Ghrelin inhibits autophagy mediated by AKT/mTOR pathway to ameliorate retinal angiogenesis induced by high glucose stress

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Abstract

● AIM: To observe the effect of ghrelin, a growth hormone-releasing peptide, on retinal angiogenesis in vitro under high glucose (HG) stress and to explore the possible mechanism of autophagy.

● METHODS: Human retinal microvascular endothelial cells (HRMECs) were treated with high concentration of glucose alone or in combination with ghrelin. The cell migration, tube formation and the expression of the autophagy-related proteins LC3-II/I, Beclin-1, p62, phosphorylated AKT (p-AKT)/AKT and phosphorylated mammalian target of rapamycin (p-mTOR)/mTOR were detected. Then, to clarify the correlation between ghrelin effect and autophagy, AKT inhibitor VIII was adopted to treat HRMECs, and cell migration, tube formation as well as the protein expressions of LC3-II/I, Beclin-1 and p62 were observed.

● RESULTS: Under HG stress, ghrelin inhibited migration and tube formation of HRMECs. Ghrelin inhibited the increases in the protein levels of LC3-II/I, Beclin-1 and the decreases in the protein levels of p62, p-AKT/AKT and p-mTOR/mTOR induced by HG stress. Moreover, under the action of AKT/mTOR pathway inhibitors, the effects of ghrelin on migration and tube formation were both reduced. In addition, the expression of LC3-II/I and Beclin-1 were significantly up-regulated and the expression of p62 was down-regulated.

● CONCLUSION: Retinal angiogenesis under in vitro HG stress can be inhibited by ghrelin through activating AKT/mTOR pathway to inhibit autophagy.

● KEYWORDS: ghrelin; retinal endothelial cell; angiogenesis; autophagy; stress

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INTRODUCTION

Diabetic retinopathy (DR) stands as a prevalent complication arising from diabetes mellitus (DM), reigning as a prominent catalyst of irreversible visual impairment within the laboring-age demographic[1]. In accordance with the pronouncement disseminated by the International Diabetes Federation (IDF), projections foretell a burgeoning surge, with an estimated 700 million individuals afflicted by DM worldwide by the year 2045[2]. This augury unmistakably underscores the persistently towering global burden of DR, both in the contemporary epoch and on the horizon. Furthermore, it warrants noting that the severity of DR lesions amplifies commensurately with the protracted duration of DM affliction and the ascent of glycemic levels. The proliferation of nascent vascular networks, occurrences of vitreous hemorrhage, and the emergence of tractional retinal detachments represent harbingers of potential vision impairment. The strides made in the administration of intravitreal injections comprising anti-vascular endothelial growth factor (VEGF) pharmaceuticals have yielded supplementary avenues for the amelioration of diabetic macular edema (DME) fraught with vision-threatening implications,
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along with the management of retinal neovascularization inherent to the realm of proliferative diabetic retinopathy (PDR). These therapeutic advancements have been pivotal in engendering enhanced visual outcomes[3-4]. Nevertheless, formidable obstacles persist, notably the weighty financial burden imposed by the therapeutic regimen and the recalcitrant nature of the malady[5]. A considerable proportion of patients have unsatisfactory response to anti-VEGF agents; 40% of eyes with DME do not respond to anti-VEGF therapy or respond suboptimally[6]. Consequently, a pressing imperative exists to illuminate hitherto uncharted pathophysiological mechanisms underlying DR and to embark upon the exploration of innovative agents targeting alternative molecular pathways. Such endeavors hold the potential to herald a revolutionary transformation within the prevailing treatment paradigm for DR[7-8].

Ghrelin, a peptide hormone, assumes a multifaceted role in eliciting appetite and feeding behavior, orchestrating carbohydrate metabolism, and maintaining equilibrium within the intricate realm of energy homeostasis[9]. Its revelation in 1999[10] marked a pivotal juncture in scientific inquiry, engendering profound intrigue surrounding its purported functions. This intrigue has resonated across diverse clinical domains, encompassing the cardiovascular system[11], neurological disorders[12], respiratory afflictions[13], and ocular maladies[14-15], where the potential therapeutic applications of ghrelin have garnered significant attention. A compendium of investigations has conspicuously underscored the capacity of ghrelin to uphold the physiological equilibrium of vascular endothelial function[16-17], reestablish the integrity of the microvascular architecture, mitigate vascular permeability, and instigate angiogenesis among endothelial cells[18]. Recent research has further unveiled the discernible glycoregulatory attributes of ghrelin within the context of diabetes[19-20]. However, there is a lack of research on the regulation of ghrelin in DR. We have proffered empirical substantiation of ghrelin’s capability to thwart retinal angiogenesis induced by elevated glucose levels in human retinal microvascular endothelial cells (HRMECs)[21]. Nonetheless, the intricacies underpinning the modulatory mechanisms through which ghrelin exerts its influence on DR have remained largely elusive. Autophagy, a self-degradative pathway intricately involved in the degradation and recycling of intracellular constituents, has emerged as a focal point of investigation in the context of DR progression, operating through diverse signaling pathways[22].

