Autophagy protects osteoblasts from advanced glycation end products-induced apoptosis through intracellular reactive oxygen species

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Abstract

Patients with type II diabetes are susceptible to fracture; however, these patients typically have normal bone mineral density. Thus, such fractures cannot be entirely explained by advanced glycation end products (AGEs)-induced osteoblast apoptosis. Autophagy is a molecular process allowing cells to degrade unnecessary or dysfunctional cellular organelles, and closely interacts with apoptosis. The aim of this study was to determine whether autophagy participated in the pathology of AGEs-treated osteoblasts, and the possible mechanism of such an involvement. Osteoblastic MC3T3-E1 cells were used. Autophagy was evaluated by detecting the level of LC3 via western blotting and immunofluorescence. p62/SQSTM1 expression was also assessed by western blotting. The autophagy inducer rapamycin (RA) and the autophagy inhibitor 3-methyladenine were used to determine whether autophagy has effect on AGEs-induced apoptosis. N-Acetylcysteine (NAC), reactive oxygen species (ROS) inhibitor, was used to determine whether ROS and mitochondrial damage were involved in autophagy regulation. The results showed that the autophagy level was increased in MC3T3-E1 cells treated with AGEs, as represented by an increase in both the total LC3 level and the LC3II/LC3I ratio, as well as a decrease in p62/SQSTM1 expression. Further inducing autophagy by RA attenuated AGEs-induced apoptosis. The antioxidant NAC suppresses AGEs-induced autophagy in osteoblastic MC3T3-E1 cells. These results demonstrate that autophagy participates in the pathology of AGEs-treated osteoblasts, and may play a protective role in AGEs-induced apoptosis in osteoblastic MC3T3-E1 cells. ROS and mitochondrial damage are essential in upregulating AGEs-induced autophagy.

Key Words
- advanced glycation end products (AGEs)
- autophagy
- apoptosis
- ROS
- mitochondrial membrane potential

Introduction

In 2014, the American Society of Bone and Mineral Research dedicated a full day before its Annual Meeting to discuss the links between diabetes mellitus and bone metabolism. As a result, osteoporotic fracture is now considered a diabetic complication (Hofbauer et al. 2016). This increased fracture risk, however, cannot be entirely explained by low bone mineral density (BMD) because the relative risk of hip fracture in type I diabetes is 6.9, which is much higher than the calculated relative risk based on BMD (Yamagishi 2011, Yamaguchi & Sugimoto 2012, Weber et al. 2015). Furthermore, BMD is normal or increased rather than...
Advanced glycation end products (AGEs) are a group of heterogeneous compounds that accumulate in the bone tissue of diabetic patients due to several factors, including increased reactive carbohydrate substrate availability, oxidative conditions favoring glycation, and impaired detoxification (Yoon et al. 2004). It is well known that AGEs participate in many pathological processes of diabetes. Human and animal experiments have shown that AGEs accumulate dramatically in bone tissue during diabetes, and are associated with bone cell dysfunction as well as bone tissue fragility (Santana et al. 2003, Yoon et al. 2004, Hein et al. 2006, Momma et al. 2012, Fajardo et al. 2014, Hofbauer et al. 2016). Several studies have demonstrated that AGEs can induce apoptosis in bone cells through MAPK, p38, caspase-8, and caspase-9 signaling pathways; however, this apoptotic response cannot fully explain the susceptibility of diabetic patients with normal BMD to fracture (Alikhani et al. 2007, Weinberg et al. 2014, Tanaka et al. 2015).

Autophagy closely interacts with apoptosis (Maiuri et al. 2007). Autophagy plays a housekeeping role in eliminating old organelles, misfolded proteins, and damaged molecules; it also plays a role in recycling limited nutrients and oxygen (Hocking et al. 2012). Autophagy is seen in all types of bone cells, and plays a complex and important role during osteogenesis (Carames et al. 2010, Hocking et al. 2012).

Whether autophagy affects the pathology of AGEs in osteoblast cells remains unclear. The aim of this study was to determine the role of autophagy in diabetes-related fracture by examining autophagy levels in osteoblasts treated with AGEs and to further explore the relationship between autophagy and AGEs-induced apoptosis.

