SPANDIDOS PUBLICATIONS

MOLECULAR MEDICINE REPORTS 16: 7080-7085, 2017

Effect of $1,25(OH)_2D_3$ on high glucose-induced autophagy inhibition in peritoneum

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Received September 16, 2016; Accepted July 14, 2017

DOI: 10.3892/mmr.2017.7408

Abstract. High glucose (HG) may damage the structure and function of the peritoneal membrane, and is considered to be one of the most important factors that leads to peritoneal fibrosis and ultrafiltration failure. Recently, $1,25(OH)_2D_3$, the active form of vitamin D, was demonstrated to protect against epithelial-mesenchymal transition and fibrosis in peritoneal mesothelium and other organs. Accumulating evidence has suggested that autophagy serves a protective role in certain diseases by regulating cell survival. The present study examined whether $1,25(OH)_2D_3$ has an effect on autophagy in peritoneal mesothelial cells. The protein level of Beclin, anti-ubiquitin-binding protein p62 (p62), microtubule-associated proteins 1A/1B light chain 3B (LC3-II), mechanistic target of rapamycin (mTOR) and phosphorylated mTOR were evaluated by western blot analysis. Autophagosomes were detected under transmission electron microscopy. It was revealed that exposure to HG inhibited autophagy in peritoneal mesothelial cells. However, $1,25(OH)_2D_3$ alleviated autophagy inhibition induced by HG in human peritoneal mesothelial cells, which activated expression of autophagy-associated genes encoding Beclin-1 and LC3-II downregulated the expression of p62 via mTOR signaling pathway. In a mouse model of HG-treated peritoneal mesothelium, autophagy inhibition was observed in peritoneum, $1,25(OH)_2D_3$ attenuated HG-induced autophagy inhibition in peritoneal mesothelium via the mTOR signaling pathway. These findings suggested that $1,25(OH)_2D_3$ may be a potential therapy for peritoneal injury.

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Key words: human peritoneal mesothelial cells, peritoneal mesothelium, $1,25(OH)_2D_3$, high glucose, autophagy, mechanistic target of rapamycin

Introduction

Peritoneal dialysis (PD) is a primary treatment of end-stage renal disease (ESRD). High glucose (HG) damages the structure and function of the peritoneal membrane, which leads to peritoneal fibrosis and ultrafiltration failure via inflammation, apoptosis, oxidative stress and epithelial-mesenchymal transition (EMT) (1-3). Recent studies have also revealed that HG regulates autophagy in certain diseases (4-6).

Autophagy, which functions to maintain cell homeostasis under various stress conditions, primarily degrades and recycles misfolded proteins and damaged organelles (7). Previous studies have demonstrated that HG modulates autophagy in multiple disease states (8-10). However, the direct effects of autophagy on HG-induced peritoneal mesothelial cells remain unknown. Macroautophagy, which is generally referred to as autophagy, is a ubiquitous, genetically programmed process. Central to this process is the formation of autophagosomes, which are double- or multiple-membrane cytoplasmic vesicles that are responsible for delivering cytoplasmic material to lysosomes (11). Several autophagy genes are involved in the process of autophagy, including microtubule-associated protein 1A/1B-light chain 3 (LC3), Beclin-1, ubiquitin-binding protein p62 (p62) and other autophagy-associated proteins (12,13). Autophagy serves a protective role in certain diseases such as renal ischemia/reperfusion, cancer, EMT and inflammation (14-16). The effects of autophagy on EMT and inflammation have also been proposed to be involved in cancer (17,18).

