Autophagy impairment aggravates the inhibitory effects of high glucose on osteoblast viability and function

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Abstract

Autophagy is a highly regulated homeostatic process involved in the lysosomal degradation of damaged cell organelles and proteins. This process is considered an important pro-survival mechanism under diverse stress conditions. A diabetic milieu is known to hamper osteoblast viability and function. In the present study, we explored the putative protective role of autophagy in osteoblastic cells exposed to an HG (high glucose) medium. HG was found to increase protein oxidation and triggered autophagy by a mechanism dependent on reactive oxygen species overproduction in osteoblastic MC3T3-E1 cells. MC3T3-E1 cell survival was impaired by HG and worsened by chemical or genetic inhibition of autophagy. These findings were mimicked by H2O2-induced oxidative stress in these cells. Autophagy impairment led to both defective mitochondrial morphology and decreased bioenergetic machinery and inhibited further osteoblast differentiation in MC3T3-E1 cells upon exposure to HG. These novel findings indicate that autophagy is an essential mechanism to maintain osteoblast viability and function in an HG environment.

Key words: autophagy, diabetes mellitus, high glucose, osteoblast, oxidative stress.

INTRODUCTION

DM (diabetes mellitus), particularly T2DM (Type 2 DM), is a common metabolic disease affecting multiple organs and tissues, including bone. Skeletal complications in DM include decreased bone mass, mainly in T1DM (Type 1 DM), and/or poor bone quality, which promotes the occurrence of fragility fractures in diabetic patients [1–3]. The mechanisms underlying the skeletal alterations in DM are, as yet, poorly understood. In adults, skeletal integrity depends on bone remodelling, a process whereby osteoclasts remove old or damaged bone tissue (bone resorption) that is subsequently replaced by new bone formed by osteoblasts [4]. In conditions in which bone resorption exceeds bone formation, as occurs after menopause and during aging, bone loss arises [5]. In vivo studies in diabetic rodent models indicate that an impaired bone formation appears to be a major contributing factor to DM-related osteopaenia [6–8]. In one of these mouse T1DM models, a decreased number of osteocytes, osteoblasts entrapped in the mineralized matrix that are considered as key modulators of bone remodelling, has been observed in the cortical tibia [9]. A low level of bone formation has also been suggested to occur in humans with T1DM [10].

Skeletal abnormalities are highly dependent on the quality of glycaemic control in diabetic subjects. Hyperglycaemia is a known cause of osteoblast dysfunction associated with a deficit in bone development and increased bone loss [11–13]. In fact, in vitro data indicate that inappropriate maturation and impaired survival of osteoblasts are consistent features when these cells are exposed to a high concentration of glucose [14]. The latter induces the formation of advanced glycation end-products and non-enzymatic glycation of type 1 collagen [15,16]. In addition, cellular exposure to elevated glucose concentrations leads to increased ROS (reactive oxygen species) production [17,18]. In this regard, augmented levels of ROS are commonly found in diabetic patients [19,20]. Increasing evidence points to ROS overproduction as playing an important pathogenic role in several scenarios associated with bone loss [39]. Hence, such a mechanism triggered by HG (high glucose) might contribute to DM-related osteopaenia.

Macroautophagy (hereafter referred to as autophagy) is a cytoprotective mechanism involving the lysosomal-mediated elimination of damaged proteins and organelles [21]. In fact, there is compelling evidence of a close interplay between autophagy and apoptosis [22]. Such a relation between both events in a pathophysiological scenario related to high oxidative stress, as represented by DM in bone tissue, remains to be explored. Of interest in this regard, previous studies indicate that autophagy may operate as a mechanism to counteract the detrimental effects of long-term glucocorticoid treatment on osteocyte death [23,24].

In the present study, we describe for the first time to our knowledge the protective role of autophagy in osteoblastic cells exposed to a high concentration of glucose. We demonstrate that impaired autophagy has deleterious effects on both mitochondrial integrity and ROS metabolism, and its consequences on osteoblast differentiation and survival in this setting.

