

# Acylated and unacylated ghrelin protect MC3T3-E1 cells against *tert*-butyl hydroperoxide-induced oxidative injury: pharmacological characterization of ghrelin receptor and possible epigenetic involvement

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**Abstract** Increasing evidence suggests a role for oxidative stress in age-related decrease in osteoblast number and function leading to the development of osteoporosis. This study was undertaken to investigate whether ghrelin, previously reported to stimulate osteoblast proliferation, counteracts *tert*-butyl hydroperoxide (*t*-BHP)-induced oxidative damage in MC3T3-E1 osteoblastic cells as well as to characterize the ghrelin receptor (GHS-R) involved in such activity. Pretreatment with ghrelin ( $10^{-7}$ – $10^{-11}$  M) significantly increased viability and reduced apoptosis of MC3T3-E1 cells cultured with *t*-BHP (250  $\mu$ M) for three hours at the low concentration of  $10^{-9}$  M as shown by MTT assay and Hoechst-33258 staining. Furthermore, ghrelin prevented *t*-BHP-induced osteoblastic dysfunction and changes in the cytoskeleton organization evidenced by the staining of the actin fibers with Phalloidin-FITC by reducing reactive oxygen species generation. The GHS-R type 1a agonist, EP1572 ( $10^{-7}$ – $10^{-11}$  M), had no effect against *t*-BHP-induced cytotoxicity and pretreatment with the selective GHS-R1a antagonist, D-Lys<sup>3</sup>-GHRP-6 ( $10^{-7}$  M), failed to remove ghrelin ( $10^{-9}$  M)-protective effects against oxidative injury, indicating that GHS-R1a is not involved in such ghrelin activity. Accordingly, unacylated ghrelin (DAG), not binding GHS-R1a, displays the same protective actions of ghrelin against *t*-BHP-induced cytotoxicity. Preliminary observations indicate that ghrelin

increased the trimethylation of lys4 on histones H3, a known epigenetic mark activator, which may regulate the expression of some genes limiting oxidative damage. In conclusion, our data demonstrate that ghrelin and DAG promote survival of MC3T3-E1 cell exposed to *t*-BHP-induced oxidative damage. Such effect is independent of GHS-R1a and is likely mediated by a common ghrelin/DAG binding site.

**Keywords** Ghrelin · Unacylated ghrelin · Ghrelin receptors · MC3T3-E1 · Oxidative stress

## Introduction

Aging is believed to be a major factor involved in the reduction of bone mass and quality leading to osteoporosis. As aging progresses, there is an increase in osteoblast and osteocyte apoptosis as well as a decrease in osteoblast number and bone formation (Manolagas 2010; Manolagas and Parfitt 2010). Thus, the amount of bone resorbed by osteoclasts is not fully restored with new bone deposited by osteoblasts, this imbalance causing bone loss (Seeman 2003).

Although the pathophysiological mechanisms underlying the adverse effects of aging on bone mass and strength are not well understood, previous studies have shown that oxidative stress resulting from excessive levels of reactive oxygen species (ROS) or dysfunction of antioxidant defense is involved in this disease (Almeida 2012; Manolagas 2010).

In mice, it has been reported that increased cellular oxidative stress induces low-turnover osteopenia (Nojiri et al. 2011) and that bone mass progressively declines in association with decreased antioxidant enzyme levels (Almeida et al. 2007). In the femora of ovariectomized rats, increased lipid peroxidation and decreased levels of

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enzymatic antioxidants were found (Muthusami et al. 2005). These experimental data fit well with clinical studies showing that increased oxidative stress is negatively associated with bone mineral density in aged men and women (Basu et al. 2001). Furthermore, elderly osteoporotic women showed high levels of lipid peroxidation (Ozgoçmen et al. 2007) and a marked decrease in plasma levels of antioxidants as compared to age-matched controls (Maggio et al. 2003).

Formation of ROS occurs primarily in the mitochondria from the escape of electrons passing through the electron transport chain during normal metabolisms. ROS are also generated during fatty acid oxidation or in response to external stimuli such as inflammation. Increased mitochondrial damage with age may result in excessive ROS production that damages proteins, lipids and DNA, leading to cell death (Muller et al. 2007; Almeida et al. 2007).

Several studies have shown that free radicals and ROS can affect osteoblast growth and function (Bai et al. 2004; Manolagas 2010), suggesting the potential use of antioxidants in the prevention and/or treatment of osteoporosis.

Ghrelin, identified in 1999 as an endogenous ligand of the growth hormone secretagogue receptor (GHS-R), derives from a 117 amino acid pre-prohormone which is cleaved into a 94 amino acid proghrelin peptide. This proghrelin peptide is further cleaved and gives rise to two major forms of 28 amino acid ghrelin (Hosoda et al. 2000). The ghrelin peptide acylated at serine-3 by ghrelin-*O*-acyltransferase (GOAT) (Yang et al. 2008), usually referred as ghrelin in the literature, and a non-acylated form (DAG) which circulates at tenfold higher levels than ghrelin (Holmes et al. 2009; Patterson et al. 2005). The octanoylation is critical for the binding to the GHS-R 1a, in inducing GH secretion, food intake (Kojima and Kangawa 2005) and inhibition of gastric acid secretion (Sibilia et al. 2006). DAG, in fact, seems to be devoid of any endocrine and gastrointestinal activities.

Indeed, DAG was reported to exert some non-endocrine action, sharing with ghrelin a protective effect on cardiomyocytes and endothelial cells (Baldanzi et al. 2002) and an inhibitory action on inflammatory pain (Sibilia et al. 2012). Therefore, the existence of a ghrelin receptor subtype different from GHS-R 1a, yet to be identified (Seim et al. 2011), that could mediate some of the effects of both peptides has been suggested.

Since ghrelin inhibits oxidative damage in various peripheral tissues (El Eter et al. 2007; Kawczynska-Drozd et al. 2006; Zhang et al. 2011; Tong et al. 2012) and in the brain (Obay et al. 2008), it has been proposed as an antioxidant agent.

The role of ghrelin, if any, in the protection against oxidative stress in osteoblasts has never been investigated previously. This is surprising, since ghrelin and synthetic GH

secretagogues play a role in bone formation. Ghrelin and its receptor GHS-R 1a are expressed in several osteoblasts and osteoblastic cell lines, and ghrelin directly promotes both osteoblast proliferation and differentiation (Kim et al. 2005; Maccarinelli et al. 2005; Fukushima et al. 2005; Costa et al. 2011). Furthermore, long-term treatment with ghrelin (Fukushima et al. 2005) or synthetic GH secretagogues increases BMD (Svensson et al. 2000) and prevents gonadectomy-induced bone loss in the rat (Sibilia et al. 1999, 2002).