Vitamin D has been demonstrated to serve an important role in the regulation of cell differentiation, cell proliferation, immunomodulation, inflammation and autophagy (19-22). $1,25(OH)_2D_3$, the active form of vitamin D, regulates bone, calcium and phosphate metabolism through the vitamin D receptor (VDR). The VDR forms a heterodimer with the retinoid X receptor and regulates gene expression in the nucleus. Research has demonstrated that $1,25(OH)_2D_3$ has protective effects in a number of diseases (23-25). It was previously revealed that $1,25(OH)_2D_3$ attenuates apoptosis, oxidative stress and inflammation in HG treated-peritoneal mesothelial cells (1). Therefore, the present study hypothesized that $1,25(OH)_2D_3$ may regulate autophagy and protect mesothelial cells during PD.

Earlier research performed by the authors of the present study revealed that HG induced peritoneal injury, apoptosis,

oxidative stress, inflammation and EMT, and $1,25(OH)_2D_3$ attenuated HG-induced peritoneal injury (1,2). In the present study, the biological function of autophagy in HG-stimulated human peritoneal mesothelial cells (HPMCs) and peritoneal mesothelium was investigated. It was also determined whether $1,25(OH)_2D_3$ attenuates HG-induced peritoneal injury via autophagy and the molecular mechanism of this effect. The present study found that $1,25(OH)_2D_3$ treatment may provide an improved solution to the peritoneal fibrosis and ultrafiltration failure.

Materials and methods

Reagents. Fetal bovine serum (FBS), penicillin, streptomycin and RPMI-1640 were all obtained from Gibco; Thermo Fisher Scientific, Inc. (Waltham, MA, USA). $1,25(OH)_2D_3$ was purchased from Sigma-Aldrich; Merck KGaA (Darmstadt, Germany). Anti-Beclin-1 (3738), anti-LC3 (4108), anti-p62 (5114), anti-mTOR (2972), anti-p-mTOR (2971), anti-β-actin (4967) and HRP conjugate anti-rabbit (5127) IgG antibodies were all purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA).

Culture of HPMCs. HPMCs were kindly provided by Professor Na Di and Professor Xu Huimian (The First Affiliated Hospital of China Medical University, Shenyang, China) and were cultured in RPMI-1640 medium supplemented with 10% FBS, 100 UI/ml penicillin and 100 μg/ml streptomycin. HPMCs were cultured as previously described (1). HPMCs were dissociated for 1 min with trypsin-EDTA and the subcultivation ratio was 1:3 or 1:4. Cells at passage 5-10 were used in all experiments. A concentration of 10^{-7} mol/l $1,25(OH)_2D_3$ was used in the present study, according to previous published research (1). HPMCs were classified into the following four groups: Control group; 10^{-7} mol/l $1,25(OH)_2D_3$ treatment only group; 126 mM HG treatment only group; 10^{-7} mol/l $1,25(OH)_2D_3$ pretreatment followed by 126 mM HG incubation.

Animals and experimental treatments. All animal procedures were approved by the Experimental Animals Ethics Committee of China Medical University (Liaoning, China). Kunming male mice (28-30 g, aged 8-12 weeks) were purchased from the Department of Laboratory Animals, China Medical University (Liaoning, China), and housed in a room with controlled temperature (22˚C) on a 12-h light/dark cycle. Food and water were given *ad libitum* throughout the experiment. Following one week to adapt to their new environment, 35 Kunming male mice were randomly assigned into seven groups (n=5/group): Control group (no dialysate or saline was infused); saline group (mice received 50 ml/kg saline by intraperitoneal (IP) injection every day for 4 weeks); low dose vitamin D group [the mice were subjected to IP injections of 1 μg/kg $1,25(OH)_2D_3$ once weekly (every Monday) for 4 weeks]; high dose vitamin D group [the mice were subjected to IP injections of 5 μg/kg $1,25(OH)_2D_3$ once weekly (every Monday) for 4 weeks]; the peritoneal dialysis (PD) group [injected IP with conventional 4.25% peritoneal dialysis fluid (PDF; Baxter Healthcare Ltd., Guangzhou, China) 50 ml/kg daily for 4 weeks]; the PD + low dose vitamin D group [mice were injected IP with conventional 4.25% PDF 50 ml/kg daily, then were subjected to IP injections of 1 μg/kg $1,25(OH)_2D_3$ once weekly (every Monday) for 4 weeks]; the PD + high dose vitamin D group [mice were injected IP with conventional 4.25% PDF 50 ml/kg daily, then were subjected to IP injections of 5 μg/kg $1,25(OH)_2D_3$ once weekly (every Monday) for 4 weeks]. At the end of the experimental period (4 weeks), the mice were starved for 12 to 13 h and then sacrificed by cervical dislocation; the visceral peritoneum was used for western blotting.

