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Inhibition of mTOR affects protein stability of OGT

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ABSTRACT

Autophagy regulates cellular homeostasis through degradation of aged or damaged subcellular organelles and components. Interestingly, autophagy-deficient beta cells, for example Atg7-mutant mice, exhibited hypoinsulinemia and hyperglycemia. Also, autophagy response is diminished in heart of diabetic mice. These results implied that autophagy and diabetes are closely connected and affect each other. Although protein *O*-GlcNAcylation is up-regulated in hyperglycemia and diabetes, and *O*-GlcNAcylated proteins play an important role in metabolism and nutrient sensing, little is known whether autophagy affects *O*-GlcNAc modification and vice versa. In this study, we suppressed the action of mTOR by treatment of mTOR catalytic inhibitors (PP242 and Torin1) to induce autophagic flux. Results showed a decrease in global *O*-GlcNAcylation, which is due to decreased OGT protein and increased OGA protein. Interestingly, knockdown of *ATG* genes or blocking of lysosomal degradation enhanced protein stability of OGT. In addition, when proteasomal inhibitor was treated together with mTOR inhibitor, protein level of OGT almost recovered to control level. These data suggest that mTOR inhibition is a more efficient way to reduce protein level of OGT rather than that of CHX treatment. We also showed that not only proteasomal degradation regulated OGT stability but autophagic degradation also affected OGT stability in part. We concluded that mTOR signaling regulates protein *O*-GlcNAc modification through adjustment of OGT stability.

1. Introduction

Autophagy is a catabolic process to degrade cellular components and organelles in response to starvation, cellular stress, and stage of development [1–3]. It has a vital function in cell growth, regulation of subcellular organelle, cell death, and aging [4–8]. Numerous evidences suggest that autophagy affects various diseases including cancer [9], neurodegenerative diseases [10], and diabetes [11]. Interestingly, beta cells of diabetic db/db mice have a large number of autophagosomes compared to control. Also, beta cell specific Atg7 knockout mice showed hypoinsulinemia and hyperglycemia. These data imply that autophagy is essential for beta cell maintenance and function [12].

O-GlcNAc modification is a dynamic posttranslational modification which occurs on Ser or Thr residues of nuclear and cytoplasmic proteins [13]. Increased cellular *O*-GlcNAcylation has been implicated in diabetes. Diabetic hyperglycemia increased *O*-GlcNAcylation on CaMKII, which resulted in cardiac mechanical malfunction and arrhythmia [14]. *O*-GlcNAcylation on endothelial nitric oxide synthase inhibits its phosphorylation and impairs its activity, leading to erectile dysfunction as well as vascular disorders [15]. Even though autophagy has a closer relationship with diabetes and hyperglycemic conditions, little is known of the relationship between *O*-GlcNAc and autophagy. A recent study showed that protein levels of beclin1 and LC3-II were diminished in heart of db/db mice and GlcNAc treated cardiomyocytes. This identified that beclin1 and bcl-2 were *O*-GlcNAcylated [16]. However, there have been no previous studies linking *O*-GlcNAc regulating enzymes and autophagy. The aim of this work is to examine the relationship between autophagy and *O*-GlcNAc modification. In this study, we used specific mTOR inhibitors in order to investigate the function of autophagy in *O*-GlcNAcylation and protein levels of OGT and OGA.

2. Materials and methods

2.1. Cell culture

Human HepG2 cells were obtained from ATCC (Manassas, VA) and cultured in complete culture medium (11095-072, Gibco) supplemented with 10% fetal bovine serum (10082-139, Gibco) and 1 mM sodium pyruvate at 37 °C.

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2.2. Western blotting, antibodies and reagents

Cells were lysed in RIPA buffer and Western blotting was performed as previously described [17]. The following primary antibodies were used: α-LC3 (152-3, MBL international), α-β-actin (#4967, Cell Signaling Technology), α-*O*-GlcNAc (RL2, MA1-072, Thermo scientific), α-OGT (H300, Santa Cruz Technology), α-MGEA5 (OGA, ab68522, Abcam), α-Atg7 (#8558, Cell Signaling Technology), and α-Atg5 (#2630, Cell Signaling Technology). Vinblastine (V1377) and cycloheximide (C-7698) were purchased from Sigma–Aldrich. PP242 (S2218) and Torin1 (S2827) were purchased from Selleckchem. MG132 (474790) was purchased from CALBIOCHEM.

2.3. Statistical analysis

Data were expressed as mean ± SE. The two-tailed Student's *t*-test was used for statistical analysis ($^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$).

2.4. Short interfering RNA (siRNA) transfection

Double-stranded siRNA targeting human ATG5 and ATG7 (purchased from Invitrogen) were administered simultaneously (30 nM each) to HepG2 cells in Lipofectamine™ RNAiMAX reagent according to the manufacturer's instructions. In all experiments, scrambled siRNA served as a control. Cells were analyzed 48 h post-transfection.

