High glucose induces autophagy of MC3T3-E1 cells via ROS-AKT-mTOR axis

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Abstract
In the present study, we investigate the function of ROS-AKT-mTOR axis on the apoptosis, proliferation and autophagy of MC3T3-E1 cells, and the proliferation of MC3T3-E1 cells after autophagy inhibition under high glucose conditions. MC3T3-E1 cells cultured in vitro were divided into the following groups: normal control group, N-acetylcysteine (NAC) group, 11.0 mM high glucose group, 11.0 mM high glucose + NAC group, 22.0 mM high glucose group, 22.0 mM high glucose + NAC group, CQ group, 22.0 mM high glucose + CQ group, 3-MA group and 3-MA + 22.0 mM high glucose group. ROS production was measured by DCFH-DA fluorescent probe. Cell proliferation was measured by MTT assay. Cells in different groups were stained with Annexin V-FITC/PI, and then apoptosis rate was detected by flow cytometry. Nucleus morphology was observed under fluorescence microscope after being incubated with Honchest33258. Protein expression was measured using Western blotting and immunofluorescence. Cell apoptosis and proliferation in high glucose group were increased and decreased, respectively, in a dose-dependent manner. Autophagy was significantly induced in high glucose group, even though different concentration of glucose induced autophagy in different stages of autophagy. ROS production in MC3T3-E1 cells was remarkably increased in high glucose group, but not in a dose-dependent manner. NAC, as an antioxidant, reduced ROS production and ameliorated cell apoptosis, proliferation abnormality and autophagy caused by high glucose. Expression of p-AKT and p-mTOR proteins were dramatically decreased in high glucose group, and NAC reversed their expression. In addition, 3-MA, an inhibitor of autophagy, significantly decreased the proliferation of MC3T3-E1 cells. When cocultured with 22.0 mM glucose that induced autophagy, proliferation of MC3T3-E1 cells was not affected compared to 22.0 mM high glucose group. Our present findings reveal that high glucose affects apoptosis, proliferation and autophagy of MC3T3-E1 cells through ROS-AKT-mTOR axis. In addition, autophagy inhibition does not affect the proliferation of MC3T3-E1 cells under high glucose conditions.

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1. Introduction

Diabetic osteoporosis (DOP) is a chronic complication of diabetes mellitus (DM) that affects the health of bones in human bodies (Albright and Reifenstein, 1948). Bone metabolic disorder caused by DM mainly includes defects in bone formation, reduced number of osteoblasts and deficiency of osteoid formation (Hofbauer et al., 2007). At last, bone metabolic disorder can result in reduced bone mass, decreased bone quality and increased bone fracture risk (Lumachi et al., 2009; Massé et al., 2010; Hamann et al., 2012). Osteoblasts and osteoclasts play important roles in the metabolism of bone cells (Ducy et al., 2000). In our previous work (Feng et al., 2011), we have found that high glucose in vitro affects osteoblast function, but the mechanism of osteoblast dysfunction caused by high glucose is still unknown.

Reactive oxygen species (ROS) is closely related to the pathogenesis of DOP, postmenopausal osteoporosis and glucocorticoid-induced osteoporosis (Isomura et al., 2004; Feng and Tang, 2014; Hamada et al., 2007). On one hand, ROS oxidizes and destroys protein, lipid, DNA, leading to altered cell function; on the other hand, it activates many adaptation signaling pathways in cells (Finkel, 2001; Genestra, 2007; Cullinan and Diehl, 2004; Li et al., 2009), in which mTOR (mammalian target of rapamycin) plays an
important role in autophagy, apoptosis, metabolism, growth, survival and aging (Roy et al., 2014; Wullschleger et al., 2006).

