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Prevention of Glucocorticoid Induced-Apoptosis of Osteoblasts and Osteocytes by Protecting against Endoplasmic Reticulum (ER) Stress *in vitro* and *in vivo* in Female Mice

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Abstract

Endoplasmic reticulum (ER) stress is associated with increased reactive oxygen species (ROS), results from accumulation of misfolded/unfolded proteins, and can trigger apoptosis. ER stress is alleviated by phosphorylation of eukaryotic translation initiation factor 2α (eIF2α), which inhibits protein translation allowing the ER to recover, thus promoting cell viability. We investigated whether osteoblastic cell apoptosis induced by glucocorticoids (GC) is due to induction of ROS/ER stress and whether inhibition of eIF2α dephosphorylation promotes survival opposing the deleterious effects of GC *in vitro* and *in vivo*. Apoptosis of osteocytic MLO-Y4 and osteoblastic OB-6 cells induced by dexamethasone was abolished by ROS inhibitors. Like GC, the ER stress inducing agents brefeldin A and tunicamycin induced osteoblastic cell apoptosis. Salubrinal or guanabenz, specific inhibitors of eIF2α dephosphorylation, blocked apoptosis induced by either GC or ER stress inducers. Moreover, GC markedly decreased mineralization in OB-6 cells or primary osteoblasts; and salubrinal or guanabenz increased mineralization and prevented the inhibitory effect of GC. Furthermore, salubrinal (1 mg/kg/day) abolished osteoblast and osteocyte apoptosis in cancellous and cortical bone and partially prevented the loss of BMD at all sites and the decreased vertebral cancellous bone formation induced by treatment with prednisolone for 28 days (1.4 mg/kg/day). We conclude that part of the pro-apoptotic actions of GC on osteoblastic cells are mediated through ER stress, and that inhibition of eIF2α dephosphorylation protects from GC-induced apoptosis of osteoblasts and osteocytes in vitro and in vivo and from the deleterious effects of GC on the skeleton.

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Key terms

Glucocorticoid-induced osteoporosis; osteoblasts; osteocytes; endoplasmic reticulum stress

INTRODUCTION

Excess of glucocorticoids (GC), either endogenous as in aging or due to glucocorticoid administration as immunosuppressants, leads to loss of bone [1]. Chronic GC therapy is prescribed for a multitude of medical conditions including autoimmune diseases such as rheumatoid arthritis, organ transplants, asthma, as a component of cancer chemotherapies, and a variety of inflammatory afflictions [2, 3]. Patients with chronic GC exposure exhibit a consistent reduced bone formation rate and histomorphometric features of increased bone resorption [1, 4, 5]. Studies with experimental animals in which GC action is blocked in osteoclasts or in osteoblasts/osteocytes, support the notion that GC-induced bone loss occurs in two phases: an early bone loss caused by osteoclast-driven bone resorption, followed by a steady decline in both bone formation and bone resorption [6–8]. Between 30 to 50% of patients experience at least one bone fracture, with the consequent morbidity and mortality. Approximately 25% of patients also develop osteonecrosis due to accumulation of apoptotic osteocytes, which increases the risk of femoral head collapse [9]. Thus, understanding the mechanisms of GC action on bone cells and designing therapeutic strategies that prevent the deleterious effects of these drugs is imperative.

Increased apoptosis of osteoblasts and osteocytes is one of the mechanisms that underlie the reduced bone formation and bone fragility that characterize GC-induced osteoporosis [1]. Apoptosis by GC is due to direct hormonal effects on osteoblasts and osteocytes [10–12] and is abolished by overexpressing in these cells the enzyme that inactivates GC, 11beta-hydroxysteroid dehydrogenase type 2 [7]. The pro-apoptotic effects of GC are mediated through the classical GC receptor and are triggered by rapid activation of the kinases Pyk2 and JNK [11]. GC-induced JNK phosphorylation lies downstream of increased reactive oxygen species (ROS) generation and subsequent activation of pro-apoptotic signaling in osteoblasts [13]. Phosphorylation of eukaryotic translation initiation factor 2α (eIF2α) by double-stranded RNA-activated protein kinase-like ER kinase (PERK) protects cells from oxidative stress and apoptosis under ER stress conditions [14]. Phosphorylated eIF2α slows global rate of protein translation to provide time for the ER to recover from the excessive protein load, allowing the cell to escape from apoptosis [14, 15]. Earlier findings showed that selective inhibition of eIF2α phosphatases with salubrinal or guanabenz protects from apoptosis induced by ER stress [16, 17]. Salubrinal increases the levels of phosphorylated eIF2α and protects PC12 pheochromocytoma cells from apoptosis induced by the ER stressor tunicamycin, in a dose dependent manner at concentrations varying from 1 – 100 μM [16]. Further, guanabenz, another eIF2α phosphatase inhibitor, antagonizes the effect of tunicamycin on several cell types at concentrations up to 50 μM [17]. We hypothesized that the pro-apoptotic effect of GC on osteoblasts and osteocytes is at least in part due to induction of ER stress, and we investigated here whether the compounds salubrinal and guanabenz that inhibit eIF2α dephosphorylation will promote osteoblastic cell viability and oppose the deleterious effects of GC *in vitro* and *in vivo*.

MATERIALS AND METHODS

Cell lines

Primary osteoblastic cells were isolated from the neonatal calvarial bones of C57BL/6 mice, as previously published [18]. Murine primary osteoblastic cells, bone marrow-derived OB-6 osteoblastic cells, and MLO-Y4 osteocytic cells were cultured as previously described [19, 20].

Quantification of cell detachment, apoptosis, and cell viability

Cell and nuclear morphology was quantified in MLO-Y4 osteocytic cells stably transfected with green fluorescent protein targeted to the nucleus (nGFP), as published [11]. Briefly, cell detachment was assessed by quantifying the number of cytoplasmic processes per cell. Cells were then categorized into one of two groups: having 3 or less processes or having more than 3 processes; and data is reported as percentage of cells with 3 or less processes. Apoptosis was evaluated by quantifying the percentage of cells with chromatin condensation and nuclear fragmentation in the same cultures. Data is reported as percentage of apoptotic cells.