Materials and methods

AGE preparation

AGE-BSA was prepared by incubating BSA (Sigma) with 500 mmol/L of D-glucose under aerobic conditions for 10 weeks at 37°C in the presence of protease inhibitors and antibiotics, as described previously (Hou et al. 2014).

Cell line and reagents

The osteoblastic cell line MC3T3-E1 was obtained from American Type Culture Collection. Cells were cultured in α-minimum essential medium containing 10% fetal bovine serum in a humidified 5% CO₂ atmosphere at 37°C and the medium was changed every other day. The inducer of autophagy rapamycin (RA), the inhibitor of autophagy 3-methyladenine (3-MA), and the reactive oxygen species (ROS) inhibitor N-acetylcysteine (NAC) (Klionsky et al. 2012) were obtained from Sigma-Aldrich. The antibodies used were as follows: LC3 (Cell Signaling Technology) and p62/SQSTM1 (Abcam). An annexin V-FITC apoptosis detection kit (BioVision) and the oxidation-sensitive fluorescent probe 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA; Beyotime, Nantong, China) were also used in this study.

Cell viability assay

Osteoblastic MC3T3-E1 cells were seeded into 96-well plates (6 × 10³ cells/well). When 60% confluence was achieved, cells were either treated with BSA–PBS solution (0, 50, 100, 150, 200, 250, and 300 mg/L) or with increasing concentrations of AGEs (0, 50, 100, 150, 200, 250, and 300 mg/L) for 24 h. Cell viability was determined using an MTT assay as described previously (Guo et al. 2012). The experiment was carried out three times with each of two different batches of AGEs.

LC3, p62/SQSTM1 expression analysis, and autophagosome quantitative analysis

Cells were treated with AGEs (200 mg/L) for 0, 4, 8, 12, 16, and 24 h. The autophagy level was assessed by detecting LC3I and LC3II using western blotting and immunofluorescence. p62/SQSTM1 expression was also assessed by western blotting. Cells were collected and lysed, then lysate protein concentrations were determined. Samples were resolved via SDS–PAGE and electroblotted onto a polyvinylidyne difluoride membrane. Membranes were then incubated with primary antibodies against LC3 or p62/SQSTM1 at 1:500 or 1:1000 dilution, respectively, overnight at 4°C. Next, membranes were incubated with goat antirabbit secondary antibodies conjugated to horseradish peroxidase (Beyotime) at a dilution of 1:3000 for 2 h at 23°C. Signals were enhanced using a chemiluminescence system.

For immunofluorescence detection, 1 × 10⁴ osteoblastic MC3T3-E1 cells were seeded into 96-well plates and treated with AGEs (200 mg/L) for 12 h. Immunofluorescence was performed according to previously described standard procedures, using the same anti-LC3 antibody that was used for western blotting at a dilution of 1:50. The number of LC3 foci was determined by manually counting foci visible in three randomly
selected fields, and nuclei were enumerated by counting DAPI-stained nuclei in the same field, imaged at the same magnification. The number of GFP-LC3 punctae per cell was evaluated as the total number of foci divided by the number of nuclei in each microscopic field. Data are presented as an average of three independent experiments.

Assessment of apoptosis after treatment with AGEs and autophagy inducer or inhibitor

Cells were treated with AGEs (200 mg/L) for 0, 4, 8, 12, 16, and 24 h before apoptosis was assessed. Apoptosis was most evident after 8 h of treatment with AGEs. Cells were thus subsequently treated with AGEs (200 mg/L) and 3-MA (2 mM), AGEs (200 mg/L) and RA (2 μM), RA (2 μM) alone, or 3-MA (2 mM) alone for 8 h. Apoptosis was analyzed by bivariate flow cytometry using a fluorescence-activated cell sorting (FACS) SCAN flow cytometer equipped with ModFit LT 3.0 (BD Biosciences) after staining cells using the Annexin V-FITC Apoptosis Detection Kit according to the manufacturer’s instructions (BioVision). The percent of apoptotic cells was calculated from the total number of cells (1 × 10⁴) using EXPo32 MultiComp software (Beckman Coulter, Brea, CA, USA). Data were presented as an average of three independent experiments.