Autophagy is a recycling process of intracellular materials mediated by lysosome. It plays an important role in cell metabolism and organelle renewal. Autophagy is a self-protection mechanism under stress, as well as a maladaptation that leads to cell death (Matsui et al., 2007; Zhu et al., 2007). Studies (Hara et al., 2006; Komatsu et al., 2006; Yang et al., 2014) have revealed that autophagy plays an important role in the onset of diabetes, metabolic bone disease, and neurodegenerative diseases such as Alzheimer’s disease and Parkinson’s disease, possibly by affecting cell growth, survival, differentiation and self-stabilization (Yang and Klionsky, 2010). Autophagy is tightly regulated by a number of pathways. The most extensively studied pathway involves mTOR, which negatively regulates autophagy (Yang et al., 2013). ROS-AKT (protein kinase B)-mTOR axis plays an important role in autophagy of cancer cells, but its role in osteoblasts is still unknown (Fiorini et al., 2015). AKT-mTOR signaling that is downstream of ROS, plays an important role in proliferation and apoptosis (Park et al., 2011). mTOR is an important negative regulator of autophagy (Wander et al., 2011), which is regulated by several upstream regulators such as PI3K-AKT and MAPK (McConnell et al., 2012). In addition, autophagy, apoptosis and differentiation are bound together under high glucose condition in osteoblasts (Bartolome et al., 2013), but the main molecular mechanism and its role on proliferation are rarely studied. In the present study, we investigate whether ROS-AKT-mTOR signaling pathway takes part in changes of autophagy level and cell function, and the relationship between autophagy and proliferation of osteoblasts under high glucose condition.

2. Materials and methods

2.1. Cells

MC3T3-E1 cells (American Type Culture Collection, Manassas, VA, USA) were grown in α-minimum essential medium (α-MEM; Hyclone, GE Healthcare, Little Chalfont, UK) supplemented with 10% fetal bovine serum (PAN Biotech, Aidenbach, Germany) and 1% penicillin/streptomycin in a humidified incubator with 5% CO2 at 37 °C. The study was divided into three parts. First, cells were divided into normal control group (cells were cultured with normal medium for 24 h), high glucose group and antioxidant group. High glucose group included 11.0 mM high glucose group (cells were cultured with 11.0 mM glucose medium for 24 h) and 22.0 mM high glucose group (cells were cultured with 22.0 mM glucose medium for 24 h). Antioxidant group included N-acetylcysteine (NAC; Beyotime, Shanghai, China) group (cells were cultured with 2 mM NAC for 24 h), 11.0 mM high glucose + NAC group (cells were cocultured with 11.0 mM glucose and 2 mM NAC for 24 h) and 22.0 mM high glucose + NAC group (cells were cocultured with 22.0 mM glucose and 2 mM NAC for 24 h). Second, in order to measure the autophagy flux, we further detected the expression of P62/SQSTM1 protein, a good indicator of autophagy suppression, and used chloroquine (CQ) as an inhibitor of the fusion of autophagosome and lysosome. The cells were divided into four groups, including normal control group, CQ group (cells were cultured with 10.0 μM CQ for 24 h), 22.0 mM high glucose group and 22.0 mM high glucose + CQ group (cells were cocultured with 22.0 mM glucose and 10.0 μM CQ for 24 h). Third, cells were divided into four groups, including normal control group, 22.0 mM high glucose group, 3-methyladenine (3-MA; Sigma-Aldrich, St. Louis, MO, USA) group (cells were cultured with 5 mM 3-MA for 24 h) and 22.0 mM high glucose + 3-MA group (cells were cocultured with 22.0 mM glucose and 5 mM 3-MA for 24 h). 3-MA, a widely used inhibitor of autophagy that inhibits the conversion of LC3B-I to LC3B-II through phosphatidylinositol 3-kinase class III (Heckmann et al., 2013). In order to test whether loss of autophagy affects the proliferation of MC3T3-E1 cells, we used 5 mM 3-MA as autophagy inhibitor (Bartolome et al., 2013).

