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Survivin regulated by autophagy mediates hyperglycemia-induced vascular endothelial cell dysfunction

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Abstract: Diabetic vascular complications are often defined by vascular endothelial lesions. However, as a plastic cell type, whether endothelial cells could transit from quiescence to hyper-active status and hamper vascular stability upon hyperglycemia stimulation and whether this process is involved in diabetic vascular complications remain obscure. Survivin has been identified as an anti-apoptotic protein in tumor or epithelial cells by either promoting proliferation or inhibiting apoptosis. Therefore, this study aims at investigating the effects of hyperglycemia on endothelial cell status and the potential involvement of survivin. We found that high glucose (25mM) did not cause endothelial injuries, instead, it evidently promotes endothelial proliferation and tube formation capacity indicating endothelial cell dysfunction upon hyperglycemia characterized by its preference to hyper-active status. Concomitantly, an upregulation of survivin was detected accompanied by the key component elevations of autophagy pathway including LC3, Beclin1, and p62. YM155, a specific inhibitor of survivin, could abrogate hyperglycemia-induced endothelial hyper-activation. Application of the autophagy inhibitor (3MA) and agonist (rapamycin) supported that survivin could be as a downstream effect or of autophagy. Thus, our results suggested that survivin/autophagy axis a potential therapeutic target in treatment of diabetic vascular complications.

Keywords: Survivin; Autophagy; Vascular endothelial cells; Hyperglycemia
INTRODUCTION

Diabetes mellitus (DM), commonly referred as diabetes, is a type of metabolic disorder characterized by high blood sugar levels (hyperglycemia). Diabetes, which has ranked the third in the risk of early death next to cancer and cardiovascular disease, is always accompanied with several diabetic complications finally leading to disability, chronic kidney diseases, blindness or cardiovascular diseases.

Diabetic vascular lesions, one of the major complications of diabetes, are further categorized into microvascular complication and macrovascular complication. The diabetic microangiopathy could occur systematically including diabetic retinopathy, diabetic nephropathy and diabetic neuropathy and macrovascular diseases mainly include coronary artery disease, stroke and peripheral vascular disease. Endothelial cells (ECs), as the major component of vasculature, its dysfunction is a common feature and leading cause of vascular complications closely related to chronic hyperglycemia. As we know, endothelial cells are plastic cells that can switch between quiescent and active status when exposed to different bioenergetic and biosynthetic stimulation. ECs divide and migrate rapidly leading to aberrant angiogenesis and increase vascular permeability in pathogenic microenvironments [1-4]. And active endothelial participation is required for monocyte adhesion and migration to the sub-endothelial space in the earliest alterations of the vessel wall of atherogenesis [5,6]. However, ECs plasticity is rarely considered in hyperglycemia induced vascular lesions. Thus, it will be worthwhile to further elucidate the effect of hyperglycemia on ECs quiescent versus hyper-active status.

Survivin, a member of inhibitors of apoptosis protein family, was found highly expressed in fetal tissues and tumors especially in malignancy but rarely present in most of the normal adult tissues [7]. The general function of survivin is to inhibit cell apoptosis and promote proliferation [8,9]. Previous studies have revealed that downregulation of survivin expression could inhibit tumor cell proliferation and viability in vitro and reduce tumor growth in mouse models [10]. Interestingly, a recent research has observed that survivin was upregulated in the retina of STZ-induced diabetic rat models [11]. In this present study, survivin expression was elevated in the EA.Hy926 endothelial cells when exposed to hyperglycemia, which enhance endothelial cell viability.

Autophagy is a catabolic process to maintain intracellular homeostasis via delivering protein
aggregates and dysfunctional organelles to the lysosome for degradation. In recent years, there are accumulating studies studying the relationship between hyperglycemia and autophagy process. Although mounting evidences have suggested the involvement of autophagy in the pathogenesis of diabetic complications [12-18], the role of autophagy in vascular endothelial cells-exposed to hyperglycemia remains unclear.

