCTACTTCTGCAATGATGT-3', Atg7-ex14-F, 5'-TCTCCCAAGACAAGACAGGGTGAA-3', Pst-Rv, 5'-CAGGACAGAGACCATCAGCTCCAC-3', product size 216 bp (WT) and 500 bp (floxed allele). Six-month-old female conditional knockout mice (Dmp1-Cre;Atg7-f/f) and their control littermates (Atg7-f/f) were subcutaneously implanted with slow-release pellets of placebo or prednisolone (2.1 mg/kg/day) (Innovative Research of America, Sarasota, FL) and sacrificed after 28 days. Mice were injected with tetracycline HCl (30 mg/kg body weight) 8 days and 4 days before sacrifice. Bone mineral density (BMD) determinations were performed on day 0 and day 28 using a Piximus densitometer (GE-Lunar Corp., Madison, WI, USA). The femoral region of interest consisted of a rectangle encompassing the entire right femur. The spinal region of interest consisted of a rectangle encompassing the 2 largest thoracic and all 6 of the lumbar vertebrae. Mice were sedated with isoflurane during scanning. Scanning of a proprietary skeletal phantom over the past 4 years has produced a mean coefficient of variation of 0.43% (n = 287). All animal procedures were approved by the Institutional Animal Care and Use Committees of the University of Arkansas for Medical Sciences.

Micro computed tomography (μ CT), biomechanical testing, and histomorphometry

Micro-CT analysis of cortical and trabecular architecture was performed in the femur and fourth lumbar vertebra, as previously described [17], followed respectively by 3-point bending and compression testing to measure biomechanical properties [18]. L1–L3 lumbar vertebrae were fixed in 10% Millonig's formalin for 24 hours and embedded undecalcified in methylmethacrylate and static and dynamic histomorphometric examination of cancellous bone was done on 5 μ m longitudinal sections with a digitizer tablet (OsteoMetrics, Inc., Decatur, GA) interfaced to a Zeiss Axioscope (Carl Zeiss, Thornwood, NY) with a drawing tube attachment, as previously described [4]. Femurs were fixed in 10% Millonig's formalin for 24 hours, decalcified in 5% formic acid, washed for 6 hours and moved to 100% ethylene glycol monoethyl ether, and embedded in paraffin before obtaining 5 μ m longitudinal sections. After removal of paraffin and rehydration, sections were stained for TRAP activity and counter-stained with methyl green and osteoclasts were enumerated on the endocortical surface. The terminology used in this study has been recommended by the Histomorphometry Nomenclature Committee of the American Society for Bone and Mineral Research [19].

TaqMan assay

L5 vertebrae and osteocyte-enriched tibia shafts were homogenized in Trizol Reagent (Life Technologies, Grand Island, NY) to extract total RNA, according manufacturer's instructions. Quantitation and 260/280 ratio of the extracted RNA were determined using a Nanodrop instrument (Thermo Fisher Scientific, Wilmington, USA). Five hundred ng of RNA was then used to synthesize cDNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystem, Foster City, CA) according to manufacturer's directions. cDNA was amplified by quantitative RT-PCR using TaqMan Universal PCR Master Mix (Life Technologies) as described previously [20]. The following TaqMan assays from Life Technologies were used: calcitonin receptor, Mm00432271_m1; osteoprotegerin, Mm00435452_m1; RANKL, Mm00441908_m1; Dmp1, Mm01208363_m1; Mepe, Mm02525159_s1; collagen 1a1, Mm00801666_g1; osteocalcin, (forward, 5'-GCTGCGCTCTGTCTCTCTGA-3', reverse, 5'-TGCTTGGACATGAAGGCTTTG-3', probe, 5'-FAM-AAGCCCAGCGGCC-NFQ-3') and the housekeeping gene ribosomal protein S2, (forward, 5'-CCCAGGATGGCGACGAT-3', reverse, 5'-CCGAATGCTGTAATGGCGTAT-3', probe 5'-FAM-TCCAGAGCAGGATCC-NFQ-3'). Gene expression was calculated using the delta Ct method [21] and ribosomal protein S2 (ChoB) levels were used for normalization.