Transmission electron microscopy. HPMCs ($2x10^7$) were fixed at room temperature with 2.5% glutaraldehyde for 2 h, then washed with 0.1 M PBS. The samples were then postfixed in 1% osmium tetroxide for 2 h at room temperature, dehydrated in a series of graded ethanol, and embedded in Epon 812 (Structure Probe, Inc., West Chester, PA, USA). Ultra-thin sections were cut (70 nm), stained with 2% uranyl acetate and lead citrate for 20 min at room temperature, and examined using transmission electron microscopy.

Western blotting. Western blot analysis was carried out as previously described (1). All experiments were repeated ≥3 times. Representative protein bands and densitometric data are displayed in the figures. Total proteins were extracted using lysis buffer (Pierce; Thermo Fisher Scientific, Inc., Waltham, MA, USA), quantified with the BCA Protein assay kit (Pierce; Thermo Fisher Scientific, Inc.) and separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Protein (50 μg) was transferred onto polyvinylidene fluoride (PVDF) membranes (EMD Millipore, Billerica, MA, USA). BSA solution (10%; Sigma-Aldrich; Merck KGaA) was used to reduce non-specific antibody binding at 37˚C for 1 h. The blots were incubated overnight at 4˚C with antibodies against anti-Beclin-1 (3738, 1:1,000), anti-LC3 (4108, 1:1,000), anti-p62 (5114, 1:1,000), anti-mTOR (2972, 1:1,000), anti-p-mTOR (2971, 1:1,000) and anti-β-actin (4967, 1:1,000) (all from Cell Signaling Technology, Inc.). Subsequently, samples were incubated with HRP conjugate anti-rabbit immunoglobulin G antibodies (5127, 1:1,000; Cell Signaling Technology, Inc.) at 37˚C for 2 h. Target proteins on the PVDF membrane were visualized using ECL Western Blotting Substrate (Pierce; Thermo Fisher Scientific, Inc.) and captured using a DNR bioimaging system (DNR Bio-Imaging Systems, Ltd., Neve Yamin, Israel).

Statistical analysis. Statistical analysis was performed using SPSS software version 18 (SPSS, Inc., Chicago, IL, USA). Data are presented as the mean ± standard error. Multiple group comparisons were made using a one-way analysis of variance followed by Tukey's multiple comparison test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Influence of $1,25(OH)_2D_3$ on HG-induced autophagy inhibition in HPMCs. To assess the potential underlying mechanism by which $1,25(OH)_2D_3$ regulates HG-induced peritoneal injury in HPMCs, cell autophagy was determined by transmission electron microscopy. Compared with the HG group, the number of autophagosomes was enhanced in