Since vascular lesions and endothelial dysfunction are widespread in diabetic complications, identifying how endothelial cell respond directly to high glucose and the underlying mechanism will be of great importance. To better understand these processes in vitro, we used a model of moderate (25mM) hyperglycemia stimulating EA.hy926 endothelial cells and investigated cell viability, proliferation, apoptosis and tube formation capacity. Involvements of survivin and autophagy were further investigated by introducing specific inhibitors. Of note, moderate hyperglycemia did not induce endothelial cell injury directly. Instead, moderate hyperglycemia promoted endothelial cell viability, proliferation and tube formation, which is mediated by autophagy/survivin axis.
MATERIALS AND METHODS

Reagent and antibody

The Cell Counting Kit-8 (CCK8) and Cytotoxicity LDH Assay Kit (LDH) were purchased from Dojindo (Kumamoto, Japan). Dulbecco’s modified Eagle medium (DMEM) and eBioscience™ AnnexinV-FITC Apoptosis Detection Kit (BMS500FI-100) were from Thermo Fisher Scientific (Waltham, MA, USA). Matrigel Matrix Growth Factor Reduced (356230) was bought from Corning Incorporated (Tewksbury, MA, USA). YM155 (S1130) was obtained from Sellect Chemicals (Houston, TX, USA). Rabbit polyclonal anti-survivin antibody (ab469) was purchased from Abcam Biotechnology (Cambridge, UK). LC3B antibody (L7543) and 3-Methyladenine (M9281) were from Sigma-Aldrich Corp. (St.Louis, MO, USA). The antibodies for Beclin1 (3738s), Bcl2 (2876), Bax (2772) and beta-actin (3700) were obtained from Cell Signaling Technology (Danvers, MA, USA). Anti-p62 antibody (PM045) was purchased from MBL International (Woburn, Massachusetts, USA). HRP Goat Anti-Rabbit/Mouse IgG (H+L) (AS014/AS003) were from ABclonal(Wuhan, HB, CN).

Cell Culture

Human umbilical vein cell line (EA.Hy926) was obtained from American Type Culture Collection (Manassas, VA). Cells were cultured in 5.6 or 25mM glucose Dulbecco’s modified Eagle’s medium (DMEM) plus 10% fetal bovine serum (FBS) at 37°C under 5% CO2 and 95% humidity.

Cell proliferation, death and apoptosis assay

Cell Counting Kit (CCK-8/WST-8) was used to measure cell proliferation of 926 according to the manufacturer’s protocol. In brief, 100μL of cell suspension (5000 cells/well) was inoculated in a 96-well plate. Then normal medium and high glucose medium were used to culture cells for 72hrs. 10μL CCK8 reagent was added into each well of 96-well plate with 100μL culture medium, and then cells were incubated for 30minutesat 37°C in a 5% CO2 incubator. Cell proliferation was monitored at a wave length of 450 nm with a Bio-Tek Synergy H1Microplate Reader (Winooski, VT, USA).

Cytotoxicity LDH Assay Kit-WST is a kit for determination of cytotoxicity by measuring a lactate dehydrogenase (LDH) activity released from damaged cells. 200 μL of the cell suspension was added to each well of a 96-well tissue culture plate overnight. Cells with different treatments were incubated for 72 hours at 37°C in a 5% CO2 incubator. Then 20 mL of the Lysis Buffer was added.
to each well of the high control for 30 minutes. The plates were centrifuged at 250 × g for 2 minutes to precipitate the cells. Then we transferred 100 μL of the supernatant from each well to each well of a new optically clear 96-well plate, followed by 100 μL of the Working Solution added to each well. After 30 minutes, 50 μL of the Stop Solution was added to each well. Cell damage was measured at 490 nm by the microplate reader.

Trypan blue dye is a vital stain used to selectively colour dead cells blue. Cells were digested with trypsin to obtain single cell, resuspended in 10% trypan blue dye buffer and counted under the microscope using a hemocytometer. Dead cells were shown as a positive staining.

Annexin V-FITC Apoptosis detection Kit was used to detect the apoptosis level of 926 endothelial cells. One to five million endothelial cells were trypsinized and resuspended in 1×Binding Buffer. 5μL of AnnexinV was added to 100μL of the cell suspension, followed by 10-15 minutes incubation at room temperature. Then cell suspension was centrifuged, washed and resuspended in 200μL of 1×Binding Buffer. At last, 5μL of Propidium Iodide Staining Solution was added in the resuspension and cell apoptosis level was analyzed by flow cytometry.