Cell culture

Bone marrow cells were harvested from long bones and plated in 12-well plates at 5×10^6 cells/well, after lysing of red blood cells, and cultured in α -MEM containing 15% fetal bovine serum, 1% penicillin/streptomycin/glutamine, and ascorbic acid (50 ug/ml). One-half of the culture medium was changed every 3 days. After 15 days, genomic DNA, protein lysates, and RNA were purified from the cultures to evaluate respectively Atg7 deletion, LC3 conversion by western blot, and osteoblast-specific gene expression. To quantify Atg7 gene deletion, the following custom Taqman assay for exon 14 was used: forward, 5'-ACCAGCAGTGCACAGTGA-3', reverse, 5'-GCTGCAGGACAGAGACCAT-3', probe, 5'-FAM-CTGGCCGTGATTGCAG-NFQ-3' in combination with a Taqman copy number reference assay, Tfr1 (catalog number 4458367), and the relative amount of Atg7 exon 14 genomic DNA was calculated using the Ct method.

Immunoblots

Proteins were extracted from humeral cortical bone after removing the ends with a scalpel, flushing the bone marrow, and removal of surface cells by scraping with a scalpel. Cortical bone fragments were then frozen in liquid nitrogen and pulverized. The bone powder was then extracted by incubation in SDS-PAGE sample buffer (50 mM Tris-HCl, pH 6.8, 2% SDS, 6% glycerol, 1% β -mercaptoethanol, and 0.004% bromophenol blue) for 10 min at 100°C. Proteins were resolved in SDS-polyacrylamide gels and electroblotted onto polyvinylidene difluoride membranes. Membranes were subsequently blocked with 5% nonfat dry milk in TBS with 0.1% Tween-20 and then incubated with primary antibodies and secondary antibodies. The following antibodies were used: anti-LC3A/B at 1:1000 (#4108, Cell Signaling, Danvers, MA), anti-p62 at 1:1000 (GP62, Progen, Heidelberg, Germany), and anti- α -tubulin at 1:2000 (#T8203, Sigma, St. Louis, MO). Blots were

developed using enhanced chemiluminescence and the intensity of the bands was quantified using a ChemDoc XRS-plus system (Bio-Rad, Hercules, CA).

Quantitation of proteins in bone marrow supernatants

The concentration of RANKL, OPG, DKK1, and sclerostin was determined in bone marrow supernatant harvested from long bones. Bone marrow supernatants were collected by removing both ends of humeri with a scalpel and then centrifuging the diaphyseal bone in a microcentrifuge tube at $850 \times g$ for 30 s. The bone marrow cell pellet and bone marrow plasma were then resuspended in 80 μ l of phosphate-buffered saline and then centrifuged at $2,300 \times g$ for 1 min. The supernatant was transferred to a fresh tube and stored at -80°C until analyzed. RANKL protein levels were determined using the Procarta immunoassay kit (Affymetrix, Santa Clara, CA, USA) while OPG, DKK1 and sclerostin were quantified using the Milliplex map mouse bone magnetic bead panel kit (EMD Millipore, St. Charles, MO), each according to the manufacturer's instructions. Data were acquired using a Luminex-200 reader (Luminex, Austin, TX, USA). A five-parameter regression formula was used to calculate the sample concentrations from standard curves using Luminex xPONENT 3.1 software. Analytes were normalized to total protein concentration determined with the BCA protein assay (Pierce Biotechnology, Rockford, IL, USA).

Visualization of the lacuno-canalicular network

Tibia were fixed in Millonig's formalin for 24 hours and decalcified in 14% EDTA pH 7.1 for one week, stored in 30% sucrose solution, and then 20 μ m thick frozen sections were obtained. Sections were rinsed 3 times in PBS for 10 minutes. The sections were permeabilized in 0.2% Triton-X100 for 20 minutes with agitation, followed by washing in PBS. The sections were then incubated in 2% BSA for 30 minutes and incubated with Alexa fluor 488 Phalloidin (Molecular Probes, Eugene, OR, USA) 0.066 μ M in 0.5% BSA for 48 hours at 4°C . At the end of the incubation, the sections were rinsed with PBS and mounted with Vectashield mounting medium (Vector Laboratories, Burlingame, CA). Confocal images and z-stacks were acquired using ZEISS LSM 510 confocal microscope and ZEN 2009 software.