Abbreviations used: Atg, autophagy related; 2,7-DCFH-DA, 2,7-dichlorofluorescein diacetate; DM, diabetes mellitus; HG, high glucose; Hsp60, heat-shock protein 60; IS, indoxyl sulfate; LC3B, microtubule-associated protein 1 light chain 3β; mTOR, mammalian target of rapamycin; NAC, N-acetylcysteine; PI, propidium iodide; ROS, reactive oxygen species; Runx2, runt-related transcription factor 2; SQSTM1, sequestosome 1; T1DM, Type 1 DM; Tom20, translocase of outer mitochondrial membrane 20.

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MATERIALS AND METHODS

Antibodies and reagents

The antibodies used were: anti-LC3B (microtubule-associated protein 1 light chain 3β; #4108) and Atg7 (autophagy related 7; #2631) (Cell Signaling Technology); anti-β-actin (Sigma–Aldrich); anti-Tom20 (translocase of outer mitochondrial membrane 20; sc-17764) and anti-(cytochrome c) (sc-13560) (Santa Cruz Biotechnology); anti-Hsp60 (heat-shock protein 60; SPA-807) (Enzo Life Sciences); anti-(ATP synthase subunit b) (ab14730), anti-(ATP synthase subunit d) (ab110275) and anti-(ubiquinol cytochrome c reductase Rieske subunit) (ab14746) (Abcam); anti-p62/SQSTM1 (sequestosome 1; GP62) (Progen); and anti-nitrotyrosine (Merck–Millipore). Chloroquine, bafilomycin, IS (indoxyl sulfate) and H2O2 were from Sigma–Aldrich.

Cell culture

Mouse osteoblastic MC3T3-E1 cells (CRL-2593; A.T.C.C., Manassas, VA) [8] were grown in α-MEM (minimum essential medium) with 10% FBS, 1% penicillin/streptomycin and 2 mM glutamine in a humidified incubator with 5% CO2 at 37°C. To generate Atg7-deficient MC3T3-E1 cells, lentivirus-mediated shRNA interference was carried out. Two different sequences targeting mouse Atg7, Atg7#1 (TRCN0000092163) and Atg7#2 (TRCN0000092164) (Sigma–Aldrich), were used. Atg7#1 had the greatest degree of interference. Subsequently, puromycin selection (1 μg/ml for 3 weeks) and subcloning were carried out. Cells were exposed to normal glucose (5.6 mM) or HG (25 mM) for 48 h. Oxidative stress was induced as described previously [51] (Supplementary Figure S1 at http://www.biochemj.org/bj/455/bj4550329add.htm). Briefly, a 2-h pulse of 100 μM H2O2 per day for 2 days was applied to cells in culture, followed by replacement with fresh medium and the addition of autophagy inhibitors on the last day.

Cell viability studies

For cell-cycle analysis, MC3T3-E1 cells were collected by centrifugation (2000 g for 5 min at 4°C) and fixed with ice-cold ethanol 70% (v/v). The cells were then washed, resuspended in PBS and incubated with RNase (Roche Applied Sciences) in PBS and incubated with 5% CO2 at 37°C. To generate Atg7-deficient MC3T3-E1 cells, lentivirus-mediated shRNA interference was carried out. Two different sequences targeting mouse Atg7, Atg7#1 (TRCN0000092163) and Atg7#2 (TRCN0000092164) (Sigma–Aldrich), were used. Atg7#1 had the greatest degree of interference. Subsequently, puromycin selection (1 μg/ml for 3 weeks) and subcloning were carried out. Cells were exposed to normal glucose (5.6 mM) or HG (25 mM) for 48 h. Oxidative stress was induced as described previously [51] (Supplementary Figure S1 at http://www.biochemj.org/bj/455/bj4550329add.htm). Briefly, a 2-h pulse of 100 μM H2O2 per day for 2 days was applied to cells in culture, followed by replacement with fresh medium and the addition of autophagy inhibitors on the last day.

Immunoblot analysis

MC3T3-E1 cells were washed with ice-cold PBS and then lysed in a buffer containing 1% (v/v) Nonidet P40, 50 mM Tris/HCl, 5 mM EDTA, 5 mM EGTA, 150 mM NaCl, 20 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin and 2 μg/ml leupeptin (pH 7.5). Cellular debris was pelleted by centrifugation at 15000 g for 15 min at 4°C, and the resulting supernatants were collected for protein determination. Samples were subjected to SDS/PAGE (8–15% gels), transferred on to Immobilon P PVDF membranes (Merck–Millipore) and subsequently incubated with specific antibodies as described previously [52].