Detection of intracellular ROS

Intracellular ROS were detected using the oxidation-sensitive fluorescent probe DCFH-DA. DCFH-DA itself has no fluorescence signal, but freely passes through the cell membrane. Intracellular DCFH-DA is oxidized into DCFH, which cannot pass through the cell membrane, by esterase. DCFH is further oxidized into 2',7'-dichlorofluorescein (DCF), which has a fluorescence signal, by intracellular ROS. (Guo et al. 2012). Therefore, the fluorescent strength of DCF reflects the level of intracellular ROS. Cells were treated with AGEs (200 mg/L) for 0, 4, 8, 16, or 24 h, or were treated with RA (2 μM) alone, 3-MA (2 mM) alone, AGEs (200 mg/L) and 3-MA (2 mM), or AGEs (200 mg/L) and RA (2 μM) for 8 h. When the treatment time was <6 h, cells were stained with 5 μg/mL DCFH-DA for 30 min before AGEs treatment. Cells were then subjected to flow cytometry using a Becton-Dickinson FACS Caliber and analyzed by CellQuest software 6 (Becton-Dickinson, San Jose, CA, USA).

Measurement of mitochondrial membrane potential

Mitochondrial membrane potential (ΔΨm) was analyzed using a JC-1 assay (Beyotime, Haimen City, China). JC-1 is a cationic dye that indicates ΔΨm by reversibly shifting its fluorescence emission between green and red. ΔΨm was analyzed using a fluorescence microscope and via flow cytometry as described previously (Guo et al. 2012). Briefly, a staining mixture (300 nM JC-1) was prepared according to manufacturer’s instructions using a JC-1 kit. Cells were divided into five groups: AGEs (200 mg/L) alone, AGEs and 3-MA (2 mM), AGEs and RA (2 μM), RA alone, and 3-MA alone. All groups were incubated in the staining mixture for 30 min at 37°C. Thereafter, cells were either assessed by fluorescence microscope or harvested and analyzed by flow cytometry.

Data analysis

Each experiment was repeated three times, and data are presented as the mean ± S.E.M. of the three independent experiments. The Statistical Package for Social Science 17.0 software (IBM) was used for statistical analysis. Comparisons among multiple groups were performed using ANOVA. Pairwise comparisons were performed using a Student’s t-test. Values of P<0.05 were considered to be statistically significant.

Results

AGEs impair osteoblastic MC3T3-E1 cell viability

Cell viability was tested in osteoblastic MC3T3-E1 cells treated with AGEs. AGEs impaired osteoblastic MC3T3-E1 cell viability in a dose-dependent manner. Cell viability in osteoblastic MC3T3-E1 cells treated with >300 mg/L AGEs was nearly eliminated. Cell viability of cells treated with 200 mg/L AGEs decreased to 50% as compared with control cells (Fig. 1). Thus, an AGE concentration of 200 mg/L was used for subsequent experiments (P<0.001 vs control cells).

The autophagy level is increased in osteoblastic MC3T3-E1 cells treated with AGEs

LC3 is the most widely detected autophagy-related protein (Klionsky et al. 2012), and remains on the membrane even after spherical autophagosomes are completely formed. The total LC3 level and the LC3II/LC3I ratio were determined to evaluate the extent of autophagy. p62/SQSTM1 is a biomarker for autolysosome degradation. Impaired autophagy is often accompanied by accumulation of p62/SQSTM1, and p62/SQSTM1 expression is negatively correlated with
autophagy level (Klionsky et al. 2012). The autophagy level was increased in M3T3-E1 cells treated with AGEs as represented by an increase in both the total LC3 level and the LC3II/LC3I ratio, as well as a decrease in p62/SQSTM1 expression (P<0.001 vs control cells). The total LC3 level and the LC3II/LC3I ratio substantially increased after 8 and 12 h of AGEs treatment, as compared with control cells. However, after >12 h of AGEs treatment, the autophagy level was increased as compared with control cells, but was not increased to the same level as seen after 12 h of AGEs treatment. p62/SQSTM1 was decreased in osteoblastic MC3T3-E1 cells treated with AGEs for >4 h (Fig. 2A and B) (P<0.001 vs control cells). Autolysosomes were detected in cultured osteoblastic MC3T3-E1 cells by intracellular LC3 redistribution, and were calculated as the average number of LC3-labeled punctae per cell. The average number of LC3-labeled punctae per cell in AGEs-treated cells was substantially increased as compared with control cells (Fig. 2C) (P<0.001 vs control cells).