**Tube formation assay**

Tube formation assay is one of the most widely used in vitro assays to model the reorganization stage of angiogenesis. Matrigel Matrix Growth Factor Reduced was thawed overnight at 4°C on ice, and 50 μL Matrigel was added in each well of pre-cooled 96-well. The plates was placed at 37°C for 30 minutes. 926 was culture in 5.6 or 25mM glucose DMEM for 72 hours. Then Matrigel may be used as a thin gel layer with 2×10^4 endothelial cells plated on top. Cells were incubated for 6 hours at 37°C in a 5% CO₂ incubator. Endothelial tube size and numbers were observed and photographed under microscope.

**Western Blotting**

Western blot analysis was performed to measure protein levels as previously described [19]. Proteins of 926 were extracted with cold RIPA buffer, separated by 12% polyacrylamide gels and blotted onto polyvinylidene difluoride (PVDF) membranes. The blots were incubated with specific primary antibodies: anti-LC3B (1:600), anti-Beclin1 (1:1000), anti-p62 (1:1000), anti-survivin (1:1000), anti-Bcl2 (1:1000), anti-Bax (1:1000), and anti-beta-actin (1:1000).

**Statistical Analysis**

Summary data are reported as Means ± SEM. The Student’s unpaired t-test was applied in the
analysis of all experimental data. Test results are two-tailed, where P less than 0.05 is considered statistically significant. All of these tests were performed using the GraphPad Prism 5.0 software (Graph-Pad Prism, Inc., San Diego, CA, http://www.graphpad.com).
RESULTS

Figure 1. Hyperglycemia could not enhance the cell damage of vascular endothelial cells but the cell viability

To determine whether glucose levels could affect the cell viability of endothelial cells, we cultured the EA.Hy926 cell line in DMEM with normal (5.6mM) and high concentration (25mM) of glucose for 72 hours. There were no positive results of cell damage and apoptosis as we expected. Instead, high glucose partially promoted the cell proliferation, reduced the cell damage and apoptosis by cell proliferation assay (CCK8), LDH assay (Fig. 1A) and Annexin V fluorescent staining (Fig. 1B). Meanwhile, endothelial cells could form more capillary-like structures on matrigel with high glucose than the control group (Fig. 1C). The results showed that high glucose could induce the cell viability promotion of EA.Hy 926 instead of cell damage.

Figure 1. Chronic exposure to high glucose (HG, 25mM) increased cell viability.
Cell proliferation and cell death were assessed by CCK8 and LDH assay in EA.Hy926 cells cultured in DMEM containing 5.6 or 25mM glucose for 72 hours, respectively. *P<0.05 and **P<0.01 vs. 5.6mM group (Ctrl). The level of apoptosis was determined by Annexin V assay in EA.Hy926 cells cultured in 5.6 or 25mM glucose medium. *P<0.05 vs. 5.6mM group (Ctrl). The endothelial cells were plated on Matrigel in DMEM containing 5.6 or 25mM glucose.

Figure 2. Hyperglycemia upregulated related protein expression of cell fate in vascular endothelial cells

In order to find out what happened to the endothelial cells in high glucose, we measured the related protein expression of cell fate including the expression of survivin, Bcl2/Bax and autophagy (LC3, beclin1 and p62) by Western Blot analysis. The results showed that the protein expression of survivin was elevated in 926 cells cultured in DMEM containing 25 or 30mM glucose (Fig. 2A). Meanwhile, significant increase of survivin was observed in 926 cells at both short term (1, 2, 16h) and long term (72h) with high glucose (25mM) treatment (Fig. 2B). LC3, Beclin1, p62 and Bcl2 were higher in high glucose (HG, 25mM), compared with that in normal glucose (Ctrl) (Fig. 2C). The results of figure 1 and 2 revealed that hyperglycemia could increase the cell viability of vascular endothelial cells, which might be some relationship with the change of related protein expression of autophagy and survivin.