3. Results

3.1. mTOR inhibitors reduce cellular O-GlcNAcylation

We first determined whether mTOR inhibition affected total *O*-GlcNAc modification in HepG2 cells. A main regulator of autophagy and cell growth is mTOR Ser/Thr kinase. Inhibition of TOR activity induced growth arrest by blocking protein synthesis and also induced autophagy by the activation of Atg1 (ULK1), which is a kinase involved in the first step of autophagy [18–20]. Induction of autophagy was monitored by the amount of LC3-II form. LC3 is a mammalian homologous of autophagy-related 8 (Atg8) and it presents a precursor form, LC3-I. Induction of autophagy induces LC3-I conversion, producing lapidated LC3-II by action of Atg12–Atg5–Atg16L complex [21]. When autophagy was induced by treatment of mTOR inhibitors PP242 or Torin1, the amount of LC3-II increased (Fig. 1A and B). We checked whether cellular *O*-GlcNAcylation changed in PP242 or Torin1 treated cells. Interestingly, total *O*-GlcNAcylation was decreased in mTOR inhibited HepG2 cells (Fig. 1C and D). To confirm the effect of mTOR inhibition on other cell lines, we treated PP242 or Torin1 in MEF and HEK 293 cells. Protein *O*-GlcNAcylation was also decreased in both cell lines (data not shown). These finding suggest that induction of autophagy by blockage of mTOR decreased *O*-GlcNAc modification of proteins.

3.2. PP242 and Torin1 decrease protein level of OGT and increase protein level of OGA

Due to the decrease of *O*-GlcNAc modified proteins in mTOR inhibition, we examined protein level of *O*-GlcNAc transferase (OGT) and *O*-GlcNAcase (OGA), which are enzymes for adding or removing UDP-GlcNAc on target proteins respectively. Protein level of OGT was decreased and protein level of OGA was increased under PP242 or Torin1 treatment (Fig. 2A and B). Both mTOR inhibitors affected protein stability of OGT and OGA in opposite directions. These data explained why global *O*-GlcNAc modification on proteins decreased under mTOR inhibition.

It is known that the inhibition of protein synthesis by CHX treatment of less than 12 h does not influence OGT proteins because of the long half-life of OGT [22]. Because mTOR inhibition also blocks protein synthesis, we treated Torin1 for less than 12 h to know whether destability of OGT occurs in short-term inhibition of mTOR or not. Torin1 treatment for more than 4 h increased OGT destability in HepG2 cells (Fig. 2C and D). This result implied that destability of OGT in mTOR inhibitor treatment for a time frame less than its half-life happens due to mainly autophagic induction.

3.3. Blockage of autophagosome formation by knockdown of ATG genes increases protein level of OGT

To know whether autophagy affects OGT degradation, we carried out transient knockdown of Atg5 and Atg7 to reduce formation of autophagosome. Conversion of LC3-I to LC3-II decreased about 23.5% in depletion of Atg5 (~72.3%) and Atg7 (~73.6%) (Fig. 3A and B). Protein level of OGT increased about 11% in Atg5 and Atg7 depletion (Fig. 3C and D). Our data suggested that reduction of autophagosome formation induces accumulation of OGT protein in the HepG2 cells.

3.4. Inhibition of autophagic degradation or proteasome degradation restores protein level of OGT under mTOR inhibition

To clarify the involvement of autophagy in regulation of the OGT protein, we designed an experiment based on the LC3 turnover assay. Vinblastine is a microtubule-disrupting agent which inhibits autophagosome–lysosome fusion, as this step requires microtubules [23]. Therefore, vinblastine treatment accumulates LC3-II in the autophagosome. We tested whether prohibition of autolysosome formation can restore the protein level of OGT after PP242 treatment. When formation of autolysosome was blocked by vinblastine treatment, protein level of OGT was not decreased in PP242 treated cells (Fig. 4C and D). These data suggest that autophagosomal degradation is involved in protein degradation of OGT when action of mTOR is inhibited.

Several groups checked the protein level of OGT after treatment of protein synthesis inhibitor cycloheximide (CHX). Because OGT has a long half-life (~12 h) [24], 3–6 h treatment of CHX did not show obvious changes in protein level of OGT [22,25]. When we treated CHX for 8 h as we treated PP242 or Torin1, protein level of OGT did not change (Fig. 4A and B). Therefore, we considered that protein destability of OGT was mainly due to the induction of autophagy, not by the inhibition of protein synthesis. However, based on previous findings, OGT has been considered to be degraded by proteasome. However, 4 h treatment of MG 132 to inhibit proteasomal degradation had no effect on endogenous OGT protein [22]. Therefore, we checked whether proteasome inhibition restored protein stability of OGT after a pre-inhibition of mTOR signaling. First, we checked OGT level after MG 132 treatment. When we treated proteasome inhibitor MG 132 in HepG2 cells, protein level of OGT was increased after 2 h. In addition, 4 h of MG132 treatment restored protein level of OGT in PP242 pre-treated cells. When comparing the amount of restored OGT between vinblastine and MG 132 treatment, more OGT proteins existed in the 8 h treatment of MG 132. Vinblastine treatment increased OGT stability, but it did not seem to perform in a time-dependent manner. On the other hand, MG 132 treatment increased protein stability of OGT in a time-dependent manner after 4 h of treatment. This implied that proteasomal degradation is the major way of degrading OGT, but that autophagy also affects the stability of OGT when mTOR signaling is blocked (Fig. 4C and D).

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