Cells were treated with the anti-oxidant agents N-acetyl cysteine (NAC, 10 mM), esbelen (20 μM), or catalase (1250 U/ml), the eIF2α phosphatase inhibitors salubrinal (1-100 μM) and guanabenz (10 μM) [16, 17], the bisphosphonate alendronate (0.1 μM), or corresponding vehicle (named control in the figures), for one hour. Subsequently, cells were exposed to the pro-apoptotic agents dexamethasone (1 μM), etoposide (50 μM), brefeldin A (2.7 μM), or tunicamycin (2.7 μM) or corresponding vehicle (named vehicle in the figures), for the indicated times. Cell viability was assessed by trypan blue uptake as previously published [11, 18]. Cells that excluded the dye were considered alive, and stained cells were considered dead. Data is reported as the percentage of dead cells.

Mineralization assay

Primary osteoblasts or OB-6 cells were plated at a density of 5,000 cells/cm^2 in growth medium consisting of MEM Alpha medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. Once cultures reached confluence, medium was replaced by osteogenic medium containing 50 μg/ml ascorbic acid and 10 mM β-glycerophosphate together with 1 μM dexamethasone or its corresponding vehicle (ethanol), and 10 μM salubrinal or guanabenz or the corresponding vehicle (DMSO). Medium was replaced every 2–3 days. Mineralization was visualized using von Kossa phosphate staining [21], Alizarin Red S (Sigma-Aldrich) staining [22], or OsteoImage Mineralization Assay Kit (Lonza). Mineralization was quantified using a microplate reader for Alizarin Red S staining (405nm absorbance) and Lonza staining (492/520nm excitation/emission fluorescence).

In vivo study

C57Bl/6 female mice (n= 7–11 per group) were purchased from Harlan (Indianapolis, IN). After a 2 week acclimation period, four-month-old mice were implanted with 60 day slow-release pellets delivering placebo, 1.4 mg/kg/d (GC1) prednisolone, or 2.1 mg/kg/d (GC2) prednisolone (Innovative Research of America, Sarasota, FL) while under isoflurane

anesthesia. For this, a small area between the shoulder blades was shaved and cleaned with 70% EtOH prior to incision. Daily subcutaneous injections of salubrinal (1 mg/kg/d, Tocris Bioscience, USA) or equal volume of vehicle (propylene glycol, Sigma-Aldrich, named control) began 3 days prior to pellet implantation and continued until experiment termination. An additional group of GC2 implanted mice (n=10) received 5.25 mg/kg/wk alendronate subcutaneous injections starting 3 days before pellet implantation. Mice were sacrificed 28 days after pellet implantation. Institutional Animal Care and Use Committee at Indiana University School of Medicine approved all animal procedures.

Bone mineral density (BMD) measurements

BMD was determined in live mice by dual-energy x-ray absorptiometry (DXA) scanning using a PIXImus II densitometer (G.E. Medical Systems, Lunar Division, Madison, WI) [23]. Experimental group assignment was randomized by basal spine BMD determined by DXA scanning performed 5 days prior to pellet implantation. DXA scanning was also performed 28 days after pellet implantation.

Bone histomorphometry and apoptosis

Distal femora were fixed in 10% neutral buffered formalin. After 48 hours in fixative, samples were transferred to 70% ethanol, and then embedded undecalcified in methyl methacryate as previously described [12]. Dynamic histomorphometry measurements were performed in 7-μm unstained bone sections under epifluorescence microscopy. For this purpose, 0.6% calcein and 1.0% alizarin red solutions were intraperitoneally injected 8 and 3 days prior to sacrifice. Histomorphometric analysis was performed with a computer and digitizer tablet (OsteoMetrics, Decatur, GA) interfaced to a Olympus BX51 fluorescence microscope (Olympus America Inc., Melville, NY) with a drawing tube attachment [24]. Apoptotic cells were detected by transferase-mediated biotin-dUTP nick end-labeling (TUNEL) reaction in undecalcified longitudinal sections of the distal femur, as previously described [12]. Analysis was performed in cancellous and cortical bone, starting 200 μm below the growth plate and ending at the mid-diaphysis.

Statistical analysis

Data is expressed as means ± standard deviation (SD). Sample differences were assessed using SigmaPlot 12.0 (Systat Software Inc, San Jose, CA), following the appropriate method for each measurement, as indicated in the figure legends. Means were considered significantly different at $p < 0.05$.

RESULTS

Glucocorticoids induce apoptosis of osteocytic and osteoblastic cells by generating ROS

The synthetic glucocorticoid dexamethasone induced retraction of osteocytic MLO-Y4 cytoplasmic processes, an early sign of cell detachment that triggers apoptosis (anoikis) [11], as revealed by a reduction in the percentage of cells exhibiting 3 or more cytoplasmic projections (Figure 1A). Dexamethasone also induced apoptosis of MLO-Y4 osteocytic cells, as quantified by evaluating chromatin condensation and nuclear fragmentation (Figure 1B and C). Further, dexamethasone increased the percentage of MLO-Y4 and OB-6

osteoblastic cells exhibiting trypan blue uptake (Figure 1D), another sign of apoptotic cell death induced by GC previously shown to be blocked by inhibiting caspase 3 activity [11, 12, 18]. Pre-treatment with the anti-oxidants NAC, esbelen, or catalase prevented GC-induced apoptosis of either cell type, although for OB-6 cells the inhibitory effect of catalase was incomplete.

Inhibition of eIF2α dephosphorylation with salubrinal and guanabenz prevents apoptosis induced by glucocorticoids, etoposide, and ER stressors in osteoblastic cells

Because ROS induce ER stress, we next investigated whether reduction of ER stress by inhibiting eIF2α dephosphorylation with salubrinal was able to prevent apoptosis induced by dexamethasone or etoposide, another proapoptotic stimulus that induces apoptosis by inhibiting topoisomerase II and DNA repair. Dexamethasone or etoposide consistently increased MLO-Y4 and OB-6 cell death (Figure 2). Salubrinal did not significantly affect cell viability, except for increasing trypan blue uptake of MLO-Y4 cells at 100 μM for 6 hours (Figure 2A). The mechanism behind the decreased viability induced by high concentrations of salubrinal is not known. However, it might be related to a transient increase in the expression of pro-apoptotic protein CHOP as found by Zhang et al in MC3T3 osteoblastic cells [25]. Further, 1 – 100 μM salubrinal prevented cell death induced by dexamethasone in both MLO-Y4 and OB-6 cells (Figure 2A and B). Salubrinal also inhibited the effects of etoposide, but with less efficiency. The 10 μM salubrinal concentration was used for subsequent experiments as it consistently prevented dexamethasone-induced apoptosis in MLO-Y4 and OB-6 cells at both 6 and 24 hour time points. Salubrinal and guanabenz, another inhibitor of eIF2α dephosphorylation, also inhibited apoptosis of OB-6 osteoblastic cells and primary osteoblasts induced by the inducers of ER stress brefeldin A, an inhibitor the ER/Golgi apparatus vesicle transport, and tunicamycin, a protein glycosylation inhibitor [16] (Figure 3A-C). In contrast, alendronate, an agent previously shown to effectively inhibit apoptosis of osteocytic MLO-Y4 cells, osteoblastic OB-6 cells, and primary calvaria derived osteoblasts induced by dexamethasone or etoposide [12, 23, 26], was unable to prevent the increase in OB-6 cell death induced by the ER stress inducers (Figure 3B).