In the present study, we investigated the effects of ghrelin on the survival of osteoblastic MC3T3-E1 cells exposed to *tert*-butyl hydroperoxide (*t*-BHP). *Tert*-butyl hydroperoxide is an organic hydroperoxide widely used as a pro-oxidant to induce oxidative stress in a variety of cells (Altman et al. 1994; Sestili et al. 1998; Haidara et al. 2008).

In order to characterize the ghrelin receptor involved in ghrelin cytoprotection, we examined the effects of DAG, which does not bind the classic GHS-R1a (Bednarek et al. 2000; Ghigo et al. 2005) and the GHS-R 1a agonist, EP1572 (Broglia et al. 2002; Sibilia et al. 2006, 2012) on *t*-BHP-induced cytotoxicity. The ability of the specific GHS-R 1a antagonist, *D*-Lys<sup>3</sup>-GHRP-6 (Carreira et al. 2006; Muccioli et al. 2007; Sibilia et al. 2006, 2012), to reverse the ghrelin protective effects against oxidative stress was also examined. The possible effect of ghrelin on the intracellular levels of H3K4me3, an important epigenetic mark, has been also evaluated in a pilot experiment.

## Materials and methods

### Drugs

Ghrelin, DAG and EP1572 (Aib-DTrp-DgTrp-CHO) were synthesized by conventional solid-phase synthesis and purified to at least 98 % purity by HPLC by Neosystem (Strasbourg, France). *D*-Lys<sup>3</sup>-GHRP-6 was purchased from Bachem AG (Budendorf, Switzerland). *Tert*-butyl hydroperoxide (*t*-BHP) and Trolox were purchased from Sigma-Aldrich Chemical, Italy. Trolox was dissolved in ethanol (70 %) with a final ethanol concentration in the medium less than 0.5 %.

### Cell culture

Mouse MC3T3-E1 osteoblastic cells (ATCC catalog number CRL-2593) were cultured in Dulbecco's modified Eagle's medium high glucose (D-MEM, Euroclone, Italy), and supplemented with 10 % fetal bovine serum (FBS, Sigma-Aldrich Chemical, Italy), 1 % L-glutamine, 100 µg/ml streptomycin and 100 U/ml penicillin at 37 °C in 5 % CO<sub>2</sub> atmosphere. This basic medium was changed every 3 days, and cells were subcultured weekly.

### Cell viability assay

Cell was plated at the density of  $3 \times 10^3$  cells/well in a 96-well culture plate. After treatment, cells were washed with phosphate-buffered saline (PBS, Euroclone, Italy) and incubated at 37 °C with 0.5 mg/ml 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide (MTT, Sigma-Aldrich Chemical, Italy) for 3 h. The conversion of the tetrazolium salt MTT to a colored formazan was used to assess cell viability. After the supernatant was removed, dimethyl sulfoxide was added to each well and the absorbance was recorded by a microplate spectrophotometer (WALLAC) at 550 nm.

Cell phenotypic observations were made using Olympus TH4-200 inverted phase contrast microscope, fitted with a digital camera Olympus C-4040 zoom to record any change during treatment.

### Hoechst staining of apoptotic cells

Morphological changes in the nuclear chromatin of apoptotic cells were detected by Hoechst-33258 (Sigma-Aldrich Chemical, Italy) staining. MC3T3-E1 cells ( $5 \times 10^3$  cells/well) were grown on 22-mm glass coverslips in 6-well plates. After treatment, the cells were fixed with 4 % formaldehyde in 0.12 M sucrose, permeabilized with 0.1 % TritonX100 in PBS for 5 min, and stained with 10 µg/ml Hoechst-33258 DNA dye for 5 min. The cells then visualized using an Axioplan fluorescence microscope. The blue fluorescent Hoechst 33258 is a cell permeable nucleic acid dye usually used to identify chromatin condensation and fragmentation by staining the condensed nuclei of apoptotic cells. Uniformly stained nuclei were scored as healthy, viable cells. Condensed or fragmented nuclei were considered as apoptotic. At least 200 cells were scored blindly without knowledge of their prior treatment.

### Cytoskeleton rearrangement

MC3T3-E1 cell growth, fixation and permeabilization were carried out by the same procedure as mentioned above. The permeabilized cells were incubated with FICT-labeled phalloidin (diluted 1:100 in PBS) for 20 min at room temperature and mounted in Vectashield HardSet Mounting Medium with 4',6-diamidino-2-phenylindole (DAPI, Vector Laboratories, Burlingame, CA). The images were obtained using an Axioplan fluorescence microscope.

### Intracellular ROS production

ROS production was measured using 5(6)-carboxy-2',7'-dichlorofluorescein diacetate (CM-DCFA, Sigma-Aldrich Chemical, Italy 10 µM) according to the method described

by Wang and Joseph (1999). The MC3T3-E1 cells were seeded in black 96-well plates and cultured for 24 h. On the day of the experiment, the cells were preincubated with ghrelin or DAG ( $10^{-9}$  M) for 2 h and loaded with CM-DCFA during the last 30 min of treatment. After CM-DCFA was removed, the cells were exposed to *t*-BHP (250 µM) for 3 h. DCF fluorescence was assessed with a spectrofluorophotometer (Victor<sup>TM</sup>, PerkinElmer, Waltham, MA, USA) at the excitation (485 nm) and emission (530 nm) wavelengths.