2.2. Flow cytometry and Inverted fluorescence microscope

DCFH-DA probe kit (BestBio, Shanghai, China) was used to detect ROS production. Cells in logarithmic growth were cultured
Fig. 2. Apoptosis rate of MC3T3-E1 cells in different groups detected by Annexin-FITC/PI staining. (A) Normal control group; (B) NAC group; (C) 11.0 mM high glucose group; (D) 11.0 mM high glucose + NAC group; (E) 22.0 mM high glucose group; and (F) 22.0 mM high glucose + NAC group. (G) Quantification of apoptotic index. *, P < 0.05 compared with normal control group; **, P < 0.01 compared with normal control group; #, P < 0.01 compared with 11.0 mM high glucose group; ※, P < 0.01 compared with 22.0 mM high glucose group.
Table 1
Comparison of optical density values in different groups.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Optical density value (means ± standard deviations)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal control</td>
<td>1.560 ± 0.152</td>
</tr>
<tr>
<td>N-acetylcysteine</td>
<td>1.990 ± 0.082*</td>
</tr>
<tr>
<td>11.0 mM high glucose</td>
<td>1.458 ± 0.129</td>
</tr>
<tr>
<td>11.0 mM high glucose + N-acetylcysteine</td>
<td>1.593 ± 0.134*</td>
</tr>
<tr>
<td>22.0 mM high glucose</td>
<td>1.183 ± 0.163*</td>
</tr>
<tr>
<td>22.0 mM high glucose + N-acetylcysteine</td>
<td>1.404 ± 0.285*</td>
</tr>
</tbody>
</table>

Note:

* P < 0.05 compared with normal control group.
* P < 0.01 compared with 11.0 mM high glucose group.
* P < 0.01 compared with 11.0 mM high glucose group.
* P < 0.05 compared with 22.0 mM high glucose group.

Fig. 3. LC3B expression detected using immunofluorescence assay. LC3B expression in (A) normal control group, (B) 11.0 mM high glucose group, (C) 22.0 mM high glucose group, (D) NAC group, (E) 11.0 mM high glucose + NAC group, and (F) 22.0 mM high glucose + NAC group was observed under inverted fluorescence microscope (magnification, ×200). (G) LC3B expression in 11.0 mM glucose group (magnification, ×400).
for 24 h, and then intervened by high glucose and NAC for 24 h. Cells were washed with phosphate-buffered saline (PBS), and then loaded with DCFH-DA (1:1000) for 30 min. Then, the cells were washed twice with basal medium. Fluorescence intensity was quantified by flow cytometry and observed by inverted fluorescence microscope. CellQuest™ software was used to analyze positive rate of ROS.

Cells were intervened for 24 h, and methanol was used to fix cells at −20 °C for 10 min. Then, the cells were washed with PBS, and loaded with Honchest33258. Fluorescence intensity was observed by inverted fluorescence microscope. In addition, Annexin-FITC/PI was used to stain cells. Apoptosis rate was detected and analyzed using flow cytometry.

2.3. 3-(4,5-Dimethylthiazole -2-yl)- 2,5-biphenyltetrazolium bromide (MTT) assay

For the detection of cell proliferation, MTT assay was used. MC3T3-E1 cells were seeded onto 96-well plates at a density of $5 \times 10^3$ per well, and cultured for 24 h. Then, the cells were exposed to high glucose and antioxidant NAC in a humidified incubator with 5% CO$_2$ at 37 °C for 24 h. Each group had 6 wells. Then, the cells were loaded with 20 µL 5 mg/mL MTT, and incubated in 5% CO$_2$ at 37 °C for 4 h. The plate was shaken for 10 min after dimethyl sulphoxide injection. Optical density values were measured using microplate reader at 570 nm.

2.4. Immunofluorescence assay

Cells in logarithmic growth were cultured for 24 h, and then intervened by high glucose and NAC for 24 h. Cells were washed with PBS, fixed with methanol and sealed with 5% bovineserum albumin (BSA) for 1 h. Then, the cells were incubated with anti-LC3B (1:50; Sigma-Aldrich, St. Louis, MO, USA) and anti-Beclin1 (1:100; Abcam, Cambridge, UK) overnight at 4 °C, followed by washing with PBS. Afterwards, the cells were incubated with red fluorescent second antibody in dark room for 30 min, before washing with PBS. Then, the cells were incubated with Honchest33258 for 5 min. Fluorescence intensity was observed by inverted fluorescence microscope.