Microscopy

For the LC3B–EGFP assay, MC3T3-E1 cells were transiently transfected with the pEGFP-LC3B plasmid (Addgene) with the use of LipofectamineTM 2000 (Life Technologies). At the end of the experiments, cells were fixed with 4% (w/v) paraformaldehyde and visualized using a SP2 confocal microscope (Leica). Other immunofluorescence assays were performed as described previously [52]. Analysis of images was performed using ImageJ (http://rsb.info.nih.gov/ij/), co-localization of red (mitochondria) with green (nitrotyrosine) pixels was calculated using the Mander’s co-localization coefficient (M) [53]. Electron microscopy assays were performed as described previously [31].

Measurement of ROS

Following HG or H2O2 (100 μM) exposure, cells were washed with PBS, and then loaded with 5 μM 2,7-DCFH-DA (2,7-dichlorofluorescein diacetate) for 10 min. Fluorescence was quantified by flow cytometry using a FACSCalibur flow cytometer (BD Biosciences) following a protocol described previously [54].

Determination of oxidized proteins

Cells were lysed in the presence of 1% (w/v) 2-mercaptoethanol. For detection of mitochondrial protein oxidation, 2×107 cells per condition were used. After subsequent trypsinization, centrifugation at 110 g for 3 min at 4°C and PBS washing, mitochondria were isolated using the Mitochondria Isolation Kit for Cultured Cells (Thermo Fisher Scientific) following the manufacturer’s instructions. All buffers were enriched with 1% (v/v) 2-mercaptoethanol to avoid further oxidation of proteins after cell lysis. Mitochondrial pelleting was performed at 3000 g for 15 min at 4°C to avoid peroxisome contamination of the mitochondrial fraction. Mitochondrial pellets were lysed in 30 μl of a buffer containing 2% (w/v) CHAPS, 10 mM Tris/HCl, 150 mM NaCl and 1% (w/v) 2-mercaptoethanol (pH 7.5). Total protein or mitochondrial protein extracts (10 μg) were derivatized using the OxyBlot kit (Merck–Millipore), and then subjected to SDS/PAGE (12% gels). After transfer on to PVDF membranes, Ponceau S (Bio-Rad Laboratories) staining was performed to confirm homogeneous protein load.

Gene expression analysis

Total RNA was extracted with TRIzol® (Life Technologies). RNA retrotranscription was carried out with 0.4–1.5 μg of total RNA performed with the cDNA High capacity cDNA reverse transcription kit (Applied Biosystems). Real-time PCR was done with an Assay-by-Design™ system, using TaqMan MGB probes (Applied Biosystems) for Runx2 (runt-related transcription factor 2), osterix and osteocalcin in an ABI PRISM 7500 system (Applied Biosystems). Melting curves were used to verify single PCR product amplification. Results were expressed in mRNA copy numbers, calculated for each sample using the cycle threshold (C T) value and normalized against 18S rRNA as described previously [54].

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Figure 1 Generation and characterization of an autophagic-deficient osteoblastic cell model

(A) RNA isolated from MC3T3-E1 cells expressing shRNA targeting Atg7 (Atg7#1 and Atg7#2) or control (scrambled shRNA) were subjected to real-time PCR. (B) Cells were treated with chloroquine (CQ; 10 μM for 15 h) or left untreated. (C) Cells were exposed to culture medium containing 5.6 mM glucose (NG) or 25 mM glucose (HG) for 48 h. Chloroquine (10 μM) was added during the last 24 h of the experiment. Autophagy markers were evaluated by Western blot analysis. Representative autoradiograms (A–C) and corresponding densitometric quantification (A and C) are shown. Values represent means ± S.D. (n=3). **P < 0.01 between the corresponding experimental conditions as indicated. (D) Cells, transiently transfected to express LC3B–EGFP fusion protein, were exposed to HG or not (control, NG) for 48 h. Representative fluorescence microscopy images are shown (scale bars, 5 μm).