### Epigenetic studies

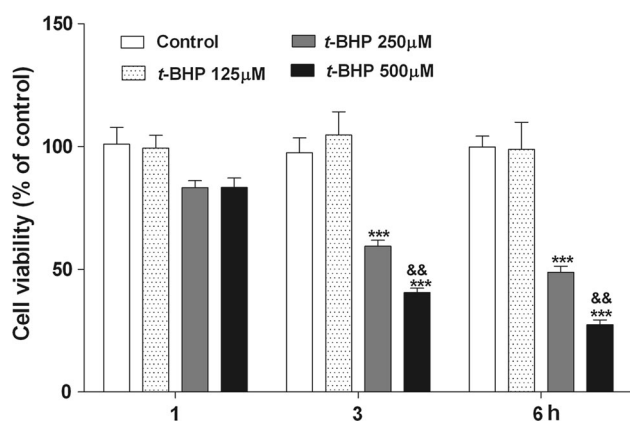
Samples for Western blotting of histones were prepared as follow: Cells were collected in PBS-EDTA, and cell pellets were homogenized in Laemmli buffer 1X (62,5 mM Tris-HCl pH 6.8, 7.5 % glycerol, 2 % SDS, 0.125 M dithiothreitol), subjected to ultrasonic bath for 10 min prior to heat samples at 95 °C for 5 min. The resulting whole cell extracts were then centrifuged at 13,200 rpm for 10 min, and supernatants were saved and used directly for immunoblotting. Protein extracts were resolved by SDS/15 %PAGE. Protein gels were electrotransferred to 0.2 µm pore nitrocellulose membranes by standard wet procedures. After transfer, membranes were stained with Ponceau S to verify the correct loading and transfer, and then blocked and probed overnight at 4 °C with the primary specific antibodies: anti-H3K4me3 (ref. Ab8580, Abcam) and anti-H4 (ref. 10158, Abcam). Membranes were processed with WesternDot<sup>TM</sup> 625 Western Blot Kits (Life Technologies) as described by the manufacturer. Fluorescent signals were quantified using ChemiDoc system (Bio-Rad).

### Statistical analysis

Statistical analysis was performed with a statistic package (GraphPad Prism5, GraphPad Software San Diego, CA, USA). All data are represented as the mean  $\pm$  SEM of four independent experiments. Differences between groups were assessed by one-way analysis of variance (ANOVA) followed by Bonferroni test when data were parametric. Nonparametric data were analyzed by a Kruskal-Wallis test followed by Dunn's test. A probability of  $p < 0.05$  was considered to be significant.

## Results

Figure 1 shows dose- and time-dependent effect of *t*-BHP on MC3T3-E1 cell viability as assessed by the MTT test. The concentration of 250 µM *t*-BHP for 3 h reduces cell viability of  $59.5 \pm 2.4$  %, as compared with control group, and this effect remains at the same extent when the

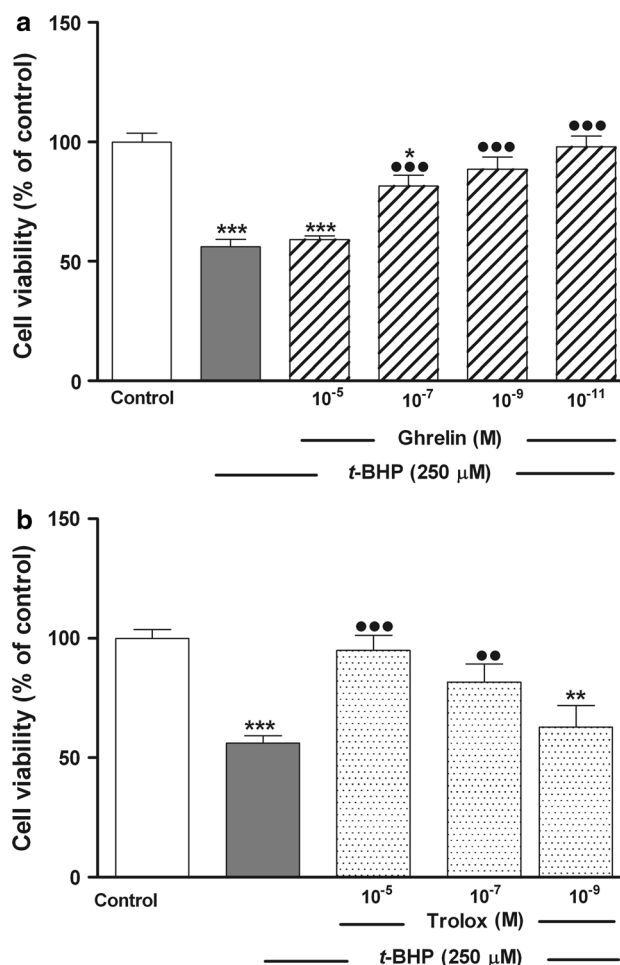


**Fig. 1** Time- and dose-dependent effects of *t*-BHP on MC3T3-E1 cell viability. Cells were treated with increasing concentrations of *t*-BHP (125–500  $\mu$ M) and incubated up to 180 min. Data are expressed as the percentage relative to control and are the mean  $\pm$  SEM of four replicates within a single experiment that was repeated at least three times. \*\*\* $p$  < 0.001 vs. control; &&  $p$  < 0.01 vs. *t*-BHP 250  $\mu$ M

incubation was carried out for 6 h. The concentration of 250  $\mu$ M *t*-BHP for 3 h was used in subsequent experiments. In our previous experiments, we have shown that ghrelin significantly increases cells viability starting from 6 h of incubation (data not shown). To avoid the confounding stimulatory action of ghrelin on cell viability, we pretreated MC3T3-E1 cells with increasing concentrations of the peptide 2 h before treatment with *t*-BHP. As shown in Fig. 2a, ghrelin exerts a maximal protective effect against *t*-BHP cytotoxicity when cells were incubated with  $10^{-9}$  and  $10^{-11}$  M. Greater ghrelin concentrations did not further increase its protective effect but, on the contrary, were less effective. We chose the concentration of  $10^{-9}$  M ghrelin for further experiments. The effectiveness of our experimental conditions has been validated by the use of Trolox, a well-known antioxidant compound (Cos et al. 2003). Interestingly, we found that a concentration of  $10^{-5}$  M Trolox is required to exert a protective action against *t*-BHP cytotoxicity comparable to that of  $10^{-11}$  M ghrelin (Fig. 2b).

We then investigated the effect of ghrelin on *t*-BHP-induced apoptosis. As shown in Fig. 3b, treatment with *t*-BHP (250  $\mu$ M) for 3 h caused a significant induction of cell apoptosis ( $28.0 \pm 3.3$  %) as compared with control ( $5.9 \pm 1.0$  % apoptotic cells) that was significantly reduced by pretreatment (2 h before) with  $10^{-9}$  M ghrelin ( $10.7 \pm 1.3$  %). The protective action of ghrelin against typical nuclear changes associated with cell apoptosis induced by *t*-BHP is reported in Fig. 3a.