Salubrinal and guanabenz ameliorate the inhibitory effects of glucocorticoids on matrix mineralization

We next investigated whether inhibitors of the ER stress alter the effects of GC on matrix mineralization. GC decreased mineral deposition in OB-6 osteoblastic cells cultured under osteogenic conditions, as shown by staining with von Kossa (that detects phosphate) or Alizarin Red S (that detects calcium) (Figure 4A and B), or in primary osteoblasts measured by hydroxyapatite accumulation (Figure 4C). Treatment with salubrinal or guanabenz increased mineralization of OB-6 cells (Figure 5A and B). Further, either compound partially prevented the decreased mineralization induced by GC in OB-6 or primary osteoblasts (Figure 5). Thus, salubrinal increased mineral content in cells treated with GC compared to GC alone after 7 and 10 days of culture (Figure 5A). However, salubrinal treatment could not block GC reductions in mineralization after 14 days of GC exposure, but guanabenz remained effective throughout the two week GC treatment period (Figure 5A-C).

Salubrinal protects against osteoblast and osteocyte apoptosis *in vivo* and partially prevents the bone loss induced by glucocorticoids

We next investigated whether inhibition of eIF2α dephosphorylation promoted bone cell viability also *in vivo*. Guanabenz appeared to be more potent in opposing the *in vitro* effects of GC compared to salubrinal. However, guanabenz is also an α2 adrenergic receptor agonist used in the treatment of hypertension [27]. To avoid potential skeletal effects of activating these receptors, we decided to use salubrinal for the *in vivo* study. C57Bl/6 female mice implanted with pellets containing two different doses of the GC prednisolone (GC1 = 1.4 or GC2 = 2.1 mg/kg/d) received daily injections of salubrinal (1 mg/kg/d). Mice treated with prednisolone exhibited increased apoptosis of osteoblasts in cancellous bone and of osteocytes in both cancellous and cortical bone (Figure 6A). Salubrinal completely blocked GC1-induced apoptosis of both osteoblasts and osteocytes, whereas it only partially prevented the increase in GC2-induced apoptosis of osteoblasts and did not inhibit GC2-induced osteocyte apoptosis. On the other hand and consistent with previous findings [23], alendronate effectively prevented GC2-induced apoptosis of both osteoblasts and osteocytes in cancellous bone, although it did not inhibit GC2-induced cortical osteocyte apoptosis. Administration of prednisolone induced a significant decrease in BMD in total body, spine, and femur, at both doses compared to placebo (Figure 6B). Mice implanted with placebo pellets and treated with salubrinal lost significantly less spinal BMD compared to those treated with vehicle. Similarly, mice implanted with GC1 pellets and injected with salubrinal lost significantly less bone compared to mice implanted with GC1 pellets and injected with vehicle. On the other hand, salubrinal did not prevent the loss of bone induced by GC2. In contrast, inhibition of resorption with alendronate not only prevented GC2-induced bone loss, as previously shown [23], but also increased BMD over placebo treated mice. Moreover, GC1 and GC2 reduced bone formation rate (BFR) in cancellous bone by a combination of reduction in MS/BS and in MAR (Figure 6C). Salubrinal reversed the decreased BFR induced by GC1 but not GC2. Alendronate reduced further GC2-mediated inhibition of BFR by decreasing both MS/BS and MAR.

DISCUSSION

Apoptosis of osteoblasts and osteocytes contributes to the reduced bone formation and increased bone fragility in glucocorticoid-induced osteoporosis [6, 7, 9]. Therefore, understanding the mechanisms by which GC induce apoptosis of osteoblastic cells is critical for the development of intervention therapies. Earlier studies demonstrated that apoptosis of osteocytes and osteoblasts is caused by loss of attachment to the extracellular matrix mediated by inside-out signaling downstream of Pyk2/JNK kinases [11] and that GC increase ROS production in bone *in vivo* and in osteoblastic cells *in vitro* [13]. We investigated in this study the effect on GC action of salubrinal and guanabenz, eIF2α dephosphorylation inhibitors that block ROS-induced ER stress [16, 17]. These compounds prevented the pro-apoptotic effect of GC on osteoblasts and osteocytes *in vitro* and the decreased in mineral deposition induced by GC in osteoblastic cell cultures. Further, salubrinal prevented apoptosis of osteoblasts and osteocytes induced by GC *in vivo* and the concomitant decrease in bone mass and bone formation.

Consistent with the current study demonstrating inhibition of apoptosis of osteoblasts and osteocytes and improved mineralization by decreasing ER stress, recent evidence demonstrates increased apoptosis of osteoblastic cells and changes in osteoblast differentiation associated with elevated ER stress *in vitro* with thapsigargin or tunicamycin [28, 29]. Remarkably, increased ER stress appears to have a time-dependent biphasic effect inducing rapid increase in osteoblast markers Runx2 and osterix, followed by a reduction in the expression of these transcription factors as well as osteocalcin [28]. Further, ER stress-mediated apoptosis of osteoblasts and impaired osteoblast differentiation was also demonstrated in a model of osteogenesis imperfecta [30] and in mice lacking the ER-localized protein Arl6ip5 in osteoblasts [31]. Consistent with the pro-apoptotic and inhibitory effect of ER stress on osteoblast differentiation, in the current manuscript we show that opposing ER stress by inhibiting eIF2α dephosphorylation prevents osteoblast and osteocyte apoptosis and the decrease in osteoblast function induced by GC in vitro and in vivo. Our findings agree with previous evidence showing that salubrinal increases the number of alkaline phosphatase positive colonies in bone marrow cell cultures [32] and osteocalcin expression in MC3T3-E1 cells [33].