2.5. Western blotting

MC3T3-E1 cells were collected, washed with cold PBS, and lysed in a buffer containing 50 mM Tris (pH7.4), 150 mM NaCl, 1% TritonX-100, 1% sodium deoxycholate, 1 mM phenylmethylsulfonyl fluoride, sodium orthovanadate, sodium fluoride, ethylene diamine tetraacetic acid and leupeptin. Cellular debris was centrifuged at 12,000×g for 12 min at 4 °C. Protein samples were subjected to SDS-PAGE (8–12%gels), and transferred onto Immobilon-P PVDF membranes (Millipore, Merck, Germany). The remaining steps were performed according to details reported previously (Yang et al., 2013). Antibodies used in the study were as follows: anti-AKT, anti-p-AKT (Thr389), anti-mTOR, anti-p-mTOR (Ser2448) (Cell Signaling Technology, Danvers, MA, USA), anti-LC3B (1:1000; Sigma-Aldrich, St. Louis, MO, USA) and anti-Beclin1 (1:500; Abcam, Cambridge, UK) and anti-P62/SQSTM1 (Abcam, Cambridge, UK).

2.6. Statistical analysis

Statistical analysis was carried out with SPSS19.0 (IBM, Armonk, NY, USA). All data were expressed as means ± standard deviations. Comparisons were performed by one-way ANOVA. Differences were considered significant if $P < 0.05$. 

![Fig. 4. Beclin1 expression detected by immunofluorescence assay. Beclin1 expression in (A) normal control group, (B) 11.0 mM high glucose group, (C) 22.0 mM high glucose group, (D) NAC group, (E) 11.0 mM high glucose + NAC group, and (F) 22.0 mM high glucose + NAC group was observed under inverted fluorescence microscope (magnification, ×200).](image-url)
3. Results

3.1. High glucose promotes the apoptosis of MC3T3-E1 cells

To study the effect of high glucose on the apoptosis of MC3T3-E1 cells, inverted fluorescence microscopy was performed. Cell nucleus morphology in high glucose group was changed significantly, showing nucleoplasm pyknosis, irregular shape, nucleolus margination, and karyotheca fracture of cell nucleus (Fig. 1). Compared with normal control group, the apoptosis rate of MC3T3-E1 cells in 11.0 mM and 22.0 mM high glucose groups was increased by 12.14% and 60.40%, respectively (both \( P < 0.01 \)). In addition, apoptosis rate of MC3T3-E1 cells in 22.0 mM high glucose group was increased by 43.04% compared with 11.0 mM high glucose group (\( P < 0.01 \)) (Fig. 2). The results suggest that high glucose promotes the apoptosis of MC3T3-E1 cells.

3.2. High glucose decreases the proliferation of MC3T3-E1 cells

To test the effect of high glucose on the proliferation of MC3T3-E1 cells, MTT assay was used. Proliferation of MC3T3-E1 cells in 11.0 mM high glucose group was 6.54% lower than that in normal control group, but without statistical significance (\( P > 0.05 \)). Compared with normal control group and 11.0 mM high glucose group, the proliferation of MC3T3-E1 cells in 22.0 mM high glucose group was significantly lower (24.17% and 18.86%, respectively; both \( P < 0.01 \)) (Table 1). The results indicate that high glucose decreases the proliferation of MC3T3-E1 cells.

3.3. High glucose induces autophagy in MC3T3-E1 cells

To investigate the effect of high glucose on autophagy of MC3T3-E1 cells, immunofluorescence assay and Western blotting were employed. After treatment with 11.0 mM glucose for 24 h,
expression of autophagy-related proteins LC3B-II and Beclin1 was increased significantly, and LC3B-II exhibited discrete distribution in the cytoplasm of MC3T3-E1 cells. When glucose concentration was increased to 22.0 mM, the expression of LC3B-II was decreased in MC3T3-E1 cells, but interestingly the expression of Beclin1 was increased significantly compared with 11.0 mM high glucose group (Figs. 3, 4 and 5A). Compared with normal control group, the expression of P62 protein in 11.0 mM high glucose group was decreased significantly, but that in 22.0 mM high glucose group was slightly increased without statistical significance (Fig. 5A). Treatment with CQ increased the expression of LC3B-II in both normal control group and 22.0 mM high glucose group, and the expression of LC3B-II in 22.0 mM high glucose + CQ group was increased significantly compared with CQ group (P < 0.01) (Fig. 5B). These results suggest that high glucose induces autophagy in MC3T3-E1 cells.

