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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).

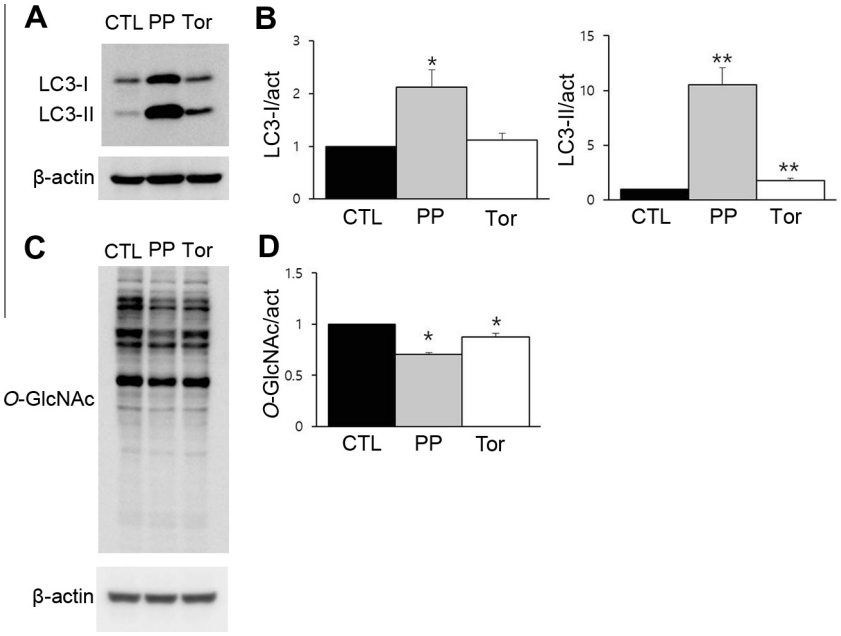

Fig. 1. *O*-GlcNAcylation decreases in autophagy inducible condition. (A) Representative Western blots showing LC3 in PP242 (PP) or Torin1 (Tor) treated HepG2 cells. HepG2 cells were treated with 10 μM PP242 or 0.1 μM Torin1 for 24 h. (B) Quantification of the amount of LC3-I and LC3-II in (A) ($^{*}p < 0.05$, $^{**}p < 0.01$, Student's *t*-test). (C) Western blots showing *O*-GlcNAcylated proteins in mTOR inhibitor treated HepG2 cells. (D) Quantification of the amount of *O*-GlcNAc bands in (C) ($^{*}p < 0.05$, Student's *t*-test). The data are presented as mean ± SE for four independent experiments.