In contrast to the protective effect of salubrinal against the action of low GC dose (GC1), salubrinal was unable to protect the skeleton from the high GC dose (GC2). Similarly, whereas salubrinal or guanabenz effectively prevented the inhibition of mineralization induced by short-term treatment of cultured osteoblasts with GC, only guanabenz was able to reverse the effects of prolonged treatment with GC. The mechanism of the different outcome of guanabenz compared to salubrinal is not known. It remains unclear also the reason for the incomplete inhibition of cortical osteocyte apoptosis by alendronate in this experiment, since in earlier studies alendronate completely prevented apoptosis of osteoblastic cells in both cancellous and cortical bone [23]. A potential explanation is that in the previous study alendronate was administered daily instead of in a weekly 7-day cumulative dose. Nevertheless, these findings suggest that the potency of the pro-apoptotic signals delivered by GC, either due to high dose or prolonged exposure, determines the ability of the protective compound to induced survival and that once a pro-apoptotic threshold is reached, salubrinal is not able to reverse it.

Consistent with the *in vitro* and *in vivo* data showing that inhibition of eIF2α dephosphorylation preserves osteoblast viability, treatment with salubrinal reversed the inhibition in BFR induced by low dose of GC mainly by reversing the decrease in MS/BS. These findings together with the demonstration that salubrinal prevents the reduction in mineralization induced by GC *in vitro* strongly suggest that the preservation of bone mineral density by salubrinal is due to its protective effects on osteoblasts. In contrast, alendronate further reduced BFR in GC treated animals, as expected due to its potent inhibitory effects on osteoclasts and resorption. Salubrinal, like alendronate, has been also shown to inhibit osteoclastogenesis [33] and to protect from ovariectomy-induced bone loss [34]. However, in the setting of GC excess, the main mechanism of salubrinal action appears to be related to osteoblasts. Thus, the bone sparing actions of salubrinal and bisphosphonates on GC excess are mediated by distinct cellular mechanisms. Whereas alendronate inhibits osteoclast

activity and reduces bone turnover, salubrinal preserves the viability and the bone forming function of osteoblasts.

Protein misfolding is a common feature of several human diseases including neurodegenerative conditions such as Alzheimer's and Parkinson's diseases, as well as type 2 diabetes [35]. Lack of functional PERK, the sensor of the unfolded protein response and a major eIF2α kinase, causes the Wolcott-Rallison syndrome (WRS) in humans, which is characterized by early onset diabetes and aberrant skeletal development [36]. PERK deletion in the mouse prevents the unfolded protein response induced by ER stressors and inhibits phosphorylation of eIF2α [37]. Homozygous PERK null mice are born normal but loose β cells rapidly and develop early diabetes [38]. As with WRS patients, PERK knockout mice develop skeletal dysplasia and defective bone mineralization [39]. Osteoblast and osteocyte number, mineral appositional rate MAR, and markers of osteoblasts and osteocytes are decreased in PERK null mice. Osteoblast survival appears not to be affected *in vitro*albeit *in vivo* studies were not shown. Together with the current study in which increased osteoblast and osteocyte apoptosis, decreased bone formation, and decreased bone mass induced by glucocorticoids are partially prevented by salubrinal, these findings suggest that inhibition of eIF2α dephosphorylation is a potential novel target for the treatment of glucocorticoid-induced osteoporosis and other conditions with deficient osteoblast differentiation and bone mineralization.

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Highlights

- GC-induced osteoblast/osteocyte apoptosis is mediated by ROS and ER stress.
- eIF2α dephosphorylation inhibitors Sal and Gz prevent ER stress induced apoptosis.
- Sal/Gz protect from GC-induced apoptosis and impaired mineral deposition in vitro.
- Sal prevented osteoblast/osteocyte apoptosis and loss of BMD induced by GC in vivo.
- eIF2α dephosphorylation is a potential target for treating GC-induced osteoporosis.