Statistics

Data were analyzed using a 2-way ANOVA to detect statistically significant treatment effects, after determining that the data were normally distributed and exhibited equivalent variances. In some cases, transformations were used to obtain normally-distributed data and equal variance. This was followed by all pairwise comparisons using Tukey's procedure. For some variables the p-values were adjusted using Holm's multiple comparison procedure. For experiments involving comparison of only two groups, Student's t-test was used. P-values less than 0.05 were considered as significant. Results in all graphs represent the mean $\pm$ s.d.

RESULTS

Glucocorticoids stimulate autophagy in osteocytes in vivo

To determine whether glucocorticoids control osteocyte autophagy in vivo and whether autophagy helps osteocytes resist the negative effects of glucocorticoids, we compared the impact of glucocorticoid administration on the skeleton of mice with and without functional autophagy in osteocytes. Mice lacking autophagy in osteocytes were generated by crossing mice harboring a conditional allele of *Atg7* with *Dmp1-Cre* transgenic mice, which express the Cre recombinase primarily in mature osteoblasts and osteocytes [13]. We have shown previously that this approach deletes *Atg7* from bone, but not soft tissues, and that it significantly reduces autophagic flux in osteocytes in vivo [13]. In the current study, 6-month-old female *Dmp1-Cre;Atg7-f/f* mice, hereafter referred to as conditional knockout mice (CKO), or *Atg7-f/f* control littermates (Ctrl) were implanted with pellets releasing placebo or prednisolone for 28 days, and then euthanized for further analysis.

To confirm suppression of autophagy in osteocytes, we measured the levels of LC3 and p62 by immunoblot analysis. LC3 is a docking protein that is incorporated into growing autophagosome membranes. This incorporation requires lipidation of the protein, which can be monitored on immunoblots by conversion of the unlipidated (form I) to lipidated (form II) version. p62 is a scaffolding protein that brings cargos to the autophagosome for degradation and may accumulate when autophagy is suppressed [22]. Comparison of conditional knockout and control mice implanted with placebo pellets revealed that deletion of *Atg7* from *Dmp1-Cre*-expressing cells inhibited conversion of LC3 and caused accumulation of p62 in osteocyte-enriched cortical bone (Figs. 1A–B), findings which are consistent with our previous report [13]. The *Dmp1-Cre* transgene did not alter abundance of the *Atg7* conditional allele or LC3 conversion in cultures of primary osteoblasts (supplemental figure 1).

We then compared autophagic flux in the four groups of mice and found that prednisolone stimulated LC3 conversion in osteocyte-enriched cortical bone of control mice but not in conditional knockout mice (Fig. 1A). The inability of prednisolone to stimulate LC3 conversion in the conditional knockout mice demonstrates that the increased conversion of LC3 observed in control mice must be occurring in *Dmp1-Cre*-expressing cells. Therefore, this result supports the idea that glucocorticoids stimulate autophagic flux in osteocytes in vivo. An increase in autophagic flux can also lead to reduced abundance of p62 [23]. Therefore, the stimulation of autophagy in control mice by prednisolone might be expected to reduce p62 levels in osteocyte-enriched bone. However, prednisolone did not alter p62 levels in control mice or conditional knockout mice (Fig. 1B).