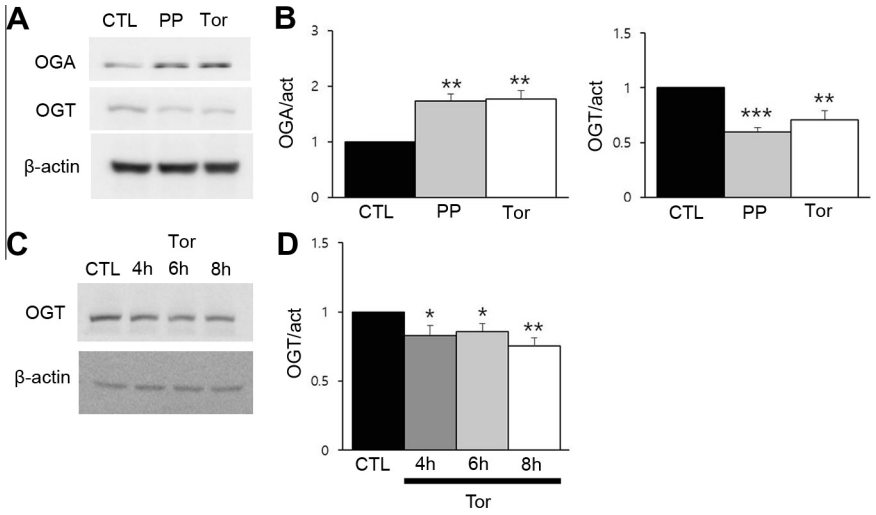

Fig. 2. Protein level of OGT and OGA changes in mTOR inhibition. (A) Lysates from HepG2 cells treated with mTOR inhibitors were analyzed by Western blotting using OGA, OGT and β-actin antibodies. (B) Quantification of OGA and OGT ($^{**}p < 0.01$, $^{***}p < 0.001$, Student's *t*-test). The data are presented as mean ± SE for four independent experiments. (C) HepG2 cells were treated 0.1 μM Torin1 for 4, 6, 8 h. After the time point, cells were harvested for immunoblotting. (D) Quantifications were performed by using Multi Gauge V3.0, mean ± S.E, $n = 4$.

4. Discussion

In this study, we showed that the induction of autophagy by mTOR inhibitors decreased OGT proteins and increased OGA proteins, leading to a total decrease in *O*-GlcNAcylation. Because OGT has a long half-life about 12 h, 6 h of protein synthesis inhibition such as CHX treatment did not change the protein level of OGT [22]. Therefore, we considered that destability of OGT protein in PP242 or Torin1 treatment of less than 12 h happens mainly due to autophagic induction and not by blockage of protein synthesis, even though mTOR inhibitors block phosphorylation of s6k and 4EBP [26]. Furthermore, knockdown of *ATG* mRNAs or inhibition of autolysosome formation showed accumulation of OGT, thus we considered autophagy to be a fundamental degradation pathway of OGT. However, previous finding showed that over-expression of OGT with ubiquitin enhanced ubiquitination on OGT, and this paper also concluded that OGT was degraded by proteasome even though 4 h of proteasome inhibition did not increase OGT proteins [22]. It has also been reported that inhibition of Hsp90 destabilized endogenous OGT in primary endothelial cells and increased proteasomal degradation of OGT. However, three kinds of proteasome inhibitor treatment did not increase OGT level in detergent (0.5% NP-40) soluble fraction. Among the three proteasome inhibitors, only MG 132 treatment accumulated OGT in the detergent-insoluble fraction [25]. Therefore, it is still ambiguous to conclude that OGT is degraded by proteasome. As both proteasomal degradation

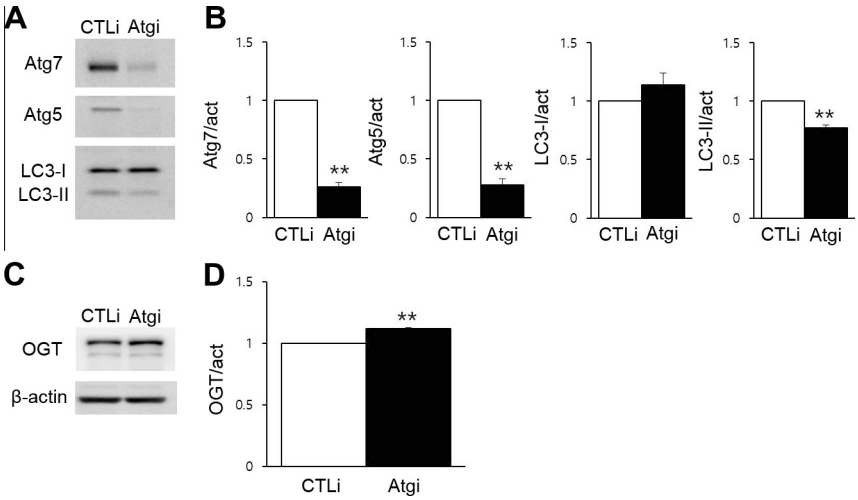

Fig. 3. OGT increases in HepG2 cells with Atg knockdown. (A) After transfection with Atg5 and 7 siRNA for 48 h, the protein level of Atg5, Atg7 and LC-3 were analyzed by Western blotting. (B) Quantifications of protein amount in (A). (C) Lysates from HepG2 cells transfected with Atg5 and Atg 7 siRNA were analyzed by Western blotting using OGT and β-actin antibodies. (D) Quantification of protein amount in (C).