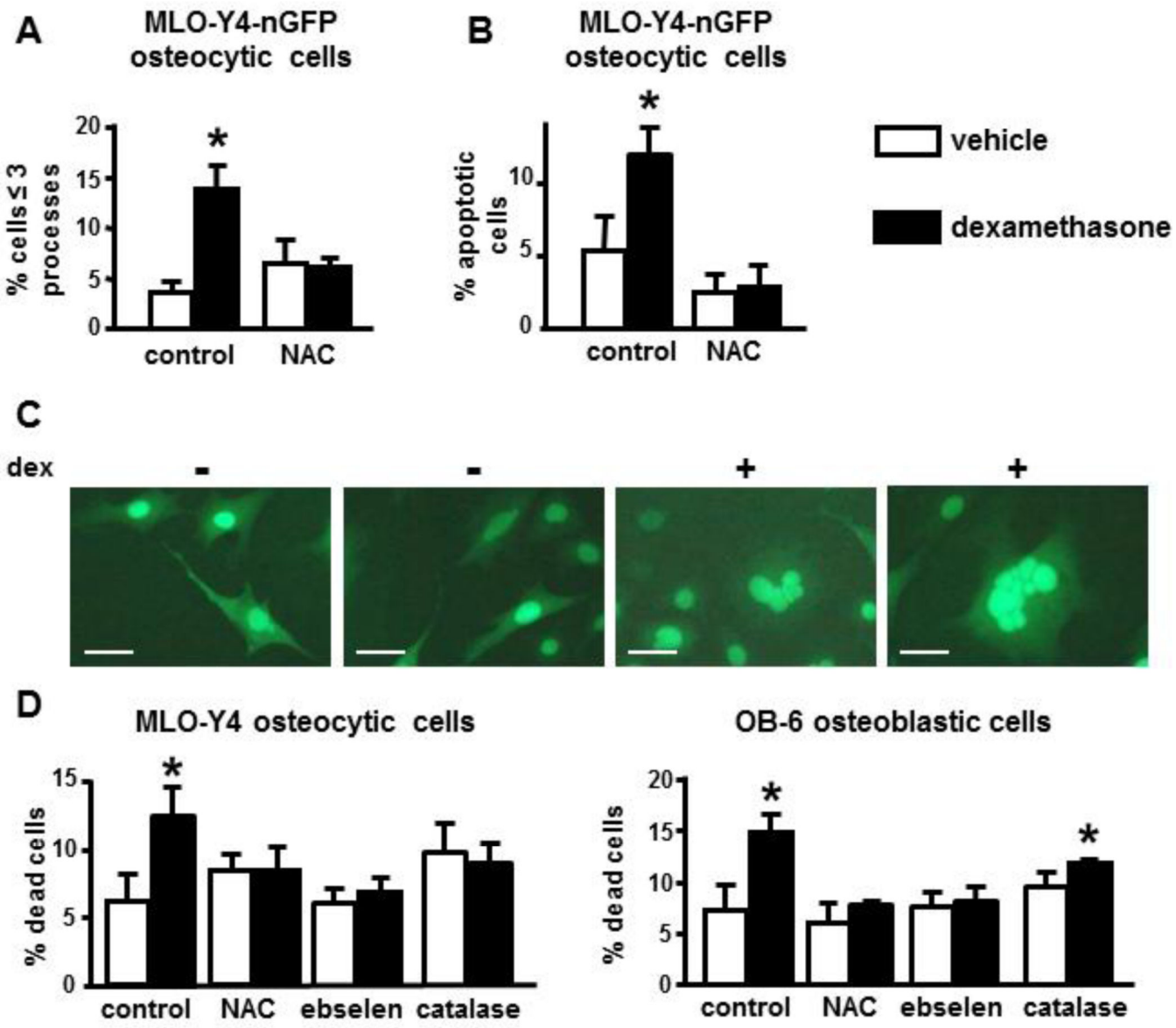

Figure 1. Glucocorticoid-induced apoptosis of osteocytic and osteoblastic cells is prevented by inhibiting ROS generation

(**A-D**) Cells were exposed to vehicle (ethanol) or dexamethasone for 6 hours. (**A**) Quantification of cytoplasmic retraction in stably transfected nGFP MLO-Y4 cells treated with or without NAC for 1 hour prior to addition of dexamethasone. (**B**) Apoptosis quantification of nGFP-transfected MLO-Y4 cells. (**C**) Representative images of nGFP-expressing MLO-Y4 osteocytic cells treated with vehicle or dexamethasone showing changes in cell morphology, chromatin condensation, and nuclear fragmentation. Lines correspond to 200 μm. (**D**) Quantification of cell death in MLO-Y4 osteocytic or OB-6 osteoblastic cells with or without pretreatment of the indicated anti-oxidant agent, assessed by trypan blue uptake. Bars represent the means ±SD of N=3 independent wells/treatment. * $p < 0.05$ vs. the corresponding vehicle-treated cells, by one-way ANOVA.

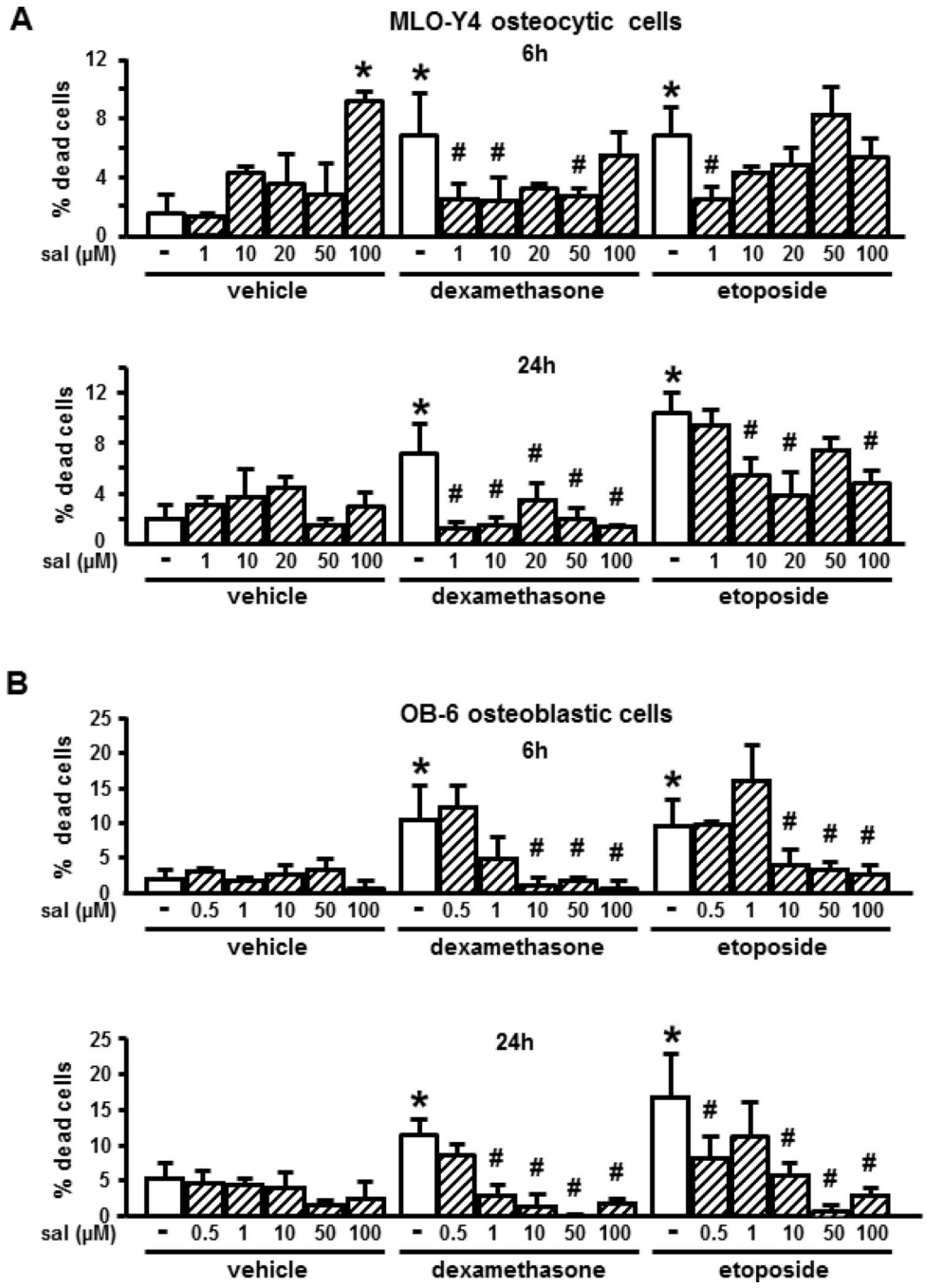

Figure 2. Protection against ER stress with salubrinal prevents dexamethasone and etoposide induced apoptosis of osteocytic MLO-Y4 and osteoblastic OB-6 cells

Cell death quantification in MLO-Y4 osteocytic (**A**) or OB-6 osteoblastic (**B**) cells treated with or without salubrinal prior to addition of vehicle, dexamethasone, or etoposide, assessed by trypan blue uptake. Bars represent the means ±SD of N=3 samples per treatment. * $p < 0.05$ vs. cells treated with vehicle without salubrinal and # $p < 0.05$ vs. corresponding apoptotic agent without salubrinal for each time point, by one-way ANOVA.