Within the ambit of this study, our principal objective was to elucidate whether the impact of ghrelin on the angiogenesis of HRMECs under conditions of high glucose (HG) stress is contingent upon the regulation of autophagic processes.

MATERIALS AND METHODS

Cell Culture HRMECs (BeNa Culture Collection, China) were routinely cultured as we previously described[21]. HRMECs in good condition were then randomly cultured in M199 medium (Gibco, USA) with 5.5 mmol/L glucose or 30 mmol/L glucose or 30 mmol/L glucose and 10 nmol/L ghrelin[17] (Glpbio, USA) to be assigned to the control group, HG group and HG+ghrelin group, respectively. In this investigation, a uniform period of 48h of treatment was administered to all cellular cohorts, with subsequent assessment of their migratory and tube-forming capabilities. Simultaneously, we conducted an examination of protein expressions encompassing autophagy markers such as LC3 (microtubule-associated protein 1 light chain 3, MAP1LC3), Beclin-1, p62 (SQSTM1), RAC-alpha serine/threonine-protein kinase (AKT), and mammalian target of rapamycin (mTOR). Furthermore, in the HG+ghrelin+AKT inhibitor cohort, HRMECs were initially cultivated in M199 medium enriched with 30 mmol/L glucose and 10 nmol/L ghrelin for an uninterrupted duration of 36h. Subsequently, a pharmacological agent, namely 2 μmol/L AKT inhibitor VIII (MCE, USA), was introduced into the medium, whereupon an additional incubation period of 12h ensued. During this interval, assessments encompassing cell migration, tube formation, as well as the quantification of protein expressions related to LC3, Beclin-1, and p62 were conducted.

Immunofluorescence Staining HRMECs underwent preliminary treatment in accordance with established protocols and were subsequently subjected to immunolabeling with a primary antibody directed against CD31 (1:100, CST, USA) at a temperature of 4°C over the course of an overnight incubation. Thereafter, all specimens underwent incubation with Cy3-labeled goat anti-rabbit IgG (1:100, Boster, China) for 1h at a temperature of 4°C over the course of an overnight incubation. Finally, the resultant images were captured utilizing a fluorescence microscope.

Cell Migration by Transwell Assay In the context of the migration assay, HRMECs were suspended within serum-free M199 medium and introduced into the upper chamber, which was a component of the Corning apparatus from the United States. The lower chambers were filled with M199 medium enriched with 10% fetal bovine serum (FBS). Following a 24-hour incubation period, the cells that had traversed to the lower chamber were subjected to fixation using a 70% iced ethanol solution for a duration of 1h. Subsequently, these cells were stained with a 0.5% crystal violet solution (Sigma, USA) for 20min and quantified under an inverted phase-contrast microscope.
Tube Formation Assay on Matrigel The in vitro endothelial cell tube formation assay was executed utilizing Matrigel as the substrate. Concisely, HRMECs were seeded onto a 24-well plate that had been pre-coated with a substantial layer of Matrigel from Corning, USA. The seeding density employed was 1×10^5 cells per well, and the cells were cultured for a duration of 12h in M199 medium. Subsequently, cellular formations were visualized through microscopy, specifically employing an IX51 Olympus microscope from Japan, and subjected to quantification and analysis utilizing Image J software (NIH, USA), with observations made at a magnification of 100×.