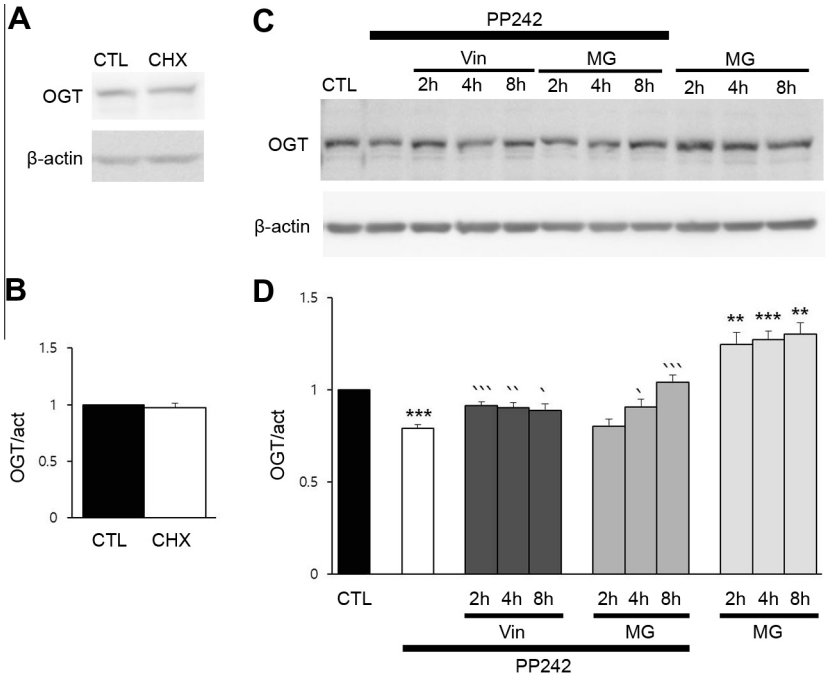

Fig. 4. Inhibition of autophagy or proteasome restores OGT level. (A) CHX alone has no effect on the expression of OGT. (B) Quantifications were performed by using Multi Gauge V3.0, mean ± S.E, $n = 4$. (C) Vinblastine and proteasome inhibitor MG 132 (MG) cause OGT accumulation in PP242 treated HepG2 cells. (D) Data are presented as mean ± S.E. from five independent experiments ($^{**}p < 0.01$, $^{***}p < 0.001$ comparing to control, $^{\cdot}p < 0.05$, $^{\cdot\cdot}p < 0.01$, $^{\cdot\cdot\cdot}p < 0.001$ comparing to pp242 treated sample, Student's t test).

and selective autophagy used ubiquitin as substrate-recognition signal, these two major degradation pathways share ubiquitin machinery. It is shown that proteasome inhibition induces autophagy and normal short-lived substrates for proteasome also accumulate under long-term inhibition of autophagy [27]. However, the physiological relevance of autophagy inhibition and proteasome inhibition is largely unknown. For these reasons, we checked whether proteasome inhibition restored protein level of OGT in PP242 pre-treated HepG2 cells as treatment with vinblastine did. More than 2 h of MG 132 treatment accumulated OGT in HepG2 cells without mTOR inhibition. Compared to this, treatment of vinblastine or chloroquine alone did not accumulate OGT (data not shown). When vinblastine was treated with mTOR inhibitors, OGT restoring event occurred. Like in vinblastine, OGT did not decrease in PP242 treated hepG2 cells when MG 132 was treated together. Interestingly, when we compare the restoring ratio between proteasome inhibition and blocking of autolysosome formation, proteasome inhibition was the more efficient way to block degradation of OGT. Vinblastine treatment increased OGT stability after 2 h of treatment but OGT was not increased time dependently. However, MG 132 treatment showed an effect after 4 h of treatment and OGT further increased after 8 h. For these reasons, we concluded that proteasomal degradation is the fundamental mechanism to degrade OGT, but autophagy is also involved in

OGT degradation when mTOR signaling is blocked. Here, we provided evidence to show a degradation mechanism of OGT by proteasome and autophagy together. Proteasome inhibition or blockage of autophagic degradation hinders the action of mTOR inhibitors on the OGT protein in HepG2 cells.

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