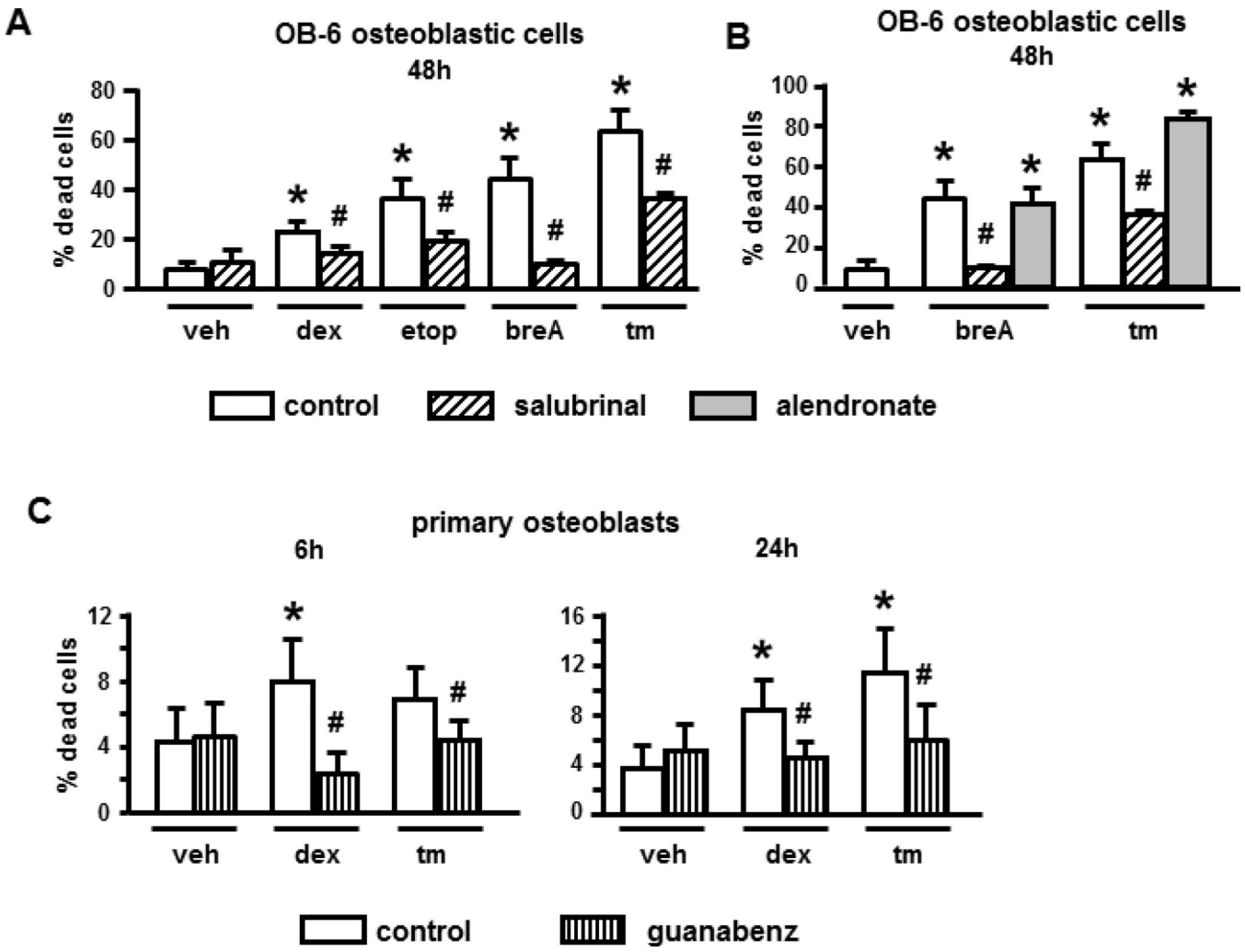

Figure 3. Salubrinal or guanabenz protect from apoptosis induced by dexamethasone, etoposide, and ER stressors in osteoblastic cells

Cell death quantification in OB-6 osteoblastic (**A** and **B**) or primary osteoblastic (**C**) cells treated with or without anti-apoptotic agents salubrinal, alendronate, or guanabenz prior to addition of dexamethasone (dex), etoposide (etop), brefeldin A (breA), tunicamycin (tm), or vehicle (veh). Bars represent the means ±SD of N=3 samples per group. *$p < 0.05$ vs. veh-treated cells and # $p < 0.05$ vs. corresponding apoptotic agent without designated eIF2α phosphatase inhibitor for each time point, by Student's t-test in (**A**) and (**C**) and by one-way ANOVA in (**B**).