Mineralization assay

Scrambled and Atg7 shRNA-expressing MC3T3-E1 cells were cultured for 10 days in the aforementioned medium with 50 μg/ml ascorbic acid and 10 mM 2-glycerophosphate, in the presence and absence of HG. Matrix mineralization was then determined by Alizarin Red S staining, as described previously [8]. Briefly, cells were washed twice with PBS, fixed with 75 % (v/v) ethanol for 30 min and then stained with 40 mM Alizarin Red S in distilled water (pH 4.2) for 30 min at room temperature (25°C). Subsequently, cell monolayers were washed five times with distilled water and the stain was dissolved with 10 % (w/v) cetylpyridinium chloride in 10 mM sodium phosphate (pH 7). Absorbance was measured at 620 nm.

Statistics

All of the data are expressed as means ± S.D. of a number of experiments performed at least in duplicate as indicated. Comparisons were performed by using an unpaired Student’s t test or a Kruskal–Wallis test followed by a post-hoc Mann–Whitney test when appropriate. Differences were considered significant at P < 0.05. Statistical analysis was performed using statistical software (GraphPad InStat™ V2.04a).

RESULTS

Establishment of an osteoblastic model with chronically impaired autophagy

In order to generate an autophagy-deficient osteoblastic cell model, two different shRNA sequences targeting Atg7 and scrambled shRNA (as a control) were used to transfect native MC3T3-E1 cells. After lentivirus-mediated transfection and antibiotic selection, cell lines stably expressing Atg7 shRNA (#1 and #2, see the Materials and Methods section) were analysed by real-time PCR, showing reduced Atg7 mRNA levels which were 95% and 50% lower respectively, compared with those in control cells (Figure 1A). Only cells with the highest degree of interference were used further. Atg7 knockdown in Atg7 shRNA-expressing MC3T3-E1 cells was also shown by Western blotting (Figure 1B). Moreover, after treatment with chloroquine, a lysosomotropic drug which raises lysosomal pH and inhibits autophagic flux, the accumulation of LC3B-II was impaired in these cells. In addition, p62/SQSTM1, an adaptor protein that accumulates under autophagy impairment conditions, was increased in Atg7 shRNA MC3T3-E1 cells, as observed in control cells upon chloroquine-mediated inhibition of autophagic flux (Figure 1B).

Figure 2 Mitochondrial abnormalities in autophagy-deficient osteoblastic MC3T3-E1 cells

(A) Representative immunoblots of cells treated with chloroquine (CQ; 10 μM for 15 h) or left untreated. Cyt c, cytochrome c. (B) Confocal microscopy images of MC3T3-E1 cells. Mitochondria are visualized with an antibody against ATP synthase subunit d (scale bars, 5 μm). (C) Representative electron micrographs showing cytoplasmic regions of MC3T3 cells (scale bars, 1 μm).
Defective autophagic flux was also evident in these cells after analysis of electron micrographs (Supplementary Figure S2A at http://www.biochemj.org/bj/455/bj4550329add.htm). Notably, concentric membranous structures were found in Atg7 shRNA MC3T3-E1 cells (Supplementary Figure S2B) as described previously in Atg7−/− mice [25].

We next sought to examine possible alterations in the mitochondria of these cells. Surprisingly, Atg7 shRNA MC3T3-E1 cells showed a marked decrease in important mitochondrial proteins implicated in oxidative phosphorylation, namely cytochrome c, cytochrome c reductase and ATP synthase, and other mitochondrial markers such as Tom20 and Hsp60 (Figure 2A). Increased fragmentation of mitochondria was also observed in these osteoblastic cells (Figure 2B). Electron micrographs also showed accumulation of abnormally swollen mitochondria in Atg7 shRNA MC3T3-E1 cells, probably resulting from a defect in autophagy which is a mechanism responsible for their degradation (Figure 2C). Atg7 shRNA MC3T3-E1 cells showed slower proliferation capability than native MC3T3-E1 cells, estimated by the proportion of cells in S/G1-M phase of the cell cycle (Supplementary Figure S3 at http://www.biochemj.org/bj/455/bj4550329add.htm).