In the studies performed to investigate the effects of *t*-BHP on the morphology of MC3T3-E1 cells, we have shown that MC3T3-E1 control monolayers are arranged in a star-like manner and present round nuclei. Many processes connect each cell with their neighboring. Treatment



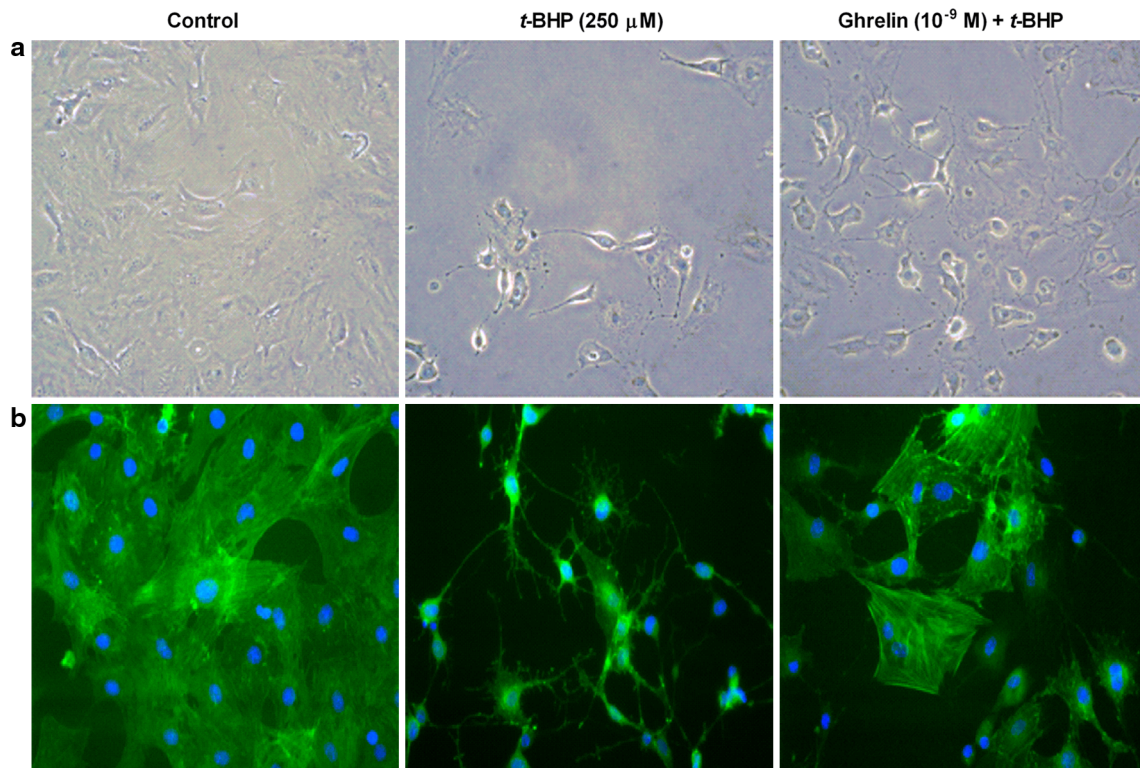
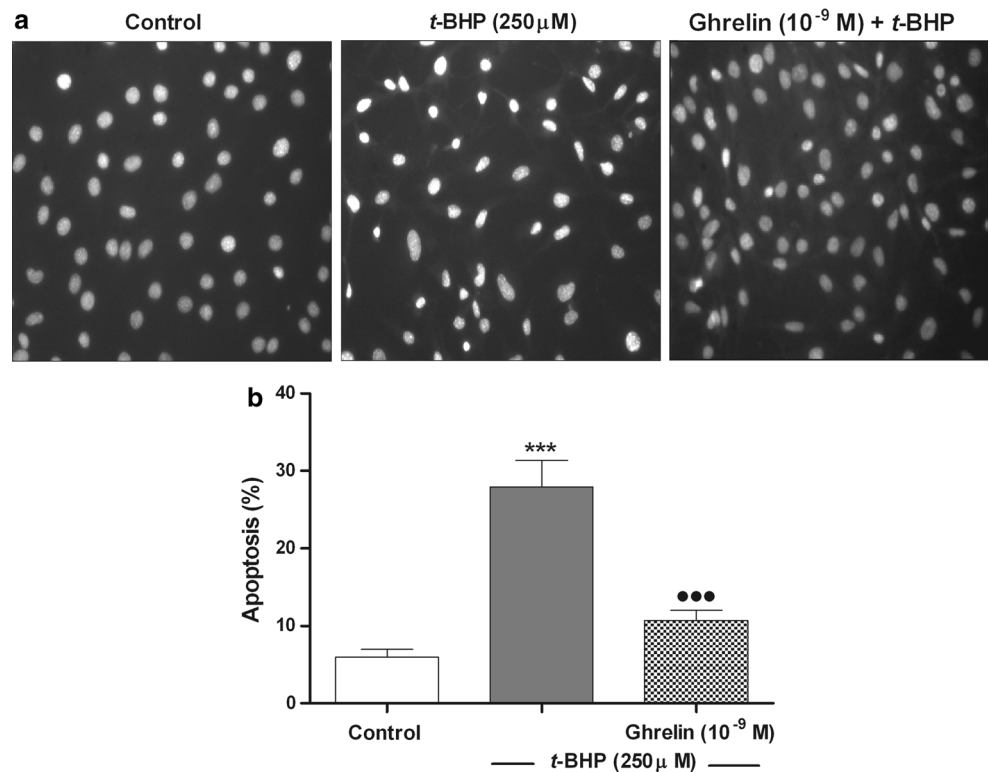
**Fig. 2** Effects of ghrelin (a) and Trolox (b) on *t*-BHP-induced cytotoxicity on MC3T3-E1 cells. Cells were preincubated with ghrelin ( $10^{-5}$ – $10^{-11}$  M) or Trolox ( $10^{-5}$ – $10^{-9}$  M) 2 h before treatment with *t*-BHP (250  $\mu$ M for 3 h). Cell viability was measured by MTT assay. Data are expressed as the percentage relative to control and are the mean  $\pm$  SEM of four replicates within a single experiment that was repeated at least three times. \* $p$  < 0.05, \*\* $p$  < 0.01, \*\*\* $p$  < 0.001 vs. control; ● $p$  < 0.01, ●● $p$  < 0.001 vs. *t*-BHP

with *t*-BHP induced cytoplasm condensation and loss of cell–cell contact. In addition, many cells died and detached from the monolayer. Pretreatment with ghrelin decreased *t*-BHP-induced morphological changes (Fig. 4a).