Suppression of autophagy in osteocytes does not accentuate the negative impact of glucocorticoids on bone

We have shown previously that suppression of autophagy in osteocytes causes low cancellous bone volume and reduces cortical thickness and that this is associated with low bone strength [13]. In the present study, we observed similar effects of autophagy suppression in osteocytes, with the exception that cancellous bone volume was not lower in

the femurs of conditional knockout mice (Figs. 2 A–C and 2E–G). However, despite the presence of these changes in the conditional knockout mice under basal conditions, administration of prednisolone reduced cortical thickness (Fig. 2A), bone strength (Fig. 2C) and femoral bone mineral density (Fig. 2D) and increased cortical porosity (Fig. 2I) to similar extents in the femurs of control and conditional knockout mice. Changes in the femoral cortical bone fraction were also similar between genotypes (supplementary figure 2). Prednisolone did not alter cortical thickness, bone strength, or BMD in the spine of either genotype (Figs. 2E–H). Administration of prednisolone did not alter cancellous bone volume in either the femur (Fig. 2B) or L4 vertebra (Fig. 2F) of either genotype, findings that are consistent with our previous observations at this dose of prednisolone in C57BL/6 mice [24].

Even though prednisolone did not alter cancellous bone volume, we and others have shown previously that it reduces osteoblast number and bone formation in this skeletal compartment [3, 5]. Therefore, we examined the effects of prednisolone administration and suppression of autophagy, both separately and combined, on the histology of cancellous bone of the spine. Similar to what we observed in our previous study, suppression of autophagy in osteocytes reduced the overall rate of bone remodeling. Specifically, osteoclast number, osteoblast number, and bone formation rate were lower in placebo-treated conditional knockout mice compared to placebo-treated control mice (Figs. 3A–G). Administration of prednisolone had no impact on osteoclast number in cancellous bone in either genotype (Figs. 3A–B). In contrast, prednisolone dramatically reduced osteoblast number and bone formation rate in control mice but did not further reduce the already low osteoblast number and bone formation rate in conditional knockout mice (Figs. 3C–D and 3E–G).

Because prednisolone reduced cortical thickness and increased cortical porosity in the femur of both genotypes, we also examined osteoclast number at the endocortical surface of the femur. In contrast to the situation in cancellous bone, osteoclast number was not different in placebo-treated control and conditional knockout mice. However, prednisolone potently increased osteoclast number in control mice and in conditional knockout mice (Fig. 4A and 4B). Consistent with these changes in cell number, expression of the osteoclast-specific gene calcitonin receptor was elevated in cortical bone of mice treated with prednisolone (Fig. 4C). Osteoclast generation and lifespan are regulated in large part by levels of the cytokine RANKL and its decoy receptor osteoprotegerin (OPG), both of which are expressed by osteocytes [25, 26]. The prednisolone-induced changes in osteoclast number in cortical bone were associated with reduced OPG protein in bone marrow supernatants and reduced OPG mRNA in osteocyte-enriched cortical bone (Fig. 4D–E) but there was no change in RANKL protein or mRNA (Fig. 4F–G). These results demonstrate that prednisolone increases bone resorption at the endocortical surface, possibly by reducing OPG production by osteocytes, but that suppression of autophagy in osteocytes does not worsen this response.

Glucocorticoid effects on osteocyte are not worse in the absence of autophagy

The results to this point indicate that suppression of autophagy in osteocytes does not worsen the impact of glucocorticoids on bone mass, strength, resorption, or formation.

Nonetheless, we wanted to determine whether effects of glucocorticoids directly on osteocytes were affected by loss of autophagy. First we counted the number of empty lacunae as a measure of osteocyte viability and found that prednisolone increased the percentage of empty lacunae in control mice but not in conditional knockout mice (Fig. 5A). Prednisolone also reduced osteocyte density in control mice but did not further reduce the already low osteocyte density in conditional knockout mice (Fig. 5B). Analysis of osteocyte-specific gene expression revealed that neither Atg7 deletion nor prednisolone administration altered Dmp1 or Mepe mRNA abundance in L5 vertebrae (Figs. 5C–D). Dkk1 and sclerostin protein were reduced by prednisolone in bone marrow supernatants, but there was no difference between genotypes (Figs. 5E–F). Lastly, we examined the impact of prednisolone on the osteocyte lacuno-canalicular network, as revealed by phalloidin-Alexa488 staining. We found no obvious impact of either prednisolone or suppression of autophagy (Fig. 5G).