Western Blotting The proteins within the samples underwent separation through an 8% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and were subsequently translocated onto polyvinylidene fluoride (PVDF) membranes procured from Millipore, USA. Post this stage, following a blockade utilizing 5% nonfat dry milk, the membranes were subjected to an overnight incubation with primary antibodies. These antibodies included anti-LC3 at a 1:2000 dilution, anti-Beclin-1 at a 1:1000 dilution, anti-p62 at a 1:10 000 dilution (sourced from Abcam, USA), as well as anti-AKT, anti-phosphorylated AKT (p-AKT), anti-mTOR, anti-phosphorylated-mTOR (p-mTOR), and anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH), all at a 1:1000 dilution (obtained from CST, USA). This extensive incubation occurred at a temperature of 4°C. Subsequent to this prolonged incubation period, a secondary antibody was employed for a duration of 2h at room temperature, followed by meticulous washing steps. The ensuing immunoreactive signals were revealed and subjected to quantification employing enhanced chemiluminescence (ECL) technology and Bandscan software version 5.0, provided by Glyko Inc., USA.

Statistics The data are presented as the mean±standard error of the mean (SEM) and are representative of a minimum of three independent experiments. Statistical analyses were conducted using SPSS 25.0 statistical software from IBM, USA. All noteworthy distinctions were determined through a two-tailed Student’s t-test, accompanied by one-way analysis of variance employing the LSD post hoc test. A P-value less than 0.05 was deemed to be indicative of statistical significance.

RESULTS
Identification of HRMECs To discern the endothelial characteristics inherent to the employed cell line, we conducted immunofluorescence staining utilizing the widely recognized endothelial marker, CD31. The outcomes unveiled a substantial presence of CD31+ cells, depicted in red, as depicted in Figure 1. This unequivocally substantiates the endothelial attributes inherent to the HRMECs utilized in the context of this investigation.

Ghrelin Inhibits HG-induced Migration and Tube Formation of HRMECs Subsequently, we executed both a transwell assay and a Matrigel assay to scrutinize the potential influence of ghrelin on HRMEC migration and tube formation. The transwell assay conspicuously revealed that exposure to HG significantly augmented the migratory capacity and tube-forming prowess of HRMECs in comparison to the control conditions. However, when subjected to combined ghrelin treatment, the cells exhibited an inverse response (Figures 2 and 3). These findings unequivocally signify that in the presence of elevated glucose levels, ghrelin exerts an inhibitory effect on both HRMEC migration and tube formation.

Ghrelin Inhibits HG-induced Autophagy of HRMECs The assessment of autophagy-related proteins, including LC3-I, LC3-II, Beclin-1, and p62, was conducted via Western blotting. As delineated in Figure 4, it is evident that HG conditions fostered an upregulation in the expression of LC3-II/I and Beclin-1 proteins, concurrently leading to a reduction in p62 protein levels. Intriguingly, the augmented expression of LC3-II/I and Beclin-1 proteins, as well as the diminished abundance of p62 protein in HRMECs subjected to HG induction, underwent pronounced reversal upon pretreatment with ghrelin. Moreover, our investigation unveiled that within the HG milieu, the AKT/mTOR signaling pathway, which serves as a pivotal regulator of autophagy inhibition, was noticeably suppressed. This inhibition was underscored by a decline in the levels of p-AKT and p-mTOR proteins (Figure 5). Notably, the restraint imposed upon the AKT/mTOR pathway by HG was substantially alleviated through the intervention of ghrelin.

Ghrelin Prevents HG-induced Migration and Tube Formation of HRMECs Through the Inhibition of Autophagy To probe whether autophagy and AKT/mTOR signaling pathways are pivotal for the inhibitory role of ghrelin concerning HG-induced HRMEC migration and tube formation, we introduced the AKT inhibitor VIII into our
As anticipated, the introduction of the AKT inhibitor led to an upregulation in the expression of LC3-II/I and Beclin-1 proteins, concomitant with a downregulation in p62 protein levels, in comparison to the ghrelin-treated group, as illustrated in Figure 6. These findings unequivocally underscore that the inhibition of the AKT/mTOR pathway can effectively stimulate autophagic processes.