3.4. High glucose induces ROS production in MC3T3-E1 cells, while treatment with NAC abolishes this effect

To examine how high glucose affects the production of ROS in MC3T3-E1 cells, immunofluorescence assay was performed. The data showed that fluorescence intensity of MC3T3-E1 cells in high glucose groups was increased and the peak of fluorescence shifted towards right compared to normal control group. After treatment with antioxidant NAC, fluorescence intensity was decreased and the peak of fluorescence shifted back towards left (Figs. 6 and 7). Production of ROS in 11.0 mM and 22.0 mM groups was increased by 35.14% and 40.13%, respectively, compared with normal control group (both P < 0.01) (Fig. 7). When being cocultured with NAC, production of ROS in 11.0 mM and 22.0 mM groups were 27.85% and 23.99% lower than that before coculture, respectively (both P < 0.01) (Fig. 7). The results indicate that high glucose induces ROS production in MC3T3-E1 cells, while treatment with NAC abolishes this effect.

3.5. High glucose affects apoptosis and proliferation of MC3T3-E1 cells via ROS-AKT-mTOR axis

To identify the mechanisms by which high glucose affects apoptosis and proliferation of MC3T3-E1 cells, we carried out flow cytometry, MTT assay and Western blotting. After treatment with NAC, the size of apoptotic cell nucleus was significantly decreased (Fig. 1). In addition, apoptosis rate of MC3T3-E1 cells in 11.0 mM and 22.0 mM high glucose groups was decreased by 15.72% and 12.61%, respectively (both P < 0.01) (Fig. 2), while cell proliferation in the two groups was increased by 16.12% and 18.68%, respectively (both P < 0.05) (Table 1). Therefore, NAC ameliorates cell dysfunction induced by high glucose. Western blotting data showed that the expression of p-AKT and p-mTOR proteins was significantly decreased in high glucose groups, while coculture with NAC significantly increased the expression of the two proteins in high glucose groups. However, NAC alone did not affect the expression of p-AKT and p-mTOR proteins (Fig. 8). NAC ameliorates cell function as well as expression of protein p-AKT and p-mTOR under high glucose circumstance. These results suggest that high glucose affects apoptosis and proliferation of MC3T3-E1 cells partly through ROS-AKT-mTOR axis.

3.6. ROS-AKT-mTOR axis plays an essential role in the regulation of autophagy under high glucose conditions in MC3T3-E1 cells

To investigate the mechanisms by which high glucose affects autophagy of MC3T3-E1 cells, we tested the expression of proteins in AKT-mTOR signaling pathway downstream of ROS. The data showed that the expression of p-AKT and p-mTOR proteins in both

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**Fig. 6. Fluorescence intensity of ROS production in MC3T3-E1 cells in different groups under inverted fluorescence microscope.** (A) Normal control group; (B) 11.0 mM high glucose group; (C) 22.0 mM high glucose group; (D) NAC group; (E) 11.0 mM high glucose + NAC group; and (F) 22.0 mM high glucose + NAC group. (Magnification, ×200).
High glucose groups was decreased significantly compared to normal control group (Fig. 8). In addition, antioxidant NAC reversed the increased level of autophagy in high glucose groups (Figs. 3–5) and the decreased levels of p-AKT and p-mTOR protein expression (Fig. 8). These results indicate that ROS-AKT-mTOR axis plays an essential role in the regulation of autophagy under high glucose conditions in osteoblasts.

3.7. Inhibition of autophagy in high glucose group does not affect the proliferation of MC3T3-E1 cells

To test whether inhibition of autophagy affects the proliferation of MC3T3-E1 cells, we further investigated the effect of 3-MA, an inhibitor of autophagy, on cell proliferation using MTT assay. We found that 3-MA significantly decreased the proliferation of MC3T3-E1 cells. Compared with 22.0 mM high glucose group, the proliferation of MC3T3-E1 cells in 22.0 mM high glucose + 3-MA
group was decreased, but without statistical significance ($P > 0.05$) (Fig. 9). The results suggest that inhibition of autophagy in high glucose group does not affect the proliferation of MC3T3-E1 cells.