To test the possibility that *t*-BHP-induced morphological changes could be due to the cytoskeleton protein modifications, we examined the cytoskeleton network by staining the actin fibers with Phalloidin-FITC.

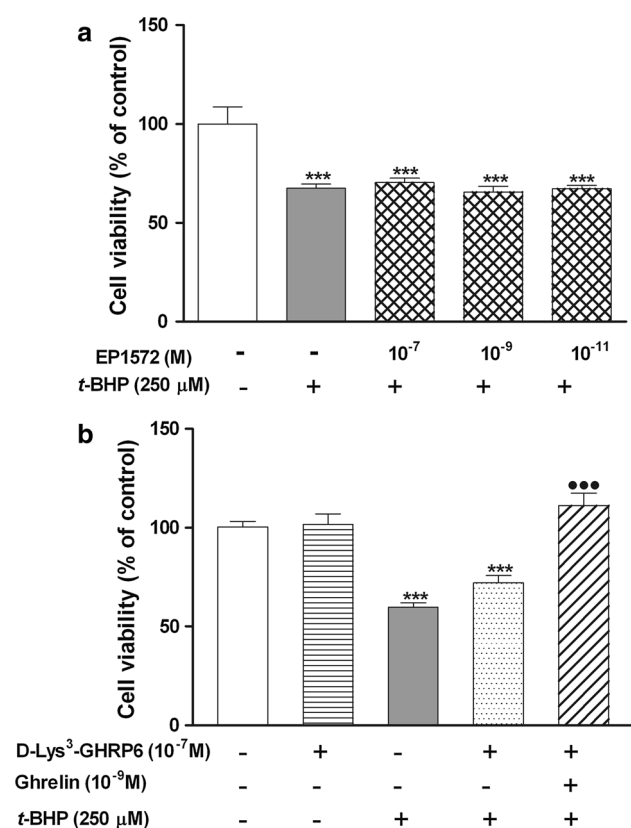
As shown in Fig. 4b, under normal conditions, MC3T3-E1 cells have actin microfilaments placed in the direction of their main axis. The MC3T3-E1 shape modification and the rearrangement of the actin network observed in the presence of *t*-BHP were prevented by ghrelin treatment. Ghrelin-treated MC3T3-E1 cells, in fact, displayed an actin filament pattern similar to that observed in control cells.

**Fig. 3** Ghrelin inhibits apoptosis induced by *t*-BHP in MC3T3-E1 cells. Cells were preincubated with ghrelin ( $10^{-9}$  M) 2 h before treatment with *t*-BHP (250  $\mu$ M for 3 h). Apoptosis was detected by Hoechst 33258 staining. **a** Panels are representative of condensed or fragmented nuclei characteristic of apoptosis. Images were taken at 20 $\times$  magnification. **b** Quantification of apoptosis. Values are the mean  $\pm$  SEM of duplicate determinations (200 cells each) of three independent experiments. \*\*\* $p$  < 0.001 vs. control; ●●● $p$  < 0.001 vs. *t*-BHP



**Fig. 4** Protective effects of ghrelin on morphological and cytoskeleton alterations induced by treatment with *t*-BHP in MC3T3-E1 cells. Cells were preincubated with ghrelin ( $10^{-9}$  M) 2 h before treatment with *t*-BHP (250  $\mu$ M for 3 h). Representative images of

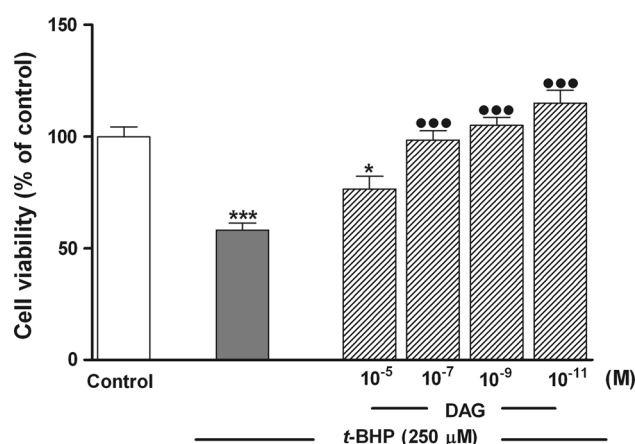
**a** morphological changes evaluated with inverted phase contrast microscope (magnification 20 $\times$ ), **b** cytoskeleton alterations evaluated with Axioplan fluorescence microscope (magnification 20 $\times$ ) detected by Phalloidin-FITC staining of the actin protein



**Fig. 5** Lack of involvement of the GHS-R 1a in the protective effect of ghrelin against *t*-BHP-induced cytotoxicity in MC3T3-E1 cells. **a** The GHS-R1a agonist, EP1572 ( $10^{-5}$ – $10^{-11}$  M) was administered 2 h before treatment with *t*-BHP (250  $\mu$ M for 3 h). **b** MC3T3-E1 cells were pretreated with the GHS-R1a antagonist, D-Lys<sup>3</sup>-GHRP-6 ( $10^{-7}$  M), 30 min before ghrelin ( $10^{-9}$  M) and subsequently incubated for 2 h before *t*-BHP (250  $\mu$ M, for 3 h). Cell viability was measured by MTT assay. Data are expressed as the percentage relative to control and are the mean  $\pm$  SEM of four replicates within a single experiment that was repeated at least three times. \*\*\* $p$  < 0.001 vs. control; ●● $p$  < 0.001 vs. *t*-BHP

To examine whether the protective effect of ghrelin against *t*-BHP-induced cytotoxicity depends on an interaction with GHS-R1a, MC3T3-E1 cells were treated with increasing concentrations of the GHS-R1a agonist, EP1572. EP1572 had no effect on cell viability when administered alone (data not shown) and did not modify the reduction in cell viability induced by *t*-BHP (Fig. 5a). Furthermore, the selective GHS-R1a antagonist, D-Lys<sup>3</sup>-GHRP-6 ( $10^{-7}$  M), applied for 30 min before ghrelin ( $10^{-9}$  M) failed to remove the protective effects of ghrelin against oxidative injury (Fig. 5b).

The possibility that the cytoprotective effect of ghrelin is linked to an interaction with a receptor different from GHS-R1a was confirmed by the results obtained with DAG which did not bind GHS-R1a. Pretreatment with increasing DAG concentrations ( $10^{-5}$ – $10^{-11}$  M) significantly increased MC3T3-E1 viability as compared with *t*-BHP-treated cells (Fig. 6).