Exposure to HG increases autophagy in osteoblastic MC3T3-E1 cells

The possible protective role of autophagy in MC3T3-E1 cells when exposed to HG conditions was then investigated. These cells were subjected to HG for 48 h, blocking the autophagic flux for the last 24 h. Upon HG exposure, this flux (as monitored by the conversion of LC3B-I into LC3B-II) was found to be higher than that observed in normal glucose conditions, and non-existent in Atg7 shRNA-expressing MC3T3-E1 cells (Figure 1C). This was confirmed by immunofluorescence analysis of MC3T3-E1
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MC3T3-E1 cells with or without an intact autophagy machinery were exposed or not (control, NG/C) to HG, in the presence or absence of the antioxidants NAC (0.4 mM) and catalase (100 units/ml), or chemical inhibitors of autophagy, chloroquine (CQ; 10 μM), bafilomycin A1 (BafA1; 2 nM) and 3-methyladenine (3-MA; 5 mM), for up to 48 h. These inhibitors were added during the last 24 h. (A and B) Annexin V-FITC/PI staining was analysed by flow cytometry. (B) Representative dot plots of MC3T3-E1 cells subjected to 100 μM H₂O₂ pulses or left untreated. Data are the percentage of annexin V-positive cells (both upper- and lower-right quadrants in representative dot plot as shown) ± S.D. (n = 3). (C) Viability was evaluated by Trypan Blue exclusion. Stimulation with two 2-h pulses of 100 μM H₂O₂ was used as positive control. Values are means ± S.D. (n = 5). *P < 0.05 and **P < 0.01 compared with the normal glucose or control value; #P < 0.05 and ##P < 0.01 compared with the corresponding HG alone value; and §§P < 0.01 compared with the corresponding scrambled shRNA value.

cells transiently transfected with EGFP–LC3B, which displayed increased fluorescent puncta under HG conditions (Figure 1D).

HG induces autophagy by increasing ROS in osteoblastic MC3T3-E1 cells

MC3T3-E1 cells grown in HG medium showed increased ROS production (Figure 3A), as well as an increased pattern of protein oxidation (Figure 3B). Of interest, autophagy-deficient MC3T3-E1 cells, after stable Atg7 shRNA expression, showed a marked increase in ROS levels and in total and mitochondrial protein oxidation even under basal conditions (Figures 3A and 3C). In addition, an increased co-localization of mitochondria with nitrotyrosine, a marker of protein oxidation, was found in these autophagy-deficient cells (Supplementary Figure S4 at http://www.biochemj.org/bj/455/bj4550329add.htm). Treatment with antioxidants, namely NAC or catalase, reduced these oxidative events induced by HG in these cells (Figures 3A and 3B). Furthermore, and supporting the notion that an excess ROS production was the underlying cause of the autophagic response to HG, NAC reversed the observed LC3B conversion triggered by HG in MC3T3-E1 cells (Figure 3D).

Autophagy has a pro-survival role under HG and oxidative stress conditions in osteoblastic MC3T3-E1 cells

The consequences of autophagy induction by HG in osteoblastic cells were examined in MC3T3-E1 cells with either chemically mediated or chronically impaired autophagy. In the former scenario, HG-mediated apoptosis was more dramatic than in native MC3T3-E1 cells, as shown by abundant annexin-V-positive cells under HG conditions in the presence of chemical inhibitors of autophagy (Figure 4A). Moreover, increased apoptosis occurred in Atg7 shRNA MC3T3-E1 cells upon both HG and H₂O₂ treatment (Figure 4B). Furthermore, consistent with the hypothesis that ROS mediate the deleterious effect of HG on osteoblast viability, NAC and catalase prevented such an effect in MC3T3-E1 cells with or without inhibition of the autophagy machinery (Figure 4C). In view of these results pointing to the important role of oxidative stress in...
Figure 5  Autophagy protects against oxidative stress-induced MC3T3-E1 cell death

MC3T3-E1 cells were treated with 100 μM H2O2 or not (control, Co), in the presence or absence of chemical inhibitors of autophagy chloroquine (CQ; 10 μM), bafilomycin A1 (BafA1; 2 nM), and 3-methyladenine (3-MA; 5 mM) for the last 24 h. (A) Representative fluorescence microscopy image of MC3T3-E1 cells expressing EGFP–LC3B after H2O2 stimulation. (B) Annexin V-FITC/PI staining was analysed by flow cytometry. Representative dot plots of MC3T3-E1 cells subjected to 100 μM H2O2 pulses or left untreated are shown. Data are percentage of annexin V-positive cells (as described in the legend to Figure 4) (n = 3). *P < 0.05 compared with the corresponding control or inhibitor alone value and #P < 0.05 compared with the H2O2 alone value.