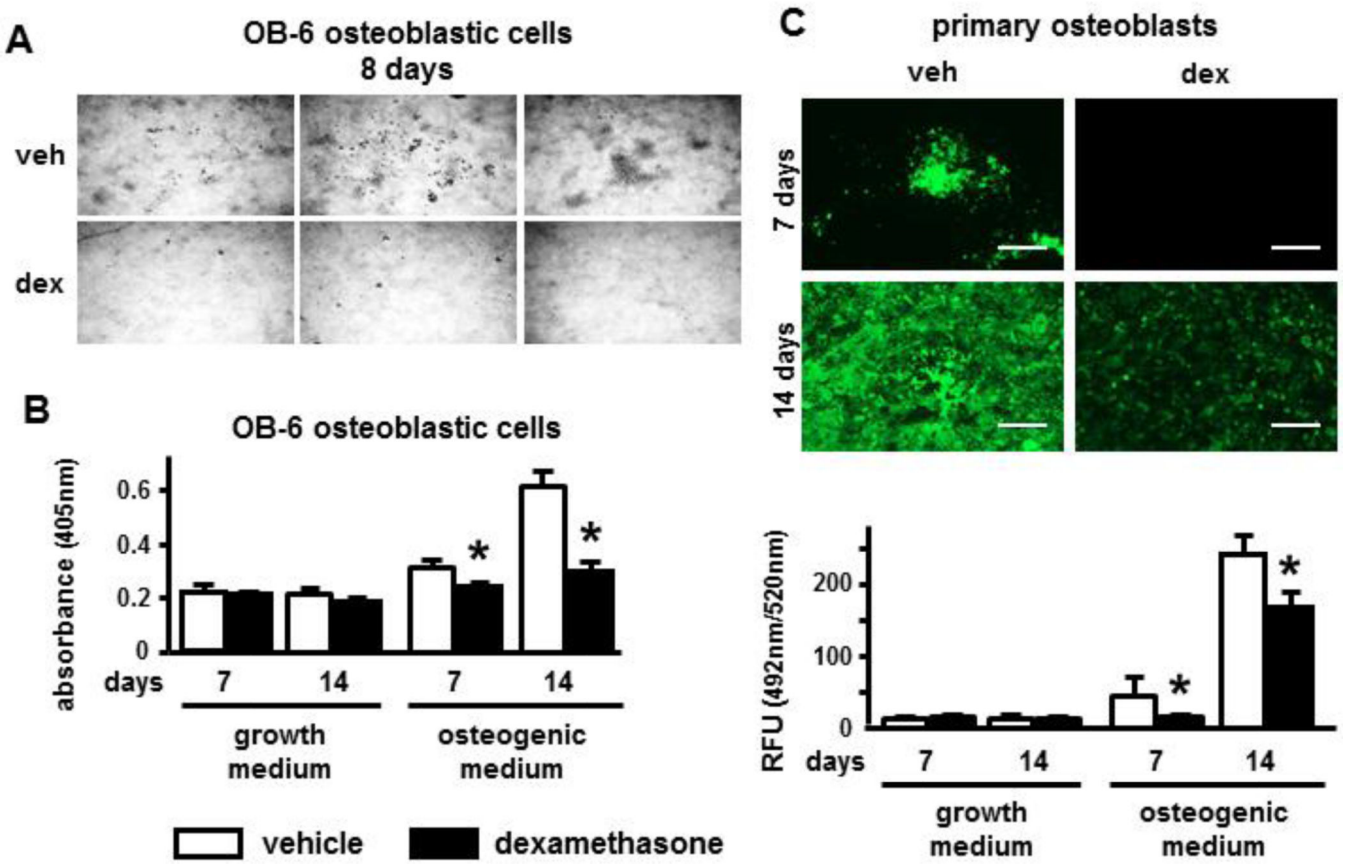

Figure 4. Glucocorticoid decreases matrix mineralization in cultures of osteoblastic cells and primary osteoblasts

(**A** and **B**) OB-6 osteoblastic cells cultured with or without dexamethasone (dex) and the degree of mineralization was assessed by von Kossa staining for 8 days (**A**) and by Alizarin Red S staining for 1 or 2 weeks (**B**). (**C**) Calvaria-derived osteoblastic cells were cultured with or without osteogenic medium for 1 or 2 weeks together with vehicle (veh) or dexamethasone (dex) and mineralization was assessed using the OsteoImage Mineralization Assay Kit. Lines correspond to 200 μm. Bars represent the means ±SD of N=3 for (**A**) and (**B**), N=6 for (**C**) samples per group. * $p < 0.05$ vs. veh-treated cells in corresponding medium condition for each time point, by two-way ANOVA.

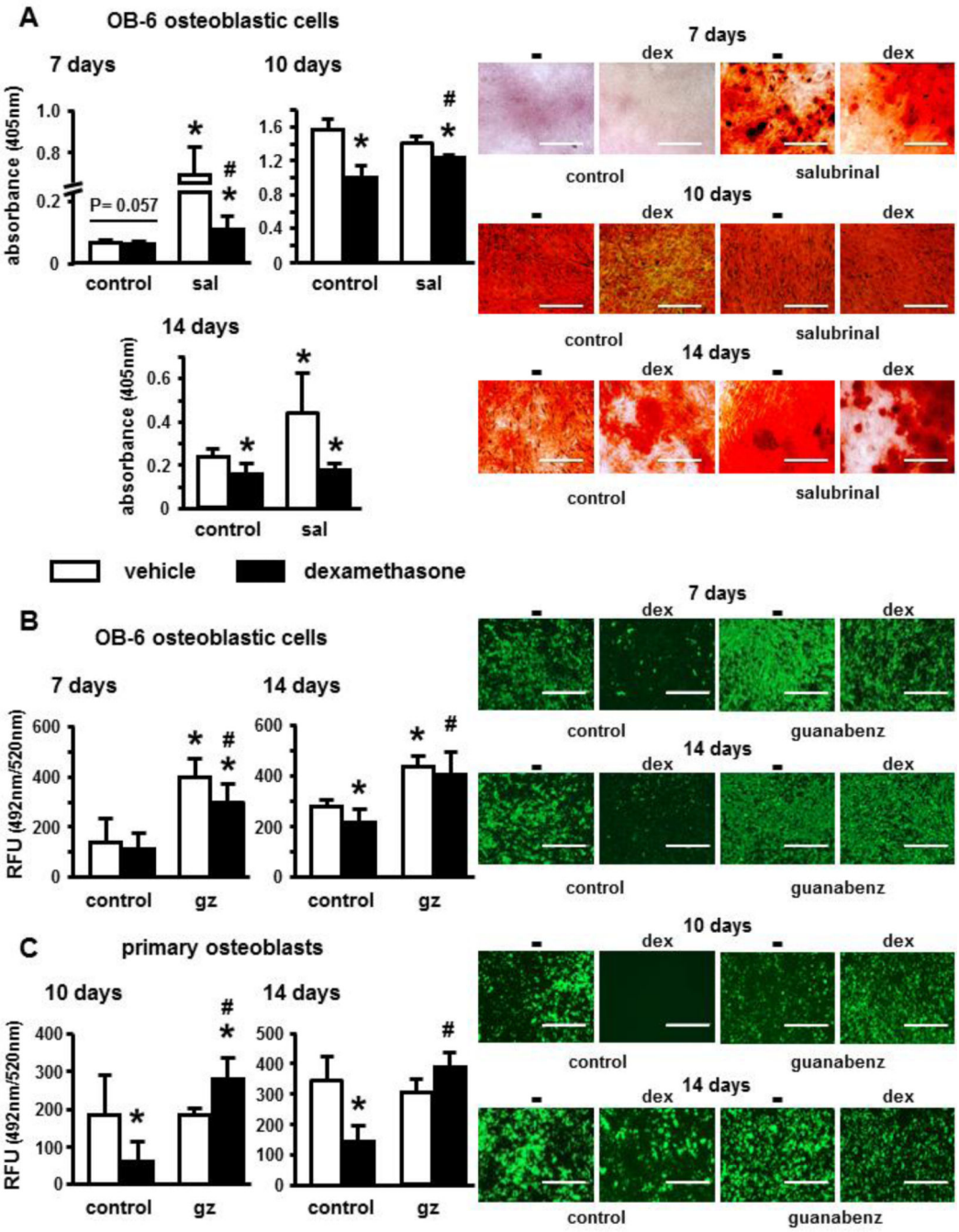

Figure 5. Salubrinal and guanabenz ameliorate the inhibitory effects of GC on matrix mineralization