DISCUSSION

Suppression of autophagy causes dysfunction and death in cells such as neurons, pancreatic beta cells, kidney epithelium, and hematopoietic stem cells [27–30]. Based on its protective role in these cell types, and on the previous observation that autophagy opposes the negative effect of glucocorticoids on osteocytes in vitro [14], we anticipated that suppression of autophagy in this cell type would worsen the impact of glucocorticoids on osteocyte function and on the skeleton. In addition, glucocorticoids induce oxidative stress in osteoblastic cells [31] and induction of autophagy acts as a key defense against oxidative stress in other cell types [32]. However, we found that the impact of prednisolone on osteocyte gene expression and on the skeleton was, for the most part, unaltered by Atg7 deletion in osteocytes. The exceptions to this were that prednisolone did not further reduce the already low bone formation caused by Atg7 deletion and the increase in osteocyte empty lacunae caused by prednisolone was prevented by Atg7 deletion. Thus, although we were able to demonstrate that glucocorticoids stimulate the process of autophagy in osteocytes, inhibition of this response did not worsen the negative impact of glucocorticoids on the skeleton.

We have shown previously that deletion of Atg7 in osteocytes does not increase the basal rate of apoptosis [13]. Consistent with this, the number of empty osteocyte lacunae, a different indicator of cell viability [33], was not altered in the conditional knockout mice under basal conditions. Using this same measure of cell viability, we found that prednisolone induced osteocyte cell death in control mice, but this effect was completely blocked in mice lacking Atg7 in osteocytes. This finding suggests that, rather than protecting cells, the process of autophagy is an essential component of the death pathway stimulated by glucocorticoids. In addition to its cell protective functions, stimulation of autophagy can also lead to cell death under a variety of conditions [34]. In some situations, the initial effect of autophagy is to promote cell survival, but if allowed to continue for prolonged periods, the effect changes to induction of cell death, often in collaboration with the apoptotic machinery [35]. In other situations, stimulation of autophagy is a necessary component of the death pathway. Indeed, similar to our observation in osteocytes, the cell death induced in lymphoid leukemia cells by dexamethasone is dependent on activation of the autophagy pathway [36]. Others have suggested that the ability of autophagy to promote cell survival

may be dependent on the dose of glucocorticoids [15]. Thus, it is possible that at different doses of prednisolone suppression of autophagy may cause a greater increase in osteocyte death than prednisolone alone. Nonetheless, the major negative effects of glucocorticoid excess, including reduced bone formation, increased cortical bone resorption, and reduced bone strength, are produced with the dose used in the current study and are not altered in the absence of osteocyte autophagy.

Prednisolone and genetic inhibition of autophagy independently suppressed bone formation in our study, but did not have an additive effect. One interpretation of this result is that both maneuvers target a common final pathway or cellular component responsible for the reduction in osteoblast number and bone formation. Several mechanisms have been proposed to explain glucocorticoid suppression of bone formation, including increased osteoblast apoptosis and elevation of Wnt signaling antagonists [37, 38]. The mechanisms responsible for the suppression of bone formation in *Dmp1-Cre;Atg7* mice are unclear. Osteoblast differentiation in vitro was unaffected in primary cultures from these mice, suggesting that the reduction in osteoblast number and bone formation is caused indirectly by action on osteocytes. However, since it is possible that *Atg7* was deleted from mature osteoblasts in vivo, direct effects on these cells cannot be ruled out. Increases in the levels of Wnt signaling antagonists *Dkk1* and *sclerostin* do not appear to be involved in the suppression of bone formation by either glucocorticoid excess or *Atg7* deletion since prednisolone suppressed these factors and the effects of prednisolone were unchanged by *Atg7* deletion. An alternative explanation for the lack of an additive effect is that prednisolone was unable to induce osteoblast death in the absence of autophagy, similar to what we observed in osteocytes. However, we were unable to directly assess this possibility in the current study because of problems with the assay we have used for measurement of osteoblast apoptosis. Specifically, the assay we have used in the past now produces an unacceptably high background and shows no difference between placebo and prednisolone-treated groups, even when using the same samples that show differences in empty lacunae.

