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The renoprotective role of autophagy activation in proximal tubular epithelial cells in diabetic nephropathy

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ABSTRACT

With intensive investigations recently, autophagy is hoped to be a potential therapeutic target to prevent or alleviate diabetic nephropathy (DN). Our previous study revealed that lipotoxicity participated in epithelial-to-mesenchymal transition (EMT) of proximal tubular epithelial cells (PTECs) under diabetic conditions. Based on evidences that autophagy and lipid metabolism are closely related, we investigated autophagy under diabetic conditions and how it contributed in the lipotoxicity and EMT. In high-glucose-cultured PTECs, we found that Beclin1 and LC3-II were elevated, while p62 was decreased. These results indicate that autophagy activity was elevated under diabetic conditions. Autophagy deficiency induced by autophagy inhibitors, chloroquine diphosphate (CQ) and 3-Methyladenine (3-MA), and by *Atg5* siRNA transfection exacerbated lipid accumulation and EMT. This supports that the elevated autophagy activity acts as a renoprotective response under diabetic conditions. Treatment of rapamycin, which is a mammalian target of rapamycin (mTOR) receptor-specific inhibitor and a known autophagy activator, attenuated high-glucose-induced lipid accumulation and EMT. The *Atg5* silence counteracted the protective effect of rapamycin. The present study deepens our understanding of the role of autophagy in DN, suggesting a complex interplay of autophagy, metabolic pathways, lipotoxicity and EMT.

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1. Introduction

Diabetic nephropathy (DN) accounts for the most common cause of end-stage kidney disease (ESRD) worldwide. It is also one of the most devastating complications of diabetes, significantly increases the morbidity and mortality in patients with diabetes. According to the International Diabetes Federation, the number of people with diabetes worldwide will increase from 382 million in 2013 to 592 million by 2035 (Shi & Hu, 2014). With the rapidly increasing prevalence of diabetes mellitus being a major global health issue, the importance of therapeutic interventions directed at preventing the development and progression of diabetic nephropathy is underscored.

Among many mechanisms underlying DN pathogenesis, autophagy attracts intensive investigations recently. Autophagy was originally thought to be an intracellular catabolic process by which cytoplasmic components such as proteins, glycogens, lipids, and nucleotides and organelles such as mitochondrial, peroxisomes, and endoplasmic reticulum (ER) are degraded under stress by the lysosomes to maintain intracellular homeostasis (Levine & Ranganathan, 2010). The autophagic pathway is composed of several steps, including initiation, vesicle nucleation, vesicle elongation, fusion and degradation. To date, more than 30 of autophagy-related genes (Atg) have been identified, which are required for autophagy and its related processes. Autophagy initiates with the formation of autophagophores, and the Beclin1-interacting complex that consists of Beclin1 and B-cell lymphoma 2 (BCL-2) family proteins is required for the initiation process. Autohagophores elongate and expand, and the elongation requires two ubiquitin-like conjugation systems: the ATG5-ATG12 conjugation system and the microtubule associated protein light chain 3 (LC3/ATG8) conjugation system. The conversion of a cytosolic truncated form of LC3 (LC3-I) to the phosphatidylethanolamine-conjugated form (LC3-II) indicates autophagosome formation. p62 serves as link between LC3 and ubiquitinated

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substrates, which becomes incorporated into the completed autophagosome and is degraded. Thus, Beclin1, LC3-II, p62 has been frequently used as a marker to assess autophagy (Klionsky et al., 2012). Accumulating evidence suggests that regulation of autophagy system may become a new therapeutic option for treatment of DN (Kume, Yamahara, Yasuda, Maegawa, & Koya, 2014).

The hyperfiltration and microalbuminuria characterize the clinical onset of DN, which are followed by sustained proteinuria with ensuing declining renal function. With the progression of the disease, tubulointerstitial fibrosis has been established as major pathological feature of DN (Remuzzi, Ruggenenti, & Benigni, 1997). And it is fairly established that compared to the glomerular damage, the injury to the tubulointerstitial compartment correlates better with the reduced renal function (Nath, 1998). These underline the investigations of tubulointerstitial fibrosis pathophysiology in DN. Under basal conditions, autophagy activity is very low in proximal tubular epithelial cells (PTECs) (Kume et al., 2014), while higher rates of autophagy are essential for cells under stresses. *In vivo* studies by deleting the *Atg5* or *Atg7* genes showed that proximal tubular cell-specific autophagy deficiency led to progressive renal damage, suggesting that sufficient basal or induction of autophagy is renoprotective during acute renal injury (Jiang, Liu, Luo, & Dong, 2010; Periyasamy-Thandavan et al., 2008) or aging (Liu et al., 2012). However, the role of autophagy in tubulointerstitial fibrosis in the pathogenesis of DN remains unclear. It is reasonable to hypothesize that autophagy deregulation in PTECs participates in the development of tubulointerstitial fibrosis under diabetic conditions with further investigation warranted.