Figure 2. High glucose increased the protein expression of survivin, bcl2 and autophagic hallmarks

(A)Survivin expression increased in endothelial cells cultured in medium containing 25 or 30mM glucose for 72 hours. *P<0.05 vs. 5.6mM group. (B)The protein level of survivin was elevated at each indicated time point. (C)Protein levels of LC3-II, beclin1, p62 and bcl2 were increased in 926 endothelial cells stimulated with 25mM HG. All western blot images were the representative image from at least three independent analyses, and beta-actin was use as a loading control.
Figure 3. Survivin could regulate the cell proliferation and tube formation of EA.Hy926

To evaluate the role of survivin in the induction of cell viability of endothelial cells by high glucose, we used its specific inhibitor (YM155) to block the survivin expression of endothelial cells in normal medium or high glucose medium. We found there was no dramatic reduction in response to YM155 treatment for short periods of time (Fig. 3A), though downregulated the cell proliferation and induced the number of cell death after 72 hours (Fig. 3B and 3C). Interestingly, the blockage of survivin can reverse the elevated cell viability (increased cell proliferation, reduced cell death and stronger tube formation) by hyperglycemia stimulation (Fig. 3B, 3C and 3D). The results implied that the cell viability regulated by hyperglycemia may depend on the expression of survivin.

Figure 3. Inhibition of survivin reversed the promotion of endothelial cell viability induced by high glucose.

(A) Effect of YM155 (100nM) on cell proliferation of 926 by cell proliferation assay for short periods of time. (B) High glucose-enhanced endothelial cell proliferation (*P<0.05 vs. Ctrl) was significantly attenuated by YM155 treatment (#P<0.05 vs. Ctrl+YM155). (C) Trypan blue exclusion assay was performed as a measurement of cell death. High glucose-reduced endothelial cell death (*P<0.05 vs. Ctrl) was significantly attenuated by YM155 treatment. (D) The
significant increase of tube formation induced by high glucose (**P < 0.001 vs. Ctrl) was completely regressed in 926 cells because of YM155 treatment. Scale bars: 200μm

Figure 4. The inhibition of autophagy attenuated high glucose-increased survivin expression and cell viability in endothelial cells.

High glucose could increase the protein expression of survivin, as well as the cell proliferation and tube formation. As shown in figure 3, we also found survivin played an important role in the process of elevated cell viability by hyperglycemia. However, we didn’t know the molecular mechanism between high glucose and survivin. So we tried to block a series of signal pathway (data not shown), and we were surprised that 3MA (an autophagic specific inhibitor) could downregulate the expression of survivin. The results of Western blotting showed that 3MA could indeed reduce the expression of related protein, including LC3, beclin1, p62 and survivin (Fig. 4A, 4B). In addition, we observed 3MA could repress high glucose (HG)-increased cell viability in endothelial cells. It could inhibit the HG-induced cell proliferation, tube formation and abrogate the decrease of cell death (Fig. 4C). Autophagy is also responsible for the promotion of endothelial cell viability.

Figure 4. Autophagy played a role in hyperglycemia-induced survivin elevation and cell viability promotion in EA.Hy926 endothelial cells.

(A) Endothelial cells were exposed to high glucose concentration (HG, 25mM) for 72 hours in presence or absence of 3-MA (5mM) and then total lysates of cells were immunoblotted with anti-LC3, anti-Beclin1, anti-p62, anti-survivin, and anti-β-actin antibodies. (B) The values of
proteins are normalized against β-actin, compared with the basal level (Ctrl). (C) Inhibition of autophagy reversed high glucose-induced cell viability promotion. *P < 0.05, **P < 0.01 and ***P < 0.001 vs. Ctrl; ##P < 0.01 and ###P < 0.001 vs. HG

**Figure 5. The expression of survivin was increased during autophagy induction**

To further demonstrate survivin regulated by autophagy, we used rapamycin (autophagy activator, Rapa). As shown in figure 5A, the results of Western Blotting revealed that rapamycin could also induce the survivin expression and the difference was statistically significant (Fig. 5B). Meanwhile, the expression of hallmark of autophagy (LC3, beclin1 and p62) were negatively affected by survivin (Fig. 5C).

![Figure 5. Survivin was elevated by rapamycin and could not regulate the expression of autophagic related proteins.](image)

(A) The protein expression of survivin was higher in rapamycin group (Rapa, 10nM). (B) The value of survivin is normalized against β-actin, compared with the basal level (Ctrl). *P < 0.05 vs. Ctrl. (C) The protein levels of LC3-II, Beclin1 and p62 have no significant differences between control (YM155-) and survivin inhibition group (YM155+).