### AGES-induced apoptosis is inhibited by the autophagy inducer RA and accelerated by the autophagy inhibitor 3-MA

AGES induced apoptosis in osteoblastic MC3T3-E1 cells in a time-dependent manner. When osteoblastic MC3T3-E1 cells were treated with AGEs (200 mg/L) for 4, 8, 12, 16, and 24 h, apoptosis was increased, as determined by annexin V and propidium iodide staining (Fig. 3A and B) (P<0.001 vs control cells). Morphological changes of the nucleus resulting from treatment with AGEs were observed by immunofluorescence after Hoechst 33324 staining. Cells treated with AGEs (200 mg/L) for 24 h showed obvious karyopyknosis as compared with control cells (Fig. 3C). AGEs-induced apoptosis was attenuated when cells were cotreated with the autophagy-inducer RA as compared with treatment with AGEs alone; total cell death was reduced from 13.56 to 7.74% (P<0.01 vs AGEs-treated cells). In contrast, apoptosis was increased in cells that were cotreated with 3-MA, an inhibitor of autophagy, as compared with treatment with AGEs alone; total cell death increased from 13.56 to 29.10% (P<0.01 vs AGEs-treated cells). However, treatment with RA or 3-MA alone had no appreciable effect on apoptosis (Fig. 3D and E).

### Autophagy regulation affects AGEs-induced intracellular ROS generation and mitochondrial damage in a time-dependent manner

The exposure of osteoblastic MC3T3-E1 cells to AGEs (200 mg/L) for 0, 4, 8, 16, or 24 h led to an increase in the number of DCF-positive cells (cells showing a DCF fluorescence signal above the threshold set by normal cells) as compared with control cells (Fig. 4A). The ratio of DCF-positive cells increased from 3.73% in the control group to 4.81, 10.50, 33.65, and 66.34% in cells treated with AGEs for 4, 8, 16, and 24 h, respectively (P<0.01 vs control cells).

The effect of AGEs on mitochondrial damage was analyzed by flow cytometry of JC-1-stained cells. Loss of ΔΨm was indicated by an increase in the number of green fluorescence-stained cells and a decrease in the number of red fluorescence-stained cells (an increase in the percent of cells in the right lower quadrant). AGEs-treated cells showed an apparent loss of ΔΨm as compared with control cells (P<0.01 vs control cells).

When AGEs-treated cells were cotreated with the autophagy inducer RA, DCF-positive cells decreased from 32.65 to 20.05%, as compared with cells treated with AGE alone (P<0.05 vs AGES-treated cells). These data indicate that intracellular ROS generation was reduced by inducing autophagy. Conversely, when AGEs-treated cells were cotreated with the autophagy inhibitor 3-MA, DCF-positive cells were increased from 33.45 to 80.46%, as compared with cells treated with AGE alone (Fig. 4D) (P<0.001 vs AGES-treated cells). In accordance with ROS data, reductions in ΔΨm were exacerbated when cells were cotreated with 3-MA (P<0.001 vs AGES-treated cells), but were attenuated by cotreatment with RA (Fig. 4E and D) (P<0.05 vs AGES-treated cells). Treatment of osteoblastic MC3T3-E1 cells with RA or 3-MA alone did not affect ΔΨm. In conclusion, these data collectively indicate that autophagy regulation affects intracellular ROS and mitochondrial damage.
The antioxidant NAC suppresses AGEs-induced autophagy in osteoblastic MC3T3-E1 cells

We found typical autophagic changes in osteoblastic MC3T3-E1 cells treated with AGEs. Since intracellular oxidative stress is known to participate in many AGEs-induced pathologic processes, the role of oxidative stress in AGEs-induced autophagy regulation was further analyzed. NAC, a well-characterized antioxidant, was used to attenuate oxidative stress. In cells pretreated with NAC (10 mmol/L), total LC3 expression and the LC3II/LC3I ratio were both statistically significantly reduced as compared with cells treated with AGEs alone using western blotting. The average number of LC3-labeled punctae per cell in NAC and AGEs-treated cells was substantially decreased as compared with control cells treated with AGEs for >4 h. Autolysosomes were detected in cultured osteoblastic MC3T3-E1 cells by intracellular LC3 redistribution, and were calculated as the average number of LC3-labeled punctae per cell. These results provide evidence that ROS may be essential to trigger autophagy in osteoblastic MC3T3-E1 cells treated with AGEs (Fig. 5).

