

Sphk1 involves in the regulation of autophagy process in cardiac myocyte cells at high glucose condition

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Abstract

Diabetic cardiomyopathy is the myocardium disorders caused by diabetes mellitus, which has become a key concern bringing heavy burden to the public health. Autophagy is one of activities involving in the pathogenesis of diabetic cardiomyopathy. Sphk1 gene plays a crucial role in cell survival and growth, and regulation of many diseases including diabetes and cardiovascular disease. However, the pathogenesis of diabetic cardiomyopathy remains poorly understood. To this aim, the study established adenovirus vectors expressing Sphk1 and shSphk1 to investigate the effects of Sphk1 on autophagy and cell survival in myocardial cells under high glucose conditions (25 mM). It was found that overexpression of Sphk1 promoted autophagy activity in H9c2 cells under high glucose treatment measured by various methodologies including qRT-PCR, western blot, fluorescence microscope, and so on. Inhibition of autophagy decreased cell vitality under high glucose condition. A broadly used medicine silibinin was demonstrated to induce autophagy in a dose-dependent manner. Herein, the findings in the present study may provide useful reference for unveiling the pathogenesis of diabetic cardiomyopathy and developing novel therapies treating diabetic cardiomyopathy.

Introduction

Diabetic cardiomyopathy (DCM) is defined as the myocardium disorders caused by diabetes mellitus, which was first to be reported in 1881 [1]. It is reported that diabetes become a key risk causing heart failure, and DCM is characterized by initial impairment of left ventricular (LV) relaxation followed by LV contractile dysfunction [2]. Notably, Finck and co-workers found that a transcription factor, peroxisome proliferator-activated receptor (PPAR)- α , closely regulated DCM in combination with its transcriptional targets [3]. This excellent study opened a window for studies towards the transcriptional mechanisms of DCM. This disease has been brought severe burden to the public health. However, the exact mechanisms underlying DCM remain unclear by most physicians, even cardiologists and diabetologists.

As an important cellular process, autophagy is reported to be crucial for various diseases. It was reported that autophagy plays an essential role in alleviating the development of insulin resistance and diabetes [4]. Autophagy also involves in muscle glucose uptake and mitigates endoplasmic reticulum (ER) stress in β cells under diabetogenic conditions [4]. As one of important components in autophagy process, lysosome is able to govern energy metabolism and its disorders disrupt autophagic activity, thus causing the accumulation of lipid within lysosomes [5]. In addition, autophagy plays an important role in maintaining intracellular homeostasis in a variety of cardiovascular derived cells including cardiomyocytes [5]. Autophagy has been confirmed to benefit for the growth of several cell types, for example, the induction of autophagy is able to promote cell survival and growth in cancer cells [6].

As a kinase producing sphingosine-1-phosphate (S1P), sphingosine kinase 1 (Sphk1), plays an essential role in cell survival and growth [7]. Sphk1 has been demonstrated to be functionally relevant to many diseases including cancer [7], Alzheimer's disease [8], inflammatory response [9], and Huntington's Disease [10]. In addition, Sphk1 is closely tied to type 2 diabetes [11]. Therefore, studies toward Sphk1

should be helpful for dissecting pathogenesis of diabetes. It is attractive to figure out the relationship between diabetes and cardiovascular disease, and ascertain the underlying mechanisms. Thus, the present study aims to explore the regulation of Sphk1 gene on autophagy in cardiac myocyte cells at high glucose circumstance, and corresponding mechanism, which should provide useful reference for developing novel therapies treating diabetic cardiomyopathy.

Materials And Methods

2.1 Chemicals and reagents

The Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS), and penicillin-streptomycin (P/S) solution were purchased from Gibco (Gibco, New York, NY, USA). Glucose was purchased from Sigma-Aldrich (St. Louis, MO, USA). Autophagy inhibitors including 3-Methyladenine (3-MA, S2767) and Z-DEVD (S7312) were bought from Selleck (Shanghai, China). Cell Counting Kit-8 (CCK-8) was purchased from Dojindo (Dojindo, Kumamoto, Japan). DMSO, MDC staining kit, trypan blue and protease inhibitor were purchased from Sigma (Darmstadt, Germany).

2.2 Cell culture

The embryonic rat heart-derived myogenic cell line H9c2 were purchased from the American Type Culture Collection (ATCC), and the cells were maintained in DMEM containing 10% FBS (V/V), 100 U/ml penicillin, and 100 µg/ml streptomycin in an atmosphere of 5% CO₂ at 37°C, as described previously [12, 13]. All cells used in the present study were below the 35th passage.