HG-induced autophagy in MC3T3-E1 cells, we aimed to further assess the consequences of inhibiting autophagy in these cells upon H2O2 exposure. This pattern of treatment induced a marked increase in puncta formation in EGFP–LC3B-transfected MC3T3-E1 cells (Figure 5A). Autophagy inhibitors or chronic inhibition of autophagy by Atg7 shRNA-potentiated H2O2-induced MC3T3-E1 cell death compared with that in the respective control cells (Figure 5B and Supplementary Figure S5). Other agents, such as IS, can induce oxidative stress in osteoblasts [55,56]. We found that IS-induced autophagy in MC3T3-E1 cells was clearly impaired in Atg7 shRNA-expressing MC3T3-E1 cells (Supplementary Figure S6A at http://www.biochemj.org/bj/455/bj4550329add.htm). In these latter cells, the IS-induced decrease in cell survival was significantly higher than that in the control cells (Supplementary Figure S6B).

Autophagy deficiency interferes with osteoblastic differentiation in MC3T3-E1 cells

MC3T3-E1 cells represent an immature osteoblastic status and thus we examined whether autophagic deficiency would aggravate the inhibitory action of HG by hampering the cell-differentiation program. As expected on the basis of previous findings in these osteoblastic cells [8], exposure to HG was found to down-regulate the expression of Runx2, ostelexer and osteocalcin, genes representing different stages of osteoblast differentiation (Figure 6A). Atg7 shRNA-expressing MC3T3-E1 cells (showing chronic autophagy inhibition) had a significantly lower ostelexin gene expression than that in control cells under normal glucose conditions. Moreover, in the former cells, a dramatic inhibitory effect was observed on the three osteoblastic gene markers tested and also on matrix mineralization upon exposure to a HG medium (Figure 6).

DISCUSSION

The current hypothesis points to the involvement of increased oxidative stress in the underlying mechanisms responsible for DM-related bone loss and dysfunction [6,26]. In fact, overexpression of the antioxidant thioredoxin-1 was found to ameliorate osteopaenia in streptozotocin-induced diabetic mice [27]. The detrimental effects of a diabetic setting represented by HG on osteoblast growth and function were previously reported by us and others [8,14,28,29]. Other diabetes-related factors, besides HG, have also been shown to impair osteoblastic function and viability [55,57]. The findings of the present study further extend these previous studies, and demonstrate that a diabetic environment elicits a protective autophagic response in a well characterized osteoblastic cell line.

The protective role of autophagy under various stress conditions is well documented [30]. Thus we have recently reported a positive outcome of autophagy stimulation challenged by different stress procedures in pancreatic β-cells [31]. On the other hand, defective autophagy has been reported to contribute to aging, insulin resistance and cancer [32,33]. However, only a few studies so far have addressed and highlighted the importance of autophagy in bone. A recent genome-wide association analysis has found a relationship between the differential expression of autophagy-regulatory genes and bone mineral density [34]; although no definite conclusions on the eventual beneficial or detrimental role of autophagy were reported in this regard. Autophagy has also been suggested to be important for mature bone health maintenance [35,36]. Moreover, deficiency in

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lyosomal sulfatases, a lysosomal storage disorder that impairs the function of autophagosomes, has been related to abnormal skeletal development [37]. In addition, progressive autophagy impairment might account for the reduced osteoblast function and bone loss associated with aging [38]. Accumulation of damaged proteins and nucleic acids together with oxidative stress are events associated with aging, in part related to an excess of glucocorticoids, and bone loss development [38,39]. Notably, it was reported in mice that glucocorticoids dose dependently triggered osteocyte autophagy as a mechanism to maintain osteocyte viability in cortical bone [23,24]. Considering the recognized role of this cell type as a true orchestrator of skeletal development [37], the present study indicates that ROS overproduction can trigger an autophagic response also in osteoblastic MC3T3-E1 cells. Mitochondria are the main source of ROS, and mitochondrial dysfunction is a major cause of deregulation of ROS homeostasis [49]. Previous reports showed the essential role of autophagy for the correct maintenance of mitochondrial structure in pancreatic β-cells [50]. Exacerbated mitochondrial fission has been recently linked to ROS hyperproduction under HG conditions [18]. In the present study, it was found that impaired autophagy in osteoblastic MC3T3-E1 cells led to accumulation of abnormal highly fragmented and oxidized mitochondria. This could explain, at least in part, the elevated sensitivity of these cells to apoptotic stimulus.