Discussion

Since 2014, osteoporotic fracture has been considered a diabetic complication (Hofbauer et al. 2016). AGEs are the most important pathogenic factors in diabetic complications, and their negative effects on bone tissue are well documented (Yamagishi 2011, Hamann et al. 2012). AGEs can impair osteoblast proliferation and mineralization as well as mature bone nodule formation (Momma et al. 2012, Yamaguchi & Sugimoto 2012). Therefore, it is important to determine the mechanism of the adverse effects of AGEs on bone cells.

AGEs can induce apoptosis in osteoblasts by inducing intracellular oxygenic stress. Many apoptosis-related signaling pathways participate in this process, including activation of MAPKs and increasing activation of caspase-3, -8, and -9 (Alikhani et al. 2007, Weinberg et al. 2014). Consistent with previous studies, apoptosis was also induced in AGEs-treated osteoblastic MC3T3-E1 cells in this study. However, if induction of osteoblast cell apoptosis is the only mechanism of AGEs pathology, it is difficult to reconcile this mechanism with the
phenomenon that diabetic patients typically have normal BMD (Hamann et al. 2012). Therefore, we hypothesized that autophagy, which interacts closely with apoptosis, was an AGES-induced adverse effect on osteoblasts.

Autophagy is also called type II cell death. However, the protective role of autophagy in cells has recently been widely accepted because autophagy-related gene (ATG) knockdown/knockout accelerates, rather than delays, cell...
Figure 4
Intracellular oxidative stress assay in AGEs-treated osteoblastic MC3T3-E1 cells. (A) AGEs increased intracellular ROS generation in osteoblastic MC3T3-E1 cells in a time-dependent manner. The ROS assay used 2',7'-dichlorodihydrofluorescein diacetate (DCF-DA), a reliable fluorogenic marker for ROS. The peaks of groups treated with AGEs for different time periods shifted to the right, indicating that DCF-positive cells (intracellular ROS-positive cells) increased. Data are representative of three independent experiments. (B and C) A decrease in ΔΨm, which represents mitochondrial damage, was determined by staining for JC-1, a well-known marker of ΔΨm. Cells with normal ΔΨm are depicted as those with high red and high green fluorescence in the upper right quadrant of the scatterplots. In contrast, cells that had decreased ΔΨm are depicted as those with high green and low red fluorescence in the lower right quadrant in response to the treatment with AGEs. Data are representative of three independent experiments. (D, E and F) Intracellular ROS levels and ΔΨm were affected by the autophagy inhibitor 3-MA (2 mM), and the autophagy inducer RA (2 μM). When AGEs-treated cells were cotreated with RA (2 μM), the number of DCF-positive cells decreased, which indicates that intracellular ROS were reduced. ΔΨm was stable, which indicates that mitochondrial damage was attenuated. In contrast, when AGEs-treated cells were cotreated with 3-MA, intracellular ROS levels were increased, and ΔΨm balance was damaged. Data are representative of three independent experiments. *P<0.05 vs AGEs-treated cells. **P<0.001 vs AGEs-treated cells.
death (Levine & Yuan 2005). Moreover, the inhibition of autophagy in various tumor cells is considered a new solution to drug resistance, leading to maximization of chemotherapeutic treatment (Carames et al. 2010, Tang et al. 2015, Wang et al. 2015). Nevertheless, the role autophagy plays in AGEs-treated osteoblasts has not been well defined.

The osteoblastic MC3T3-E1 cell line was demonstrated to have the ability to differentiate into premature and mature osteoblasts, and has been used as a model system for osteoblast differentiation and biological calcification (Sudo et al. 1983, Hong et al. 2010). In this study, we found typical autophagy overexpression in osteoblastic MC3T3-E1 cells treated with AGEs as compared with control cells. Further increases in autophagy induced by the known autophagy inducer, RA, reversed AGEs-induced apoptosis. Conversely, the proportion of cells undergoing apoptosis was aggravated when autophagy was inhibited by 3-MA. These results suggest that autophagy might play a self-protective role in osteoblastic MC3T3-E1 cells during AGEs-induced apoptosis.