**Figure 6. Schematic diagram**
High glucose can increase the expression of survivin and autophagy at the same time. Both survivin and autophagy regulate the cell viability of ECs. Survivin decreased along with autophagy inhibition and increased with autophagy induction, but not the reverse. Hyperglycemia promoted EC hyperactivity (cell proliferation and tube formation), which is mediated by autophagy/survivin axis. The increase of EC hyperactivity induced by hyperglycemia could possibly cause diabetic vascular complications.
DISCUSSION AND CONCLUSION

Diabetic vascular lesions are the leading cause of morbidity and mortality among people suffering diabetes mellitus, which often begins with endothelial dysfunction [20-22]. Therefore, to reveal how endothelial cells respond to hyperglycemia directly and the precise underlying molecular mechanisms will be fundamental to identify novel therapeutic targets for diabetic related vascular complications. However, the effect of hyperglycemia on endothelial cells have been studied extensively but appeared to be context and cell type dependent. Several studies have demonstrated that hyperglycemia can result in endothelial cell injuries, including decreased cell viability, aggravated apoptosis [23,24] and inflammation [25]. In contrast, other reports suggested that there was a correlation between hyperglycemia and excessive endothelial cell proliferation [26] and migration [27-30]. In the present work, ECs were exposed to a moderate hyperglycemia-culture medium with 25mM D-glucose for 3 days, in which we demonstrated that moderate high glucose did not induce endothelial cell injury but instead, enhanced the cell viability (promote cell proliferation and reduce cell death and apoptosis). The hyper-activated ECs could possibly cause angiogenic components enhancement and vascular instability [1-4,31-34]. Under this circumstance, for the first time, we have shown that 25mM glucose could trigger the elevation of the antiapoptotic protein-survivin and major components of autophagy pathway compared to normal glucose. Moreover, we demonstrated a novel survivin-dependent mechanism for hyperglycemia-increased endothelial viability, which is mediated by autophagy process.

The major finding of this study indicates that hyperglycemia could increase the protein expression of survivin, which mediated hyperglycemia induced endothelial viability. Several studies have demonstrated that survivin is critical in anti-apoptosis and promoting proliferation. It is found highly expressed in fetal tissues and tumors with high malignant potential but absent in most of the normal adult tissues [7,35,36]. Down-regulating the expression of survivin could efficiently decrease the proliferation and viability of tumor cells and therefore inhibit the tumor growth[10]. Survivin could also maintain stem cells at less differentiated stage with higher proliferation potential [37,38]. In our previous study, we have demonstrated that survivin was highly expressed in pterygium tissues (one proliferative disease of ocular surface) and positively correlated to pterygium staging and progression, in which silencing of survivin could inhibit proliferation of pterygium epithelial cells [19]. Similarly, some studies also identified abundant
survivin expression in endothelial cells with high proliferative capacity which promoting endothelial cell proliferation, survival and angiogenesis [39,40]. Knockdown of survivin could efficiently abrogate angiogenic process in nude mice xenografts [41]. Therefore, survivin could function as a positive regulator of endothelial cell proliferation and angiogenesis. In consistent, as it is shown in Figure 3, YM155 could inhibit the viability and tube formation capacity of endothelial cells when cultured in normal glucose concentration (5.6mM), which supported that survivin was required for normal endothelial behavior. Combining with previous reports demonstrating survivin, as an anti-apoptotic factor, was required for cell survival, it was not surprising to observe an inhibitory effect of survivin on endothelial survival and tube forming irrespective of glucose concentration. In our study, we revealed that survivin is significantly upregulated by hyperglycemia which directly contributed to increased endothelial proliferation and viability. Of particular importance, YM155, the specific inhibitor of survivin could efficiently reverse hyperglycemia-induced endothelial proliferation and viability which indicated its therapeutic potential on diabetic vascular complications.