**Fig. 6** DAG protects MC3T3-E1 cells from *t*-BHP-induced cytotoxicity. The cells were preincubated with DAG ( $10^{-5}$ – $10^{-11}$  M) for 2 h before treatment with *t*-BHP (250  $\mu$ M for 3 h). Cell viability was measured by MTT assay. Data are expressed as the percentage relative to control and are the mean  $\pm$  SEM of four replicates within a single experiment that was repeated at least three times. \* $p$  0.05, \*\*\* $p$  0.001 vs. control; ●● $p$  < 0.001 vs. *t*-BHP

DAG  $10^{-9}$  M significantly reduced the proportion of apoptotic cells ( $13.1 \pm 1.1$  %) as compared with *t*-BHP-treated cells ( $26.0 \pm 3.0$  %, Fig. 7) and prevented both the typical morphological changes (Fig. 8a) and the disassembling of actin filaments (Fig. 8b) induced by the cytotoxic effect of *t*-BHP.

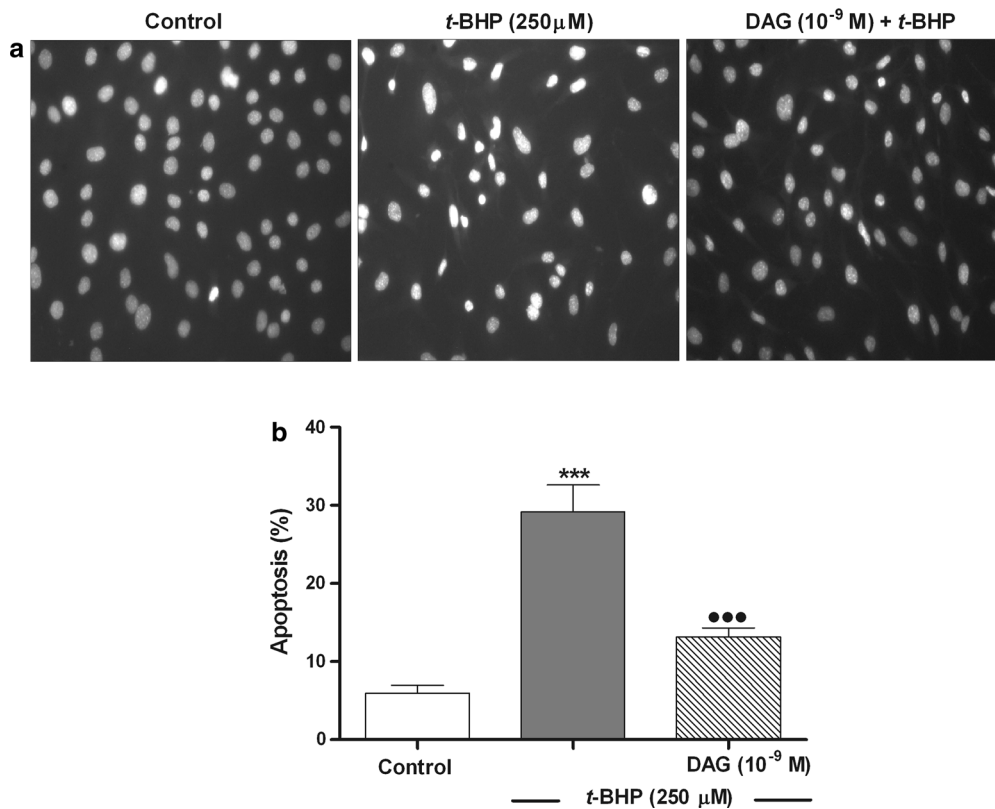
Intracellular ROS production plays an important role in *t*-BHP-induced oxidative injury. To study whether ghrelin and DAG exert their protective action against *t*-BHP by reducing ROS production, the intracellular ROS concentration was measured. CM-DCFA assay showed that treatment with *t*-BHP significantly increased intracellular ROS production in MC3T3-E1 cells. In the presence of ghrelin or DAG, ROS production was similar to the one detected in untreated control cells (Fig. 9).

In the last series of experiments, we studied the possible effect of ghrelin on the cellular content of H3K4me3 in MC3T3-E1 cells treated with *t*-BHP. As shown in Fig. 10, ghrelin significantly increased the amount of trimethylation of lys4 on histones H3, an important epigenetic mark of gene activation.

## Discussion

This study was aimed to evaluate the effects of ghrelin on *t*-BHP-induced cytotoxicity and to determine whether GHS-R1a mediates its protective effects against oxidative injury.

For this purpose, we used ghrelin, synthetic analogs acting as GHS-R1a agonist (EP1572) or antagonist, (D-Lys<sup>3</sup>-GHRP-6), and the natural ghrelin isoform DAG which does not bind GHS-R1a.



**Fig. 7** DAG inhibits apoptosis induced by *t*-BHP in MC3T3-E1 cells. Cells were preincubated with DAG (10<sup>-9</sup> M) 2 h before treatment with *t*-BHP (250 μM for 3 h). Apoptosis was detected by Hoechst 33258 staining. **a** Panels are representative of condensed or fragmented nuclei characteristic of apoptosis. Images were taken at