In contrast to our findings in cells treated with AGEs for < 24 h, we also found that the protective effect of autophagy against AGEs-induced apoptosis was limited after 24 h. Osteoblastic MC3T3-E1 cells eventually died after exposing to AGEs for 24 h. Autophagy functions via formation of the autophagosome, which is a double membrane structure. Transformation of LC3 proteins, which exist on the membrane of the autophagosome, from the unlipidated species (also known as form I) to the lipidated species (form II) occurs when an autophagosome is formed. When autophagy is induced, LC3 levels (including LC3I and LC3II) are usually upregulated in different patterns (Klionsky et al. 2012). We found that the total LC3 level and the LC3II/LC3I ratio increased significantly in osteoblastic MC3T3-E1 cells treated with AGEs for 8 and 12 h as compared with control cells. p62/SQSTM1, a biomarker of the degradation of autolysosomes with its expression negatively correlated with autophagy level, was decreased in osteoblastic MC3T3-E1 cells treated with AGEs, which is in accordance with the change of LC3 level. After over 12 h, however, autophagy was still increased, but was not increased to the same level as seen after 12 h of AGEs treatment. In the early stages of autophagy, both the formation of the intracellular preautophagic double membrane and the transformation of the double membrane are increased. Our results might indicate that before intracellular autophagosomes are fully formed, further induction of autophagy could reverse AGEs-induced apoptosis.
However, once autophagosomes are fully formed, which means the protection of autophagy had reached its limit, apoptosis is inevitable if harmful factors remain present.

Previous research on the pathological mechanism of AGEs showed that AGEs induced ROS production, representing a main mechanism for many of the complications associated with diabetes. In addition, in vitro and in vivo studies have reported that increased oxidative stress has a negative impact on bone formation by modulating the differentiation and survival of osteoblasts (Schroder 2015). Loss of nuclear factor (erythroid-derived 2)-like 2, a master transcription factor that regulates the induction of antioxidant gene expression and Phase II antioxidant enzymes, leads to increased oxidative stress and increased susceptibility to radiation-induced bone loss. Our results demonstrate that ROS levels were increased in AGEs-treated osteoblastic MC3T3-E1 cells, and ΔΨm (a reflection of mitochondrial membrane stability; the decline of ΔΨm indicates mitochondrial damage) was decreased. We further interrogated ROS and ΔΨm changes by regulating autophagy with 3-MA and RA. Autophagy induction was accompanied by a decrease in both ROS levels and mitochondrial damage, whereas the autophagy inhibition was accompanied by a further increase in ROS production and mitochondrial damage. Therefore, we hypothesized that the protective function of autophagy against apoptosis may depend on the ability of autophagy to clear ROS and to alleviate damaged mitochondria.

Additionally, we pretreated cells with NAC, a well-known antioxidant, before AGE exposure. We found that AGEs-induced autophagy was inhibited in osteoblastic MC3T3-E1 cells. We hypothesized that ROS was not only a clear target of autophagy, but also necessary to trigger autophagy. In other words, ROS and autophagy interact closely with each other, and ROS might be a bridge between autophagy and apoptosis. Previous studies have presupposed that autophagy and apoptosis might be triggered by common upstream signals. On a molecular level, this means that the apoptotic and autophagic response mechanisms share common pathways that either link or polarize cellular responses (Maiuri et al. 2007, Zhang et al. 2011). ROS could induce mitochondrial damage; conversely, mitochondrial damage is also a major source of intracellular ROS. When mitochondrial ROS are abundant and the mitochondrial membrane is damaged, leakage of ROS and various apoptosis-related factors such as cytochrome c and apoptosis-inducing factor into the cytosol has been observed (Guo et al. 2012). Cytosolic ROS can induce severe damage in many organelles, eventually leading to induction of apoptosis in conjunction with apoptosis-related factors. We hereby propose that increased ROS production is a common upstream signal of autophagy and apoptosis in osteoblasts treated with AGES.

Conflict of interest statement
None of authors in this paper has any financial or personal relationships with other people or organizations that could influence (bias) the research results. No conflicts exist with the authors of this study.

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