Although there is debate recently, epithelial-to-mesenchymal transition (EMT) is still considered to be central to the process of tubulointerstitial fibrosis by many researchers (Hills & Squires, 2010), and the mechanisms underlining EMT has been studied intensively. Our previous studies revealed that lipotoxicity participates in EMT (Xu et al., 2014), suggesting a link between disturbances of lipid metabolism and cell function maintenance in PTECs. As a mechanism that is closely regulated by nutrient sensing, autophagy is related to lipid metabolism in many ways. It has been shown that in *c. elegans*, autophagy genes are required for normal lipid levels (Lapierre et al., 2013). Studies in liver demonstrated that autophagy mediated the breakdown of lipid droplets and autophagy inhibition led to the development of a fatty liver (Singh et al., 2009). A recent study showed that acute liver injury would activate hepatic stellate cells to undergo autophagy and promote loss of lipids (Hernandez-Gea et al., 2012). In PTECs, the role of autophagy in lipotoxicity as well as in EMT needs to be further studied.

In the development of diabetes mellitus and its complications, hyperglycemia plays a critical role by triggering formation of advanced glycosylation end products (AGE), activation of protein kinase C (PKC), and so on. Meanwhile, in the treatment of diabetes, glycaemia control remains challenging despite of enormous efforts. The present study aims at evaluating the role of autophagy in diabetic nephropathy with focus on hyperglycemia. Based on evidences that autophagy and lipid metabolism are closely related, the aim of the present study was to investigate autophagy under diabetic conditions and its role in lipotoxicity and EMT. Previous studies showed that cellular autophagy was inhibited in proximal and distal tubular cells in streptozotoxin (STZ)-model (Han, Zhou, & Pfeifer, 1997) or in patients (Yamahara et al., 2013). Based on these results, we first hypothesized that autophagy is blunted in PTECs under diabetic conditions, and the deficiency of autophagy accounts for the lipotoxicity and EMT. However, inspection of autophagy-related proteins in high-glucose-cultured PTECs as the *in vitro* model of hyperglycemia showed elevated autophagy activity. This prompts our alternative hypothesis that elevated autophagy activity acts as a renoprotective response under diabetic conditions and autophagy deficiency in PTECs will exacerbate the high-glucose-induced lipotoxicity and EMT.

2. Materials and methods

2.1. Cell culture and treatment

HK-2 cells, a proximal tubule cell line immortalized by transduction with human papillomavirus type 16 E6/E7 (Cell Culture Center of School of Basic Medicine Peking Union Medical College, Institute of Basic Medical Sciences Chinese Academy of Medical Sciences), were cultured in Dulbecco's MEM (Gibco, USA) containing 10% FBS as described previously (Xu et al., 2014). All cell lines were maintained at 37 °C in a humidified atmosphere of 5% CO_2. Two groups of culture conditions were investigated, which were normal glucose medium (5.5 mmol/L glucose) and high-glucose medium (30 mmol/L glucose). Pictures were obtained using Leica 090–135.001 microscope (Leica, Wetzlar, Germany) and graphed using Nikon coolpix 4500 (Nikon, Japan) at various time points as indicated.

2.2. Antibodies and reagents

Chloroquine diphosphate (CQ) and 3-Methyladenine (3-MA) were purchased from Sigma. LC3 and E-cadherin (E-cad) antibody was obtained from Abcam. p62 antibody was purchased from Cell Signaling Technology. Anti-Atg5-Atg12 antibody was purchased from Novus Biologicals. Beclin1 and Vimentin antibody were obtained from Santa Cruz Biotechnology. β-actin and horseradish peroxidase-conjugated secondary antibodies were purchased from ZSGB-BIO. Oil-red O was from Beijing Solarbio Science & Technology.

2.3. Knockdown of Atg5 by small interfering RNA (siRNA) transfection

siRNA oligonucleotides targeting human *Atg5* were designed by Invitrogen (CAAAGAAGUUUGUCCUUCUGCUAUU and AAUAGCAGAAGGACAAACUUCUUUG). The siRNA was transfected into HK-2 cells using Lipofectamine® 3000 transfection reagent (Invitrogen) according to the manufacture's protocol. The knockdown efficiency of the *Atg5* was confirmed by real-time quantitative PCR (RT-qPCR) using sequence-specific primers for *Atg5* (forward 5'-TTT GCA TCA CCT CTG CTT TC-3' and reverse 5'-TAG GCC AAA GGT TTC AGC TT-3'), which were designed by TaKaRa.

2.4. Oil-Red O staining

Oil-Red O was usually used to stain the neutral fat such as triglyceride (Jun et al., 2009). Briefly, at the end of incubation, cultured cells were washed three times with PBS and fixed for 30 min with 4% paraformaldehyde. Then the cells were stained for 20 min in 1% Oil red O (in 60% isopropanol). In order to remove the background staining, the cells were washed with 60% isopropanol for 5 s. Finally, the sections were counterstained with hematoxylin for 10 s and then rinsed in tap water for 60 s. The glycerol jelly-mounting medium was used to fix the sections, and the stained sections were imaged with Leica DM4000B microscope (Leica, Wetzlar, Germany).

2.5. Real-time quantitative PCR (RT-qPCR)

Total RNA was extracted using RNAiso Plus, and synthesized cDNA using PrimeScript RT regent Kit with gDNA Eraser. Real-time PCR was performed using LightCycler 480 (Roche Diagnostics) with SYBR® Premix Ex TaqTM(2×). All the RT-qPCR reagents were obtained from TaKaRa Biotechnology (Dalian).

2.6. Western blot analysis

The procedure of western blot analysis was carried out as described previously (Delfin et al., 2011). At the end of the incubation, HK-2 cells were washed three times with PBS. Then attached cells

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