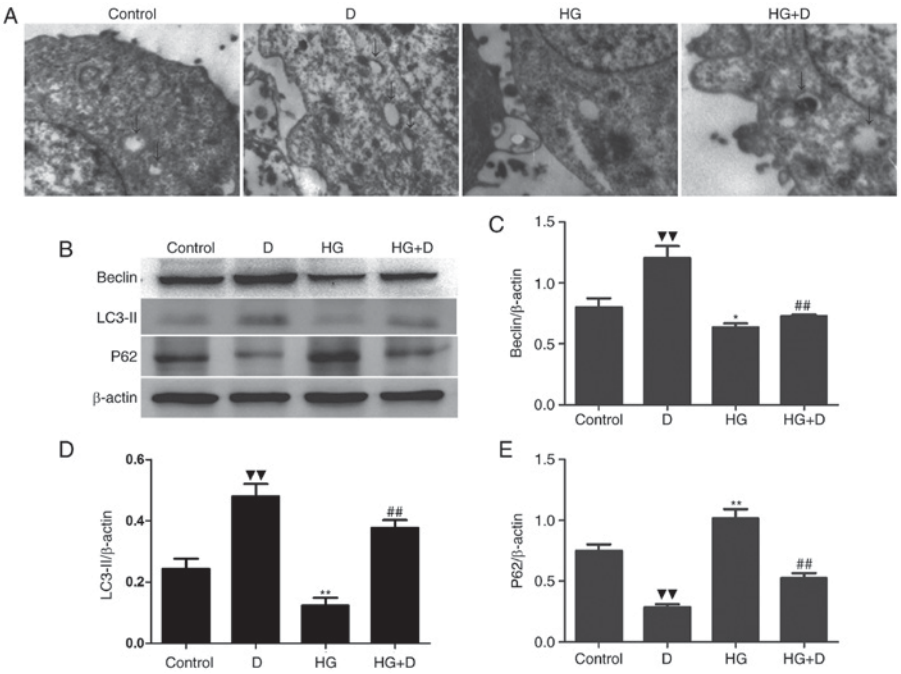

Figure 1. 1,25(OH)$_2$D$_3$ attenuated HG-induced autophagy inhibition. (A) HPMCs were exposed to 126 mM HG in the presence or absence of 10^{-7} mol/l 1,25(OH)$_2$D$_3$ pretreatment. Representative transmission electron microscopy (x4,000) images are displayed. Arrows, autophagosomes. (B) Western blotting was performed with the antibodies of Beclin-1, LC3-II and p62. (C-E) Protein expression levels were assessed using densitometry and are expressed as relative intensities. Each value represents the mean ± standard error (n=3). ▼▼P<0.01 vs. control; *P<0.05 vs. control; **P<0.01 vs. control; ##P<0.01 vs. HG group. HG, high glucose; HPMCs, human peritoneal mesothelial cells; LC3-II, microtubule-associated proteins 1A/1B light chain 3B; p62, ubiquitin-binding protein; D, 1,25(OH)$_2$D$_3$; HG + D, high glucose and 1,25(OH)$_2$D$_3$.

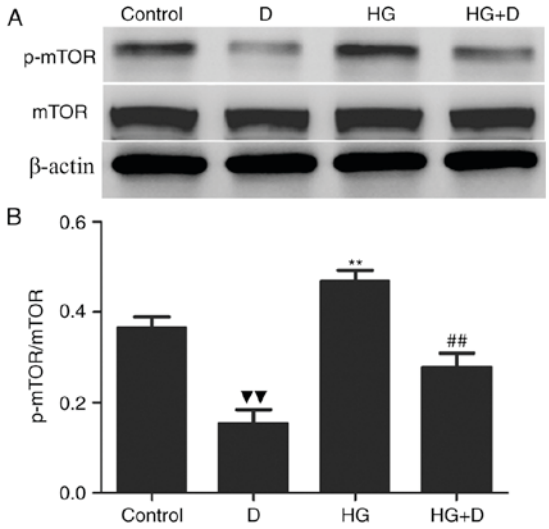

Figure 2. Effects of 1,25(OH)$_2$D$_3$ on the mTOR signaling pathway in HG-treated HPMCs. HPMCs were exposed to 126 mM HG in the presence or absence of 10^{-7} mol/l 1,25(OH)$_2$D$_3$ pretreatment. (A) Western blotting was performed with mTOR and p-mTOR antibodies. (B) Protein expression levels were assessed using densitometry and were expressed as relative intensities. Each value represents the mean ± standard error (n=3). ▼▼P<0.01 vs. control; **P<0.01 vs. control; ##P<0.01 vs. HG group. mTOR, mechanistic target of rapamycin; HG, high glucose; HPMCs, human peritoneal mesothelial cells; p-mTOR, phosphorylated mechanistic target of rapamycin, D, 1,25(OH)$_2$D$_3$; HG + D, high glucose and 1,25(OH)$_2$D$_3$.