We have shown previously that blockade of glucocorticoid action on osteoblasts and osteocytes prevented the increase in cancellous osteocyte apoptosis caused by prednisolone and that this was associated with preservation of bone strength even in the presence of reduced bone mass [4]. Based on these results, we speculated that osteocytes may contribute to bone strength independently of bone mass. In the current study, the suppression of autophagy also prevented the increase in cancellous osteocyte death, albeit measured differently. However, in this experiment prednisolone did not cause a significant decline in bone strength in vertebral bone in either genotype. Thus the increase in osteocyte death observed at this site was not associated with altered bone strength. Although the two experiments were performed in different strains of mice and cell death was measured differently, the current results do not support the idea that osteocyte viability, in-and-of-itself, is a major contributor to bone strength.

Prednisolone increased osteoclast number on the endocortical bone surface but not the cancellous bone surface. This finding is consistent with the loss of cortical bone but not cancellous bone and with the loss of femoral BMD. Measurement of BMD in murine femurs is heavily influenced by cortical bone while vertebral BMD is a measure of both cortical and

cancellous bone. Even though osteoclast number did not increase on cancellous bone, osteoblast number and the bone formation rate were notably reduced in this compartment. It is unclear why this imbalance between osteoblast and osteoclast number did not lead to cancellous bone loss. Teitelbaum and colleagues have shown that glucocorticoids can directly inhibit osteoclast function by inhibiting cytoskeletal organization [39]. Thus one possibility is that even though osteoclast number was normal on cancellous bone in the prednisolone-treated mice, these osteoclasts had a reduced ability to resorb bone. If that were the case, then osteoclasts at the endocortical surface either responded differently to prednisolone or their numbers were increased to such an extent that even with reduced function they were still able to resorb a significant amount of bone.

The mechanisms underlying the increase in endocortical osteoclast number in response to prednisolone are unclear, thus an explanation for the lack of an increase in osteoclast number on cancellous bone is not evident. Higher doses of prednisolone, use of different mouse strains or analysis after shorter durations of treatment has shown that prednisolone can increase cancellous osteoclast number [40, 41]. Nonetheless, a failure to increase cancellous osteoclast number in C57BL/6, the strain used in our study, has been a consistent finding under the conditions used here [24]. We have shown previously that glucocorticoids increase osteoclast lifespan in vitro [40], and this may have contributed to the increase in cortical osteoclast number. In addition, the reduction in OPG protein levels observed in bone marrow supernatants may also have contributed. Perhaps more importantly, if the increase in osteoclast number is one of the main factors responsible for glucocorticoid-induced bone loss, then loss of autophagy in osteocytes would not be expected to alter this response unless factors produced by osteocytes mediate the effects on osteoclasts. Osteocytes have been proposed as an important source of OPG [26]. However, if this turns out to be the case, then autophagy does not appear to control production of OPG by this cell type.

In conclusion, we have shown that glucocorticoid excess stimulates the process of autophagy in osteocytes but inhibition of this phenomenon does not alter the negative impact of glucocorticoids on the skeleton. These results suggest that elevated autophagy is not a self-protective mechanism used by osteocytes to oppose the stress induced by glucocorticoid excess.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- Excess glucocorticoids cause bone loss and stimulate autophagy in osteocytes.
- Genetic suppression of autophagy in osteocytes did not accentuate the impact of glucocorticoids on bone.
- Osteocyte autophagy does not oppose glucocorticoid action on this cell type.
- Promotion of osteocyte autophagy is unlikely to prevent the negative impact of glucocorticoids on the skeleton.

Figure 1.

Prednisolone stimulates autophagy in osteocytes. A, protein lysates extracted from osteocyte-enriched humeri shafts of 6-month-old female Ctrl and CKO mice (n=3–4 animals per group) treated with placebo or prednisolone for 28 days, were subjected to immunoblot to detect LC3 and B, p62. The intensity of the LC3-I and LC3-II bands was quantified and plotted as the ratio of LC3-II to LC3-I. p62 expression was quantified and normalized to tubulin. *P< 0.05.