Furthermore, the inhibitory effects of ghrelin on HG-induced HRMEC migration and tube formation were notably attenuated in the presence of the AKT inhibitor, as visually depicted in Figures 7 and 8. Collectively, these outcomes suggest that the activation of autophagy can mitigate the inhibitory influence of ghrelin on the migration and tube formation of HRMECs under HG conditions. This insight implies that ghrelin exerts its protective role against HG-induced HRMEC migration and tube formation by inhibiting the autophagic process.
DISCUSSION

DR stands out as one of the most severe complications arising from DM. Current standards of care have demonstrated their efficacy in preserving and markedly enhancing visual function among numerous individuals grappling with DR\[^{23}\]. The sustained elevation of blood glucose levels in diabetic patients exacts a toll on the delicate retinal microvessels, thereby catalyzing the onset and progression of DR. Despite notable advancements in diagnostic modalities that have significantly advanced our capabilities in DR prevention and management, there remains a conspicuous dearth of awareness regarding proactive measures. Consequently, the disease continues to proliferate, ushering in a stage marked by a constellation of grave complications\[^{24}\]. In the terminal phase of this exceptionally intricate retinal malady, neovascularization is spurred by the aberrant production and release of VEGF. Currently, anti-VEGF agents have emerged as the primary therapeutic option for addressing DR. Nevertheless, the
landscape remains fraught with challenges, prompting ongoing exploration of novel approaches in this domain\(^\text{[8]}\). Given that the primary instigator of DR lies in the prolonged exposure of retinal endothelial cells to elevated glucose levels\(^\text{[25]}\), our experimental approach involved the utilization of HRMECs exposed to HG to emulate the \textit{in vitro} conditions akin to DR. Ghrelin, an intricately structured 28-amino acid peptide hormone, has garnered recognition for its protective properties in safeguarding vascular endothelial cells\(^\text{[16]}\). In the context of our study, we administered ghrelin treatment at a concentration of 10 nmol/L for a duration of 48h, drawing upon insights from our prior investigations\(^\text{[21]}\) as a point of reference. Once again, our experimentation has substantiated the inhibitory potential of ghrelin treatment when it comes to curtailing the transwell migration and tube formation of HRMECs. These two techniques, widely acknowledged and employed within the realm of angiogenesis research\(^\text{[26-27]}\), bear testament to the robustness of ghrelin’s action even under the taxing conditions of elevated glucose levels. The pronounced inhibitory influence exerted by ghrelin on pathological retinal angiogenesis in the presence of HG lends credence to the notion that ghrelin may hold promise as a therapeutic agent in the context of PDR.

Autophagy stands as a remarkably conserved catabolic pathway with a pivotal role in the regulation of critical biological functions encompassing cell survival and cellular metabolism\(^\text{[28]}\). It further serves as an adaptive mechanism, responding adeptly to a gamut of stressors, including but not limited to hypoxia, ischemia/reperfusion injury, nutrient scarcity, and oxidative stress\(^\text{[29]}\). The intricate interplay between autophagy and DR has emerged as a focal point of research, with accumulating evidence suggesting that autophagy may exhibit a dualistic role that evolves over time. In the early stage of DR, autophagy acts as an adaptative response with pro-survival and anti-apoptotic effects under mild stress; while in the late phase of DR, dysregulated autophagy due to long-term damage under severe stress contributes to the apoptotic retinal cell death exacerbating the damage\(^\text{[30]}\). The transformation of soluble LC3 (LC3-I) into its membrane-bound counterpart, LC3-II, through conjugation with phosphatidylethanolamine (PE), represents a pivotal event in the intricate machinery of autophagy\(^\text{[31]}\). Within this intricate process of autophagosome formation, mammalian Beclin-1 assumes a central role by orchestrating critical steps. It accomplishes this feat through the activation of a diverse array of Beclin-1-binding

![Figure 7](image7.png) **Figure 7** Representative images of migration (A, bar=100 μm) of HRMECs at 48h of treatment by Transwell assay in different groups, and the statistical comparison of the number of migrated cells (B) \(n=3\), \(^*\)\(P<0.05\) vs control group; \(^{a}\)\(P<0.05\) vs HG group; \(^{b}\)\(P<0.05\) vs HG+ghrelin group. HG: High glucose.