the HG + 1,25(OH)$_2$D$_3$ group (Fig. 1A). To further confirm the influence of 1,25(OH)$_2$D$_3$ on autophagy, western blotting was performed to detect Beclin-1, LC3-II and p62 expression. Compared to the control group, HG downregulated the expression levels of Beclin-1 and LC3-II, and upregulated the expression levels of p62 (Fig. 1B-E). However, it was revealed that 1,25(OH)$_2$D$_3$ itself induced autophagy, and HG-induced autophagy inhibition may be attenuated by co-treatment with 10^{-7} mol/l 1,25(OH)$_2$D$_3$.

Effects of 1,25(OH)$_2$D$_3$ on the mTOR pathway in HG-treated HPMCs. The mTOR signaling pathway has been reported to be involved in autophagy and EMT (17); however, whether 1,25(OH)$_2$D$_3$ regulates autophagy via the mTOR pathway in HPMCs remains unknown. The present study therefore examined the effect of 1,25(OH)$_2$D$_3$ on the mTOR signaling pathway in HPMCs.

mTOR activation was measured by western blotting with a p-mTOR antibody. As demonstrated in Fig. 2A and B, when cells were exposed to HG alone, mTOR phosphorylation was increased compared to the control group, whereas p-mTOR expression was significantly decreased when cells were co-treated with 1,25(OH)$_2$D$_3$. These results suggested that 1,25(OH)$_2$D$_3$ regulates autophagy via the mTOR pathway in HPMCs.

Influence of 1,25(OH)$_2$D$_3$ on HG-induced autophagy inhibition in peritoneal mesothelium. A previously published report by the authors of the present study revealed that 1,25(OH)$_2$D$_3$ protects HPMCs from inflammation and apoptosis (1). The present study used a mouse model to further assess the underlying mechanism by which 1,25(OH)$_2$D$_3$ influences HG induced peritoneal injury. Compared to the control group, HG PDF significantly downregulated the expression levels of Beclin-1 and LC3-II, and upregulated the expression levels of