(**A**) Mineralization was determined in vehicle (veh) or dexamethasone (dex) treated differentiated OB-6 cells without (control) or with salubrinal (sal) pre-treatment for 7, 10, and 14 days by Alizarin Red S staining. (**B**) and (**C**) Quantification of mineralization in OB-6 (**B**) and calvaria-derived (**C**) osteoblastic cells treated with vehicle or dexamethasone with or without guanabenz (gz) for the indicated incubation periods. Hydroxyapatite accumulation was measured by OsteoImage Mineralization Assay Kit. Lines correspond to

400 μm. Bars represent the means ±SD of N=3 for (**A**) and N=12 for (**B**-**C**). *$p < 0.05$ vs. veh-treated control cells and # $p < 0.05$ vs. dex-treated control cells, by one-way ANOVA.

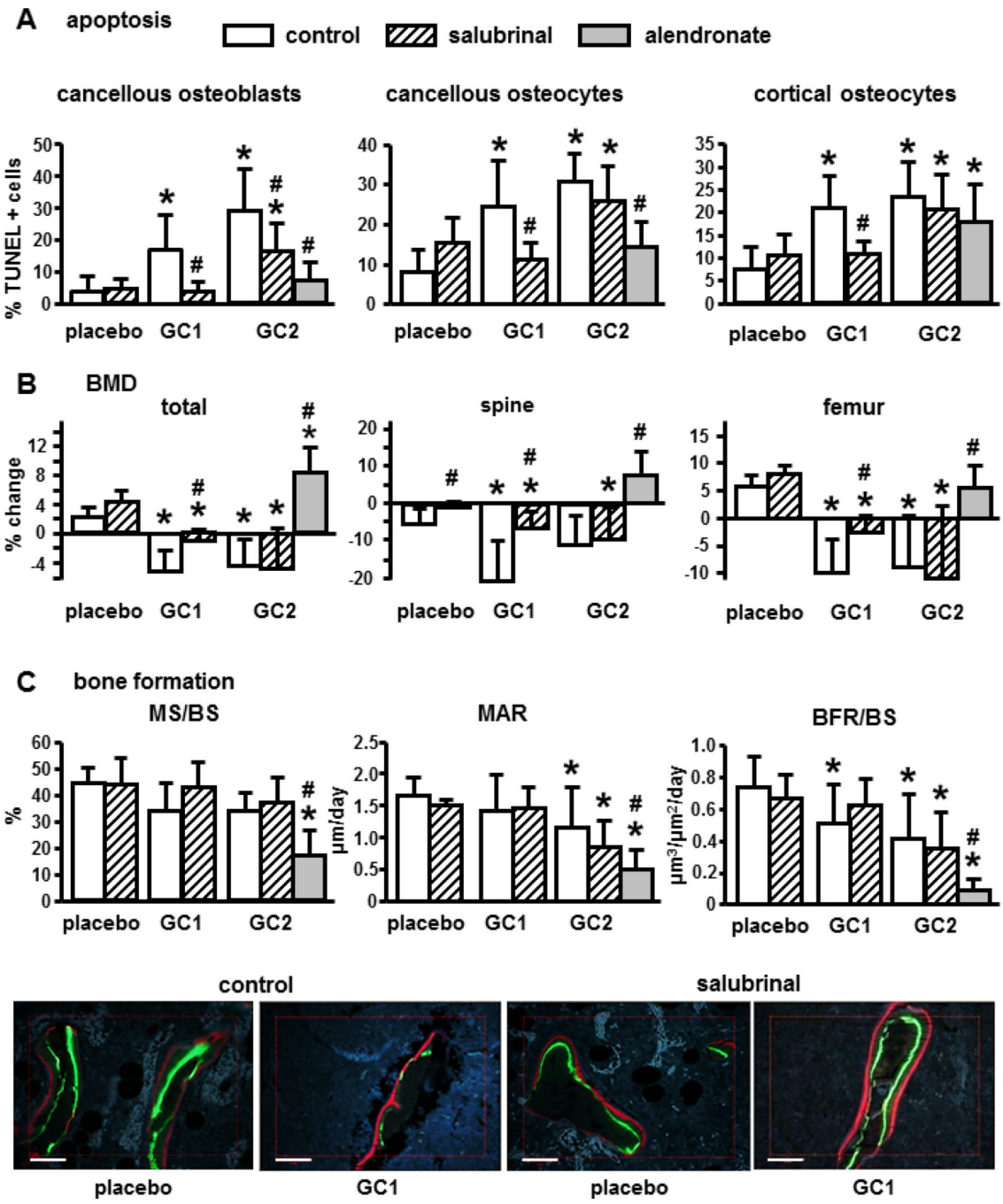

Figure 6. Salubrinal protects against glucocorticoid-induced apoptosis and consequent bone loss *in vivo*

(**A**) TUNEL and (**C**) dynamic histomorphometric data was obtained from longitudinal distal femur sections. Representative images of fluorochrome labeled bones are shown. Bars correspond to 50 µm. (**B**) BMD percent changes for placebo, 1.4 mg/kg/d prednisolone (GC 1), or 2.1 mg/kg/d prednisolone (GC 2) pellet implanted mice with or without salubrinal intervention were determined by DXA analysis. Bars represent the means ±SD of N=7-10 for (**A**) and (**C**) and N=6-10 for (**B**). Statistical analysis for placebo, GC1, and GC2 treated

mice treated with vehicle (control) or salubrinal was performed by two-way ANOVA. *$p < 0.05$ vs. placebo mice injected with vehicle (control) or salubrinal. #$p < 0.05$ vs. the corresponding GC treated mice injected with vehicle (control). The effect of alendronate on GC2 treated mice was analyzed by comparing placebo, GC2, and GC2 plus alendronate by one-way ANOVA. *$p < 0.05$ vs. placebo mice injected with vehicle (control) and #$p < 0.05$ vs. GC2 treated mice injected with vehicle (control).