In summary, our present findings disclose the role of autophagy as an important mechanism to protect osteoblast survival and function in a diabetic environment represented by HG. These results suggest that targeting autophagy in osteoblasts might be an alternative strategy for the therapy of diabetic osteopaenia.

AUTHOR CONTRIBUTION
Alberto Bartolomé and Ana López-Herradón performed all of the experiments. Sergio Portal-Núñez, Pedro Esbrit and Carlos Guillén supervised the experiments. All authors were involved in planning the experiments, analysing the experimental data and/or commenting on the paper. Alberto Bartolomé, Pedro Esbrit and Carlos Guillén wrote the paper.

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REFERENCES


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57 Ogawa, N., Yamaguchi, T., Yano, S., Yamauchi, M., Yamamoto, M. and Sugimoto, T (2007) The combination of high glucose and advanced glycation end-products (AGEs) inhibits the mineralization of osteoblastic MC3T3-E1 cells through glucose-induced increase in the receptor for AGEs. Horm. Metab. Res. 39, 871–875

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SUPPLEMENTARY ONLINE DATA

Autophagy impairment aggravates the inhibitory effects of high glucose on osteoblast viability and function

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**Figure S1** Schematic representation of H2O2 stimulation of osteoblastic MC3T3-E1 cells

**Figure S2** Cell ultrastructure of control and autophagy-deficient MC3T3-E1 cells

(A) Electron micrographs of exponentially growing MC3T3-E1 cells, with or without intact autophagy machinery, treated with chloroquine (CQ; 10 μM) or left untreated (control) for 15 h. Quantification of the cytoplasmic area occupied by autophagic vacuoles, which are indicated by arrowheads in the images, is also shown. Values are means ± S.D. (n = 3). *P < 0.05 between the conditions indicated. (B) Detailed images of Atg7 shRNA MC3T3-E1 cells showing concentric membranous structures, indicated by arrowheads. Framed region is magnified in the right-hand image.
**Figure S3** Cell-cycle profile of control and autophagy-deficient MC3T3-E1 cells

Representative plots showing MC3T3-E1 cell-cycle profiles by PI staining and flow cytometry are shown. Percentages of S–G2/M cells are represented as means ± S.D. (n = 3). *P < 0.05 compared with the corresponding scrambled (Scr) shRNA value.

**Figure S4** MC3T3-E1 cells without intact autophagy machinery show co-localization of cytochrome c and nitrosylated tyrosine residues

Representative confocal microscopy images showing co-localization (white) of cytochrome c (Cyt c) (red) and nitrosylated tyrosine residues (green); nuclei are depicted in blue by DAPI staining. Histogram represents the Manders’ co-localization coefficient (M1) red/green, showing proportion of red/green pixels over total red pixels. Values are means ± S.D. per field, corresponding to evaluation of several fields in two independent experiments. *P < 0.05 compared with the corresponding scrambled shRNA value.

**Figure S5** Autophagy inhibition induces changes in morphology of MC3T3-E1 cells

Representative light microscopy images from either native MC3T3-E1 (upper panels) in the presence or absence of chemical autophagy inhibitors as indicated in the text, or these cells without intact autophagy machinery (lower panels) submitted to H2O2. BaflA1, bafilomycin A1; CQ, chloroquine; 3-MA, 3-methyladenine.

**Figure S6** Autophagy deficiency renders MC3T3-E1 cells more vulnerable to IS-mediated cell death

(A) Representative Western blotting of LC3B in both scrambled or shRNA-expressing MC3T3-E1 cells in response to different IS concentrations. (B) Percentage of surviving cells after 24 h exposure to 1 mM IS. Values are means ± S.D. (n = 3). *P < 0.05 compared with the corresponding value in scramble shRNA. C, control.