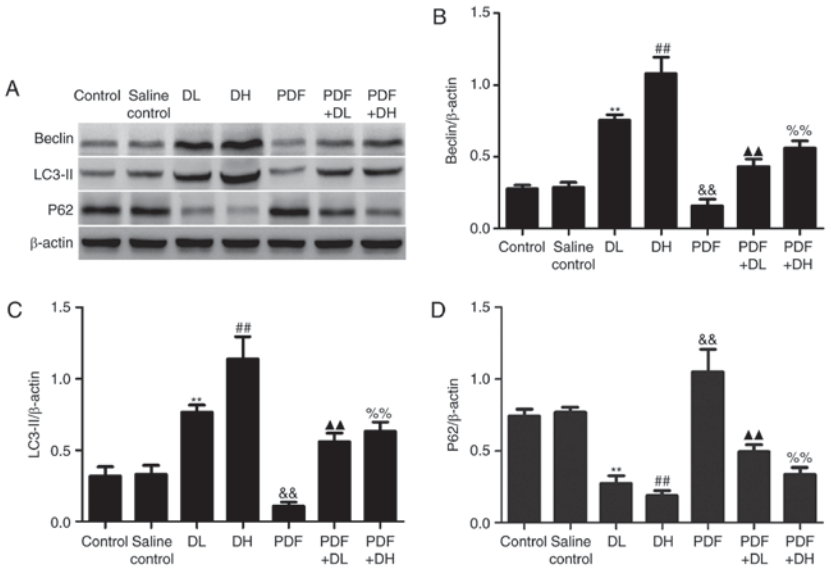

Figure 3. Effects of 1,25(OH)$_2$D$_3$ on autophagy in HG PDF-treated peritoneal mesothelium. DL, vitamin D low-dose group [1 μg/kg 1,25(OH)$_2$D$_3$]; DH, vitamin D high-dose group [5 μg/kg 1,25(OH)$_2$D$_3$]; PDF + DL, PDF + low dose vitamin D [1 μg/kg 1,25(OH)$_2$D$_3$] group; PDF + DH, PDF + high-dose vitamin D [5 μg/kg 1,25(OH)$_2$D$_3$] group. (A) Western blotting was performed using the antibodies of Beclin-1, LC3-II and p62. (B-D) Relative expression levels of Beclin, LC3-II and p62 were calculated and normalized to the loading control by densitometric analysis. Each value represents the mean ± standard error (n=5). **P<0.01 vs. control; $^{\#\#}$P<0.01 vs. control; $^{\&\&}$P<0.01 vs. control; $^{\blacktriangle\blacktriangle}$P<0.01 vs. PDF; $^{\%\%}$P<0.01 vs. PDF. HG, high glucose; PDF, peritoneal dialysis fluid; LC3-II, microtubule-associated proteins 1A/1B light chain 3B; p62, ubiquitin-binding protein.

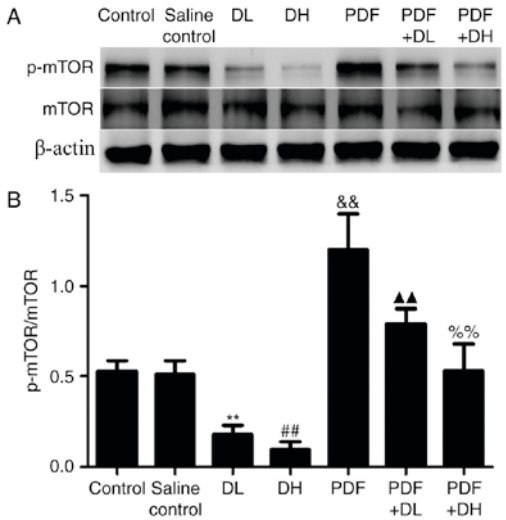

Figure 4. Effects of 1,25(OH)$_2$D$_3$ on the mTOR signaling pathway in HG PDF treated peritoneal mesothelium. DL, vitamin D low dose group [1 μg/kg 1,25(OH)$_2$D$_3$]; DH, vitamin D high-dose group [5 μg/kg 1,25(OH)$_2$D$_3$]; PDF + DL, PDF + low-dose vitamin D [1 μg/kg 1,25(OH)$_2$D$_3$] group; PDF + DH, PDF + high dose vitamin D [5 μg/kg 1,25(OH)$_2$D$_3$] group. (A) Western blotting was performed with mTOR and p-mTOR antibodies. (B) p-mTOR protein levels were assessed using densitometry and were expressed as relative intensities. Each value represents the mean ± standard error (n=5). **P<0.01 vs. control; $^{\#\#}$P<0.01 vs. control; $^{\&\&}$P<0.01 vs. control; $^{\blacktriangle\blacktriangle}$P<0.01 vs. PDF; $^{\%\%}$P<0.01 vs. PDF. mTOR, mechanistic target of rapamycin; HG, high glucose; PDF, peritoneal dialysis fluid; p-mTOR, phosphorylated mechanistic target of rapamycin.

p62 (Fig. 3A-D). However, 1,25(OH)$_2$D$_3$ induced autophagy, and HG-induced autophagy inhibition may be attenuated by co-treatment with 1 and 5 μg/kg 1,25(OH)$_2$D$_3$.