4. Discussion

Both type I DM and type II DM can affect bone metabolism and finally lead to osteopenia, decreased bone strength and bone fracture (Lumachi et al., 2009; Massé et al., 2010; Hamann et al., 2012). However, the main mechanism is still unknown. Osteoblasts play an important role in bone remodeling (Ducy et al., 2000), and its function is significantly changed under high glucose conditions (Hamann et al., 2012). According to diagnostic criteria of diabetes by WHO in 1999, hyperglycemia is defined as fast blood-glucose (FBG) $\geq 7.0$ mmol/L and 2-h postprandial blood-glucone (PBG) $\geq 11.1$ mmol/L, and clinical FBG and 2-h PBG should be controlled at 4.4–6.1 mmol/L and 4.4–8.0 mmol/L, respectively (World Health Organization, 1999). In the present study, we chose 5.5 mM as normal control group and defined 11.0 mM and 22.0 mM as high glucose. We found that apoptosis of MC3T3-E1 cells was increased significantly in a dose-dependent manner, which is in accordance with what we have found before (Feng et al., 2011). However, Zhen et al. 2010 have shown different results. These authors found that apoptosis of osteoblasts under 11.0 mM high glucose did not change statistically compared with normal group, but was significantly increased when glucose concentration increased. In addition, we found that cell proliferation in 11.0 mM high glucose group was decreased, but without statistical significance. When glucose concentration was increased to 22.0 mM, cell proliferation was decreased significantly, concuring with the observations in some other studies (Shao et al., 2014; Ma et al., 2014; Dong et al., 2014). However, some others found that cell proliferation under high glucose first increased and then decreased, when glucose concentration was extremely high (Zhen et al., 2010; Cheng et al., 2008). The discrepancy in these studies is probably due to different cells and culture time.

The process of autophagy covers a series of steps, including the formation of phagophore, the elongation and expansion of the phagophore, the closure and completion of autophagosome, autophagosome maturation and fusion with an endosome and/or lysosome, breakdown and degradation of autophagosome inner membrane and cargo inside the autolysosome, and recycling of the
resulting macromolecules through permeases (Pattinigg et al., 2008). There are many essential markers in different stages, such as Beclin1, LC3B and P62. Beclin1 is involved in autophagosome formation at an early stage, rather than the expansion step (Pattinigg et al., 2008). LC3B-II is closely correlated with the number of autophagosomes, serving as a good indicator of autophagosome formation (Kabeya et al., 2000). P62, an alternative marker to detect autophagic flux and a good indicator of autophagy suppression, can bind LC3B-II and hence, serving as a selective substrate of autophagy (Nakai et al., 2007). In the present study, we found that expression of Beclin1 and LC3B-II was increased and expression of P62 was decreased in 11.0 mM high glucose group, indicating that 11.0 mM high glucose induces autophagy, which is in accordance with other studies (Bartolome et al., 2013; Ravikumar et al., 2003). When glucose concentration was 22.0 mM, we found that P62 expression was increased and LC3B-II protein was significantly degraded on the degradation stage of autophagy, also suggesting the induction of autophagy. However, Kobayashi et al. 2012 reported that high glucose inhibited autophagy in cardiomyocytes, which was a beneficial adaptive response that protects cardiomyocytes against high glucose toxicity. In addition, decreased autophagy under high glucose was partly due to increased mTOR signaling, which is a main regulator of autophagy.

ROS are mainly represented by superoxide radical anion (O2−), hydrogen peroxide (H2O2) and hydroxyl radical (OH−) in cells, which play an important role in cell function (Liang et al., 2012; and autophagy via ROS. These findings are in accordance with other studies (Bartolome et al., 2013; Zhen et al., 2010; Guo et al., 2012). mTOR, a non-classical Ser/Thr protein kinase, belongs to phosphatidylinositol kinase (PIKK) family, and plays a vital role in cell apoptosis, survival, proliferation and autophagy (Roy et al., 2014; Wullschleger et al., 2006). mTOR is regulated by many upstream regulators, such as PI3K,AKT, and AMPK (Hay and Sonenberg, 2006). mTOR is regulated by many upstream signaling, which is a main regulator of autophagy. Akt/mTOR axis in osteoblasts might be an alternative strategy for the treatment of DOP.

Disclosures
All authors declare no financial competing interests. All authors declare no non-financial competing interests.

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