Figure 2.

Suppression of autophagy in osteocytes does not alter the impact of glucocorticoids on the skeleton. Bones from 6-month-old female Ctrl and CKO mice treated with placebo or prednisolone for 28 days were analyzed by μ CT. A–D, cortical thickness, bone volume over tissue volume (BV/TV), strength and percent change of BMD were measured in the femur and E–H, in the spine (n=9–11 animals per group). I, representative μ CT images showing femoral cortical porosity. Quantification of cortical porosity (mean $\pm$ s.d.) is shown in the insets. *P<0.05 vs placebo-treated Ctrl and # P< 0.05 vs placebo-treated CKO.

Figure 3.

Cellular changes induced by prednisolone were not worse in the absence of autophagy. Static and dynamic histomorphometric analyses were performed in longitudinal section of the lumbar vertebra 1–3 harvested from 6-month-old female Ctrl and CKO mice (n=4–5 animals per group) treated with placebo or prednisolone for 28 days. A–G, osteoclast number per bone perimeter (Oc.N/B.Pm); osteoclast perimeter per bone perimeter (Oc.Pm/B.Pm); osteoblast number per bone perimeter (Ob.N/B.Pm); osteoblast perimeter per bone perimeter (Ob.Pm/B.Pm); mineralizing perimeter per bone perimeter (M.Pm/B.Pm); mineral apposition rate (MAR) and bone formation rate (BFR). *P< 0.05.

Figure 4.

Prednisolone stimulates endocortical resorption and suppresses OPG production. A, osteoclast number per endocortical bone perimeter (Oc.N/Ec.Pm) measured by histomorphometric analysis starting from the region under the primary spongiosa of decalcified femurs from 6-month-old female Ctrl and CKO mice treated with placebo or prednisolone for 28 days (n=5 animals per group). B, histological sections of femurs stained for TRAP activity (osteoclasts are stained red) and counterstained with methyl green (scale bar = 50µm) in endocortical bone. C, real-time quantitative PCR of calcitonin receptor in tibial cortical bone (n=6 animals per group). D–E, OPG protein levels in humeri bone marrow supernatants (n=9–10 animals per group) and OPG mRNA in osteocyte-enriched tibia shafts of 6-month-old female Ctrl and CKO mice treated with placebo or prednisolone for 28 days, measured by real-time quantitative PCR (n=3–6 animals per group). F–G, RANKL protein levels in humeri bone marrow supernatants (n=9–10 animals per group) and RANKL mRNA in osteocyte-enriched tibia shafts of 6-month-old female Ctrl and CKO mice treated with placebo or prednisolone for 28 days, measured by real-time quantitative PCR (n=3–6 animals per group). All mRNA levels were normalized to ribosomal protein S2 mRNA levels. *P< 0.05.

Figure 5.

Suppression of autophagy does not worsen the effects of prednisolone on osteocytes. A, percentage of empty lacunae and B, osteocytes density (#/mm²) measured in the cancellous bone of L1–L3 from 6-month-old female Ctrl and CKO mice treated with placebo or prednisolone for 28 days (n=5 animals per group). C–D, Dmp1 and Mepe mRNA in L5 vertebrae of 6-month-old female Ctrl and CKO mice treated with placebo or prednisolone for 28 days, measured by real-time quantitative PCR (n=6 animals per group). All mRNA levels were normalized to ribosomal protein S2 mRNA levels. E–F, DKK1 and sclerostin protein levels in humeri bone marrow supernatants (n=9–10 animals per group). G, representative confocal Z-stack images showing the lacuno-canalicular network stained with Alexa fluor 488 Phalloidin (scale bar= 20 μm) in 20 μm frozen sections of tibias obtained from 6-month-old female Ctrl and CKO mice treated with placebo or prednisolone for 28 days. *P< 0.05.