Autophagy promotes cell survival and proliferation by delivering cytoplasmic components to the lysosome for degradation to reuse. The role of autophagy in diabetes due to “hyperglycemia” has attracted much attention. In the Rhesus choroid-retinal endothelial cells, inhibition of autophagy could efficiently abrogate hyperglycemia-induced cell migration and tube formation which strongly suggested the involvement of autophagy in endothelial angiogenic potential exposed to hyperglycemia [42]. In contrast, elevated LC3-II/I ratio, Beclin1 and p62 were detected in human mesenchymal stem cells (MSCs) incubated with serum from type 2 diabetes patients, however, with impaired angiogenic potential [43]. Thus, the role of autophagy in diabetic vascular complications has not been determined. Our data demonstrated that the key components of autophagic pathway, including LC3-II, Beclin1, and p62, were elevated together with the cell viability enhanced by high glucose. Treatment with 3-MA, a specific inhibitor of autophagy could attenuated high glucose-increased cell viability as well as survivin expression. It will be of great interest to discuss and further elucidate how hyperglycemia regulates autophagic response and how autophagy regulates survivin and cell viability. Autophagy is a key mechanism in the maintenance of homeostasis and integrity of cells. Elevated autophagy activity induced by high glucose acts as a protective response under diabetic conditions. We also noted that irrespective of
high glucose, 3-MA could abrogate endothelial survival, tube formation and promote cell death and apoptosis when cells were exposed to normal glucose concentration (Figure 4). Although there have been mounting evidences showing the involvement of autophagy in cell viability and proliferation, in our circumstance, combing with our data showing that survivin is required for endothelial viability, which was significantly inhibited by 3-MA, it is rationale to speculate that the inhibitory effect of 3-MA on endothelial viability and function was partially dependent on the decrease on survivin. In the present study, we also demonstrated that the expression of p62 was slightly but stably upregulated in EA.Hy926 upon hyperglycemia stimulation. P62 is a multi-functional and ubiquitin binding protein integrated in both ubiquitin-proteasome system (UPS) and autophagy-lysosome system. It is widely involved in the tumorgenesis via promoting tumor cell proliferation, metastasis as well as apoptosis and inflammation [44-49] by modulating the NF-kappaB and NRF2 pathways. Therefore, it’s rational to predict that accumulation of p62 induced by hyperglycemia in the endothelial cells might also promote endothelial cell viability. Moreover, a correlation of Beclin 1 and survivin has also be reported in human glioma cells in which knock-down of Beclin 1 could downregulate survivin [50]. In the present study, we found treatment with 3-MA could attenuated high glucose-induced survivin accumulation. As previously mentioned, survivin could play an important role in regulating the endothelial cell proliferation and apoptosis, especially in the environment of hyperglycemia. Survivin mediated the process of cell viability promotion by autophagy (Fig. 6).

In conclusion, hyperglycemia triggered the increase of survivin expression as well as the induction of autophagy and treatment with 3-MA or rapamycin could change survivin expression, while reduction of survivin could reverse high glucose-induced cell viability promotion. It’s easy to see that hyperglycemia contributes to the promotion of vascular endothelial cell viability by regulation of autophagy-survivin-mediated signaling pathway. To our knowledge, this study is the first evidence of the effect of hyperglycemia on autophagy/survivin pathway in vascular endothelial cells, and our findings suggest a potential strategy for the treatment of diabetic vascular complications.
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Author Contributions: Y.-X. Xu, S.H. Liu and S.Y. Yang conceived and designed the experiments; Y.-X. Xu, C.X. Huang, M.Y. Liu, N.N. Chen, W.T. Chen and C. Y performed the experiments; Y.-X. Xu, Y. Zhao, X.J. Li and J.G. Duan analyzed the data; S.Y. Yang and S.H. Liu contributed reagents/materials/analysis tools; Y.-X. Xu, S.H. Liu and S.Y. Yang wrote the paper.

Conflicts of Interest: The authors declare no conflict of interest.
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Highlights

- High glucose did not induce endothelial cell injury directly, but increased cell viability after 72hrs.
- High glucose could induce endothelial cell dysfunction by promoting its hyper-active capacity as determined by proliferation and tube formation assay.
- High glucose triggered upregulation of anti-apoptotic protein survivin expression as well as key components of autophagy pathway.
- Survivin regulated high glucose-induced vascular endothelial cell dysfunction mediated by autophagy pathway.