Effects of 1,25(OH)$_2$D$_3$ on the mTOR pathway in HG-treated peritoneal mesothelium. A mouse model was used to further assess the association of the mTOR signaling pathway and autophagy. When mice were exposed to HG PDF, mTOR phosphorylation was significantly increased compared with the control group; however, p-mTOR was decreased when co-treated with 1,25(OH)$_2$D$_3$. These results suggested that 1,25(OH)$_2$D$_3$ regulates autophagy via the mTOR signaling pathway in peritoneal mesothelium.

Discussion

PD is generally accepted as an important renal replacement therapy for the treatment of ESRD. However, long-term PD may lead to peritoneal membrane failure. A previous study published by the authors of the present study indicated that HG induced peritoneal inflammation, apoptosis, oxidative stress and EMT, which were involved in the development of peritoneal dysfunction (1,2). Studies have demonstrated that HG may also affect autophagy in certain diseases (8-10).

Autophagy is emerging as a key factor in various physiological and pathological events. A previous study showed that autophagy serves a protective role in diseases such as renal ischemia/reperfusion, cancer, fibrosis and inflammation (14-16,26). It is understood that high glucose levels may affect autophagy in numerous cell types (9,27,28).

Accumulating evidence suggests that 1,25(OH)$_2$D$_3$ affects organ fibrosis, exhibits antioxidant properties and induces autophagic capabilities (17,29,30). Research has also demonstrated that 1,25(OH)$_2$D$_3$ may induce autophagy in human monocytes and macrophages (17). Previous research has focused on the autophagic induction of vitamin D$_3$ primarily in cancer cells (31-33), suggesting the potential use of vitamin D$_3$ as an anticancer drug. However, the pathophysiological role of autophagy in peritoneal injury and whether 1,25(OH)$_2$D$_3$ regulates autophagy in peritoneum remains unknown.

In the present study, *in vitro* and *in vivo* experiments revealed that high glucose induced autophagic inhibition, due to increased expression of Beclin and LC3-II, and decreased expression of p62. However, $1,25(OH)_2D_3$ induced autophagy and attenuated HG-induced autophagy inhibition in HPMCs and peritoneal mesothelium. Earlier research by the authors of the present study demonstrated that HG induced peritoneal injury includes apoptosis, oxidative stress, inflammation and EMT, and $1,25(OH)_2D_3$ attenuated HG induced peritoneal injury (1,2). In the present study, HG decreased autophagy and $1,25(OH)_2D_3$ induced autophagy. Therefore, HG may induce peritoneal injury by decreasing autophagy and $1,25(OH)_2D_3$ exhibits a protective effect by increasing autophagy.

The mTOR signaling pathway is a classic pathway in autophagy that has been extensively studied (18,34). A previous study demonstrated that $1,25(OH)_2D_3$ protected β cells in the pancreas against high glucose-induced apoptosis via the suppression of the mTOR signaling pathway (35). Jang *et al* (36) found that vitamin D protects against rotenone-induced neurotoxicity by enhancing autophagy via the mTOR pathway. In the present study, the association between the mTOR signaling pathway and peritoneal autophagy after HG treatment was investigated. Results demonstrated that treatment with HG activated the mTOR pathway, induced peritoneal autophagy inhibition, and these alterations may be attenuated by $1,25(OH)_2D_3$ pretreatment.

In conclusion, it was found that HG induced autophagy inhibition in peritoneum, $1,25(OH)_2D_3$ induced autophagy and attenuated the HG-induced autophagy inhibition in peritoneum, possibly via the mTOR signaling pathway. Further investigation in this area will generate novel insights into the critical role of $1,25(OH)_2D_3$, and will provide an experimental basis for its clinical use in the treatment of PD. One limitation of the present study is that we only measured mTOR and p-mTOR, which is only one protein involved in the mTOR signaling pathway. Measuring the expression levels of other proteins in this pathway will be considered for future studies.

Acknowledgements

The present study was supported by the National Natural Science Foundation of China (grant nos. 81300636 and 81370865).

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