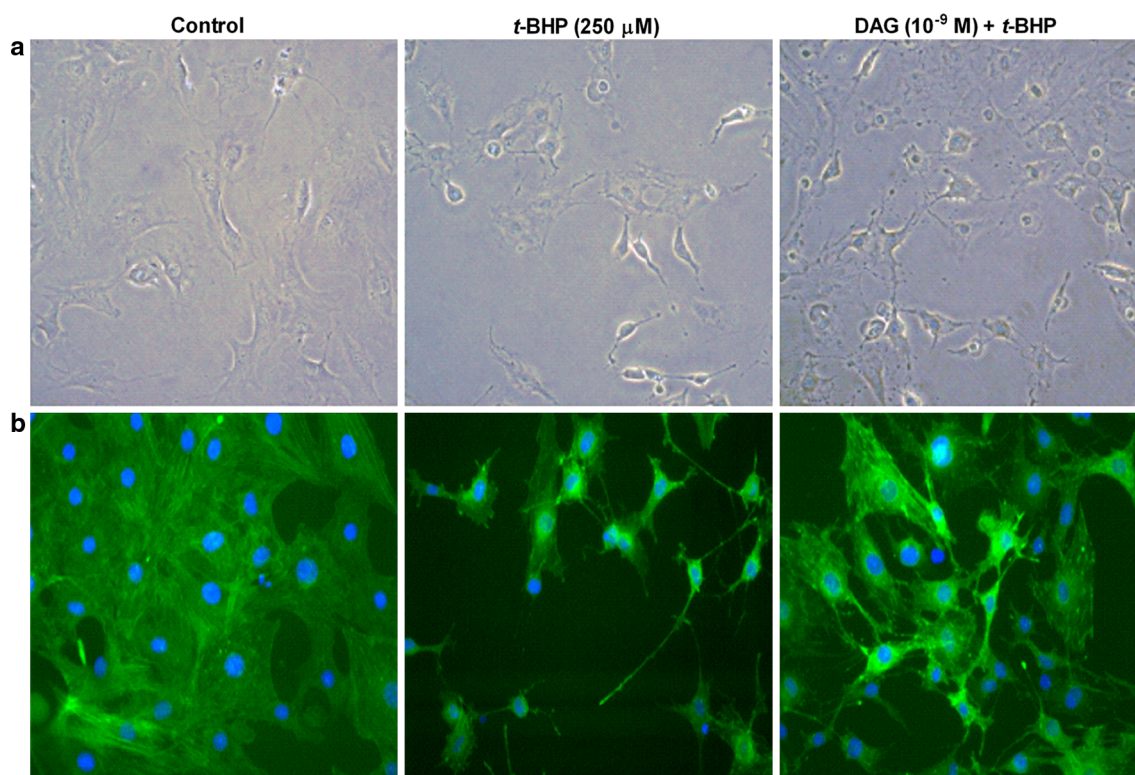
20× magnification. **b** Quantification of apoptosis. Values are the mean ± SEM of duplicate determinations (200 cells each) of three independent experiments. \*\*\**p* < 0.001 vs. control; ●●●*p* < 0.001 vs. *t*-BHP

We have shown that ghrelin, in either the acylated or unacylated form, protects MC3T3-E1 cells from *t*-BHP-induced toxic activity raising the possibility that its protective effect could be independent of the presently known GHS-R1a. Accordingly, EP1572 has no effect on *t*-BHP-induced oxidative injury and the selective GHS-R1a antagonist, D-Lys<sup>3</sup>-GHRP-6, did not prevent the protective effect elicited by ghrelin treatment.

In recent years, the beneficial effects of ghrelin on bone tissue have received increasing attention (Cocchi et al. 2005; van der Velde et al. 2008; Nikolopoulos et al. 2010; Delhanty et al. 2013).

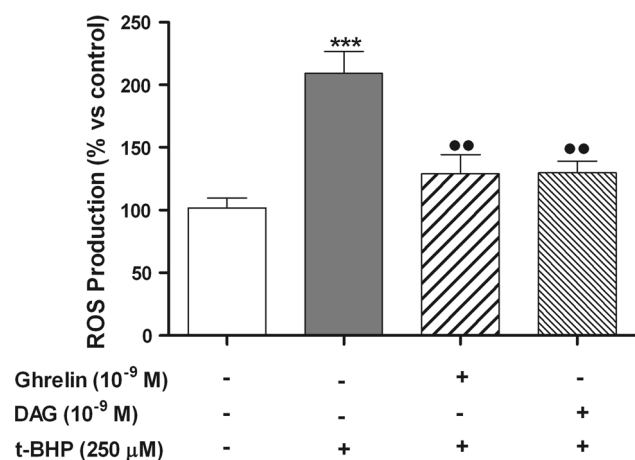
It has been reported that ghrelin stimulates proliferation and differentiation (Fukushima et al. 2005; Kim et al. 2005; Maccarinelli et al. 2005) and inhibits apoptosis of MC3T3-E1 cells (Kim et al. 2005; Liang et al. 2013). These results together with previous studies, showing that ghrelin decreases oxidative injury in various peripheral tissues (El Eter et al. 2007; Kawczynska-Drozd et al. 2006; Iseri et al. 2008; Zhang et al. 2011) and in the brain (Obay et al. 2008), lead us to examine the possibility that ghrelin could protect osteoblast against *t*-BHP-induced oxidative damage.

Oxidative stress plays an important role in the pathogenesis of age-related bone loss not only in rodents (Banfi et al. 2008) but also in humans (Basu et al. 2001; Maggio et al. 2003) leading to increased osteoblast and osteocyte apoptosis and reduced rate of bone formation (Manolagas and Parfitt 2010). In line with these reports, *t*-BHP reduced osteoblastic viability and increased their apoptosis. In this study, we showed that ghrelin protects MC3T3-E1 cells against *t*-BHP-induced oxidative stress. Interestingly, ghrelin increased viability of *t*-BHP-treated cells in a way that was not directly dose-dependent. In fact, the maximal protective activity of ghrelin was observed at low concentrations. This pattern, previously shown in other reports (Maccarinelli et al. 2005; Kim et al. 2005; Costa et al. 2011), could be due to the existence of multiple ghrelin receptor subtypes with differential affinities for the peptide. By increasing ghrelin concentrations, the peptide could recognize receptor subtypes having different or no activity on cell viability thus tempering the protective effect prevailing at low concentrations. Previous studies have shown that ghrelin protects MC3T3-E1 cells from apoptosis induced by TNF-α (Kim et al. 2005) and serum deprivation (Liang et al. 2013).



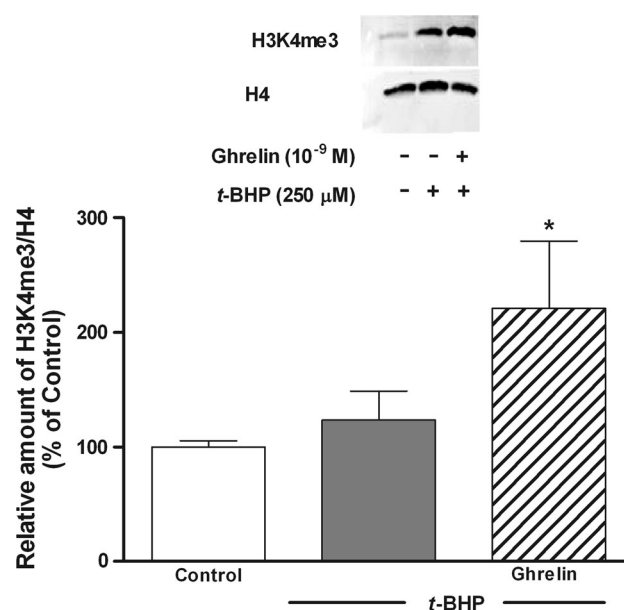
**Fig. 8** Protective effects of DAG on morphological and cytoskeleton alterations induced by treatment with *t*-BHP in MC3T3-E1 cells. Cells were preincubated with DAG ( $10^{-9}$  M) 2 h before treatment with *t*-BHP (250  $\mu$ M for 3 h). Representative images of