![Figure 8](image8.png) **Figure 8** Representative images of tube formation (A, bar=100 μm) of HRMECs at 48h of treatment by Matrigel assay in different groups, and the statistical comparison of the number of tube (B) \(n=3\), \(^*\)\(P<0.05\) vs control group; \(^{a}\)\(P<0.05\) vs HG group; \(^{b}\)\(P<0.05\) vs HG+ghrelin group. HG: High glucose; HRMECs: Human retinal microvascular endothelial cells.
proteins\textsuperscript{[32]}. As part of the autophagosome formation process, p62/SQSTM1, serving as a connecting link between LC3 and polyubiquitinated proteins, is selectively enclosed within the autophagosome. Subsequently, it undergoes degradation at the hands of proteolytic enzymes residing within the autophagolysosome\textsuperscript{[33]}. Consequently, it is imperative to recognize that the expression level of p62 protein exhibits an inverse relationship with the activity of autophagy. In our study, conducted under the duress of elevated glucose levels, the observed surge in the expression levels of autophagic proteins, as indicated by the LC3-II/I ratio and Beclin-1, accompanied by a decrement in p62 levels, distinctly underscores the activation of autophagy in HRMECs. These findings harmonize with prior research conducted on retinal endothelial cells\textsuperscript{[34-35]}. Moreover, in various experimental models pertaining to DR, an apparent trend emerges wherein overactivation of autophagy appears to be associated with the progression of DR. This is manifested through the upregulation of LC3 and Beclin-1, coupled with the downregulation of p62\textsuperscript{[36-39]}. However, notwithstanding the existing body of research, the pivotal question regarding whether autophagy serves as a counteracting or favoring force in the evolution of autophagy, the pivotal question regarding whether autophagy can play a protective role in DR remains cloaked in ambiguity\textsuperscript{[40]}. Within the intricate landscape of growth and metabolism regulation, mTOR, a serine/threonine-protein kinase, occupies a pivotal role by acting as a negative regulator of autophagy\textsuperscript{[40]}. AKT, hailing from the serine/threonine-protein kinase family, wields the ability to dampen autophagy through the activation of mTOR\textsuperscript{[41]}. The prevailing consensus posits that the AKT/mTOR signaling pathways play a central role in potentiating autophagy within the endothelial cell milieu, even in challenging conditions such as elevated glucose levels\textsuperscript{[35,42]}. The AKT pathway is inhibited in the early stage of DR and this inhibition leads to retinal cell apoptosis and structural destruction. However, during the late phase of DR, the AKT signaling pathway is overactivated in retinal cells and retinal capillary endothelial cells due to compensatory vascular hyperplasia\textsuperscript{[43]}. In addition, activation of the AKT/mTOR signaling pathway can play a protective role in DR\textsuperscript{[44]}. In our investigation, ghrelin administration elicited an increase in the expression of p-AKT and p-mTOR, consequently leading to a reduction in autophagy levels, when contrasted with HRMECs subjected solely to HG treatment. Furthermore, the influence of ghrelin was underscored by a significant reduction in the levels of LC3-II/LC3-I and Beclin-1 proteins, coupled with an increase in the abundance of p62 protein. Intriguingly, the introduction of an AKT/mTOR inhibitor effectively reversed these effects, shedding light on the mechanism through which ghrelin operates. Hence, our findings indicate that ghrelin mitigates autophagy levels under the HG milieu by stimulating the AKT/mTOR pathway. Furthermore, it is noteworthy that the migration and tube formation of HRMECs, prompted by HG exposure, were substantially ameliorated by prior treatment with ghrelin. These effects, too, were conspicuously reversed upon pretreatment with an AKT/mTOR inhibitor. In summation, our study’s findings offer an initial glimpse into the prospect that ghrelin can curtail autophagy, mediated through the AKT/mTOR pathway, ultimately alleviating retinal angiogenesis induced by HG conditions. While DR has traditionally been perceived primarily as a microvascular disorder, it is becoming increasingly evident that neurodegeneration and neuroinflammation play pivotal roles in its pathogenesis. Our study, while for the first time shedding light on the interplay between ghrelin and autophagy in the context of retinal vascular endothelial cell migration and tube formation, acknowledges that DR encompasses multifaceted pathological processes. Since the pathogenesis of DR is complex, whether ghrelin plays a role through other signaling pathways and how to use ghrelin for DR treatment are still unknown. Consequently, further research endeavors should be undertaken to probe the potential impact of ghrelin in various other facets of DR and to explore the pharmacological potential of ghrelin pathway modulation for the treatment of DR. Moreover, the ghrelin receptor (GHSR-1a) plays a crucial role in restoring and maintaining metabolic homeostasis, precise determinations of its molecular pharmacology and pathway-specific physiological effects will enable discovery of GHSR-1a drugs to provide safer and more effective treatments for DR\textsuperscript{[45]}. As our understanding of the complex interconnections within DR deepens, the role of ghrelin as a potential therapeutic target may usher in novel approaches for ameliorating the disease and mitigating its multifarious complications.

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