**a** morphological changes evaluated with inverted phase contrast microscope (magnification 20 $\times$ ), **b** cytoskeleton alterations evaluated with Axioplan fluorescence microscope (magnification 20 $\times$ ) detected by Phalloidin-FITC staining of the actin protein



**Fig. 9** Ghrelin and DAG reduce the generation of intracellular ROS induced by *t*-BHP in MC3T3-E1 cells. Cells were preincubated with ghrelin ( $10^{-9}$  M) or DAG ( $10^{-9}$  M) 2 h before treatment with *t*-BHP (250  $\mu$ M for 3 h). The intracellular ROS concentration was measured using CM-DCFA assay. Data are expressed as the percentage relative to control and are the mean  $\pm$  SEM of six replications. \*\*\* $p$  < 0.001 vs. control; \*\* $p$  < 0.01 vs. *t*-BHP

In this study, we provided the first direct evidence that ghrelin reduced *t*-BHP-induced nuclear morphological changes and chromatin condensation associated with cell



**Fig. 10** Western blot analysis of H3K4me3 levels in MC3T3-E1 cells. Cells were preincubated with ghrelin ( $10^{-9}$  M) 2 h before treatment with *t*-BHP (250  $\mu$ M for 3 h). The relative amount of H3K4me3 was normalized by the signal obtained for total H4 with an anti-H4 antibody. Data are expressed as the percentage relative to control and are the mean  $\pm$  SEM. \* $p$  < 0.05 vs. control



apoptosis as shown by Hoechst 33258 staining. Further studies will be required to examine the cell death pathway involved in the *t*-BHP cytotoxicity and the endocellular mechanisms involved in the protective effect of ghrelin against oxidative stress in MC3T3-E1 cells.

*t*-BHP-induced cytotoxicity is accompanied by osteoblast dysfunction and changes in the cytoskeleton organization with destruction of actin fibers.

Ghrelin treatment reduced the osteoblastic dysfunction induced by *t*-BHP and partially prevented the disruption of the cytoskeleton. Further studies will be required to examine whether longer ghrelin incubation time (24 and 48 h) could completely prevent *t*-BHP-induced cytoskeletal disruption.

Ghrelin and GHS-R1a are expressed in MC3T3-E1 cells (Fukushima et al. 2005; Maccarinelli et al. 2005), and many biological actions of ghrelin are linked to the binding of ghrelin to GHS-R1a. Therefore, the association of GHS-R1a with ghrelin's protective action against *t*-BHP-induced cytotoxicity was examined. Treatment with EP1572, acting as GHS-R1a agonist, did not prevent the reduction in cell viability induced by *t*-BHP. Furthermore, in the presence of the GHS-R1a antagonist, D-Lys<sup>3</sup>-GHRP-6, ghrelin was still able to exert its protective effect against oxidative injury.

The lack of the involvement of GHS-R1a in mediating the protective effects of ghrelin is in line with the results obtained with DAG which does not bind GHS-R1a (Muccioli et al. 2007).

It is unlikely that DAG could exert its protective effect in MC3T3-E1 cells through an interaction with specific binding sites not recognized by ghrelin, as previously reported in cardiomyocytes (Lear et al. 2010). Our data, in fact, showed that both ghrelin and DAG were equally protective against oxidative damage, suggesting the existence of an unknown ghrelin receptor, different from GHS-R1a, that DAG might share with ghrelin.

Evidence for an alternative ghrelin receptor has been previously reported. Ghrelin and DAG have been shown to stimulate cell signaling in cells that do not express GHS-R1a and in GHS-R-knockout animal models (Baldanzi et al. 2002; Delhanty et al. 2006; Granata et al. 2007). Furthermore, DAG shares with ghrelin an inhibitory action on inflammatory pain (Sibilia et al. 2012) and stimulatory effect on adipogenesis (Thompson et al. 2004).

Furthermore, Costa et al. (2011) have shown that ghrelin promotes rat osteoblast proliferation through a GHS-R1a-independent pathway, confirming the data previously reported by Delhanty et al. (2006) in bone cells from humans.

Several protective effects have been proposed to explain the anti-oxidant activity of ghrelin in various peripheral tissues and in the brain (Obay et al. 2008; Tong et al. 2012). Our data showing that both ghrelin and DAG significantly reduce ROS levels in *t*-BHP-treated cells indicate

that the peptides could enhance the endogenous antioxidant defense mechanisms by up-regulating intracellular antioxidant enzymes.

Further studies will be designed to clarify the signaling pathways involved in the protective effect of both peptides against *t*-BHP.

Ghrelin binding to GHS-R1a has been shown to activate different intracellular pathways including PI3 K, PKA, MAPK and AMPK (Baldanzi et al. 2002; Malagón et al. 2003; Carreira et al. 2004). Interestingly, Granata et al. (2007) demonstrated that activation of cAMP/PKA and PI3K/Akt pathway signaling is involved in the cytoprotective effects of both ghrelin and DAG on pancreatic  $\beta$ -cells via a specific ghrelin/DAG binding site, that is not GHS-R1a.

Our preliminary results showing that the levels of H3 K4me3 are significantly increased by ghrelin, suggest a possible involvement of epigenetic modifications in the protective effect of ghrelin against oxidative injury. It is noteworthy that Huang et al. (2012) showed a significant correlation between this epigenetic mark and the high expression of genes involved in the peroxisome proliferator-activated receptors  $\alpha$  (PPAR $\alpha$ ) and PPARs, which regulate ROS production (Aleshin and Reiser 2013, in press).

In conclusion, we have shown for the first time, that ghrelin may protect MC3T3-E1 cells from oxidative stress induced by *t*-BHP by reducing the intracellular ROS levels. This effect is independent from GHS-R1a, and it is likely mediated by a common ghrelin/DAG binding site. Even if the mechanism involved in ghrelin antioxidant activity remains to be clarified, this study provides a theoretical basis for exogenous application of ghrelin to maintain osteoblast activity during aging.

The increase in ROS levels, in fact, may represent a key pathogenic event in the dramatic decrease in bone formation observed in age-related bone loss.

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