2.3 Plasmids, Ad vectors, and transfection

Adenovirus (Ad) vectors including Ad-Sphk1 and Ad-shSphk1 were constructed by using an improved *in vitro* ligation method [14]. Briefly, FLAG epitope-tagged WT human sphingosine kinase 1 (Sphk1) cDNA (GenBank accession no. AF200328) was sub-cloned into pcDNA3* vector, followed by generating adenoviruses. All Ad vectors (Ad-Sphk1 and Ad-shSphk1) were purified as described previously [14]. The virus particles (VPs) were measured using an Adeno-X-rapid titer kit (Clontech, Mountain View, CA, USA).

Before transduction, H9c2 cells cultured in T75 flask were passaged into six-well plates (2x10⁵ cells/well), and cells were cultured overnight to reach 70–80% confluence. Afterwards, adenoviruses of Ad-Sphk1 and Ad-shSphk1 with multiplicity of infection (MOI) of 100 were used to transduce cells, followed by being selected in growth medium containing 1 mg/mL G418. All experiments were conducted in the absence of serum at 50% confluence. The transduction was verified by western blot and observation under fluorescence microscope.

2.4. The mRNA expression using quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from H9C2 cells using TRIzol (Invitrogen) according to the manufacturer's instructions [15, 16]. The RNA reverse transcript (RT) was executed using TransScript One-Step gDNA Removal and cDNA Synthesis SuperMix kit (TransGen Biotech, Beijing, China) following the manufacturer's protocol and 500 ng RNA was used to generate cDNA. The cDNA products were subjected to qRT-PCR using TransStart Top Green qPCR SuperMix (TransGen Biotech, Beijing, China) according to the manufacturer's protocol. The protocol for thermal cycling consisted of 60 s at 95 °C, followed by 40 cycles of 15 s at 95 °C and 60 s at 63 °C. Relative mRNA levels of target genes were normalized to the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). The results are based on cycle threshold (Ct) values. We calculated differences between the Ct values for experimental and *GAPDH* and graphed as a percent of each RNA to the calibrator sample. All primers used in the study were listed in Table 1.

2.5. Western blot

The total protein was extracted with RIPA solution from *in vitro* cultured cells, and protein samples were quantified using BCA Protein Assay Kit (Beyotime Biotechnology). The protein (20 µg) was subjected to 10% SDS-PAGE gel electrophoresis, followed by transferring to a PVDF membrane. Then, the PVDF membranes containing protein were blocked using blocking buffer (Beyotime Biotechnology), followed by being washed for 3 times with PBS containing 0.5% TWEEN-20 (PBST). Afterwards, PVDF membranes were incubated with primary antibodies, including rabbit anti-Sphk1 (1: 1000, 10670-1-AP, Proteintech), anti-Beclin1 (1:1000, bs-1353R, Bioss, Beijing, China), anti-p-LC3B (1:1000, BM4827, Bioss, Beijing, China), and anti-GAPDH (1: 1000, ab9845, Abcam) overnight at 4°C. The next day, membranes were washed for 3 times with PBST, 5 min each time, followed by incubation with anti-rabbit IgG-HRP secondary antibody (1: 5000, Proteintech, Wuhan, China) at room temperature for 1 h. Then, the blocks were washed with PBST for 5 min, followed by imaging with ECL method (Beyotime Biotechnology).

2.6. Lentiviral expression of green fluorescence protein bound to LC3

H9c2 Cells were cultured on a round cover slide on the bottom of well of a 6-well-plate with culture medium and transduced with lentiviral particles carrying a construct of TagGFP2-LC3 driven by the elongation factor-1 promoter (Millipore LentiBrite) for 48 h, followed by replacing cell culture medium containing drugs and treated for indicated time period. Green punctate representing autophagy was assessed by taking images using fluorescence microscope (Mshot, Guangzhou, China).

2.7. Monodansylcadaverine (MDC) staining

To assess autophagy, H9c2 Cells (10^5 cells/well) were plated in wells of 6-well plates and were treated with indicated treatments for indicated time. Then, cells were subjected to MDC staining buffer for 45 min at room temperature (Nanjing KeyGen Biotech co., Ltd.) according to the manufacturer's protocol. Subsequently, the fluorescence was measured using a fluorescence microscope (Mshot, Guangzhou, China). The activated autophagosome represented acidic vesicles, which was tested by measuring the level of green fluorescence.

To further measure autophagy, after that cells were stained using MDC, the cells were analyzed by a flow cytometry (Altra; Beckman), and 10000 events were recorded per sample at wave length of 355 nm, and data were analyzed by Flow Jo software (Version 7.6.1).

2.8. Cell vitality measurement

Cell growth and vitality were evaluated by 3-(4,5)-dimethylthiazolium (-z-y1)-3,5-di-phenyltetrazolium bromide (MTT) assay or trypan blue according to the manufacturer's protocol (Nanjing KeyGen Biotech co. Ltd.). Briefly, Cells (10^3 /well) were seeded into wells of 96-well plates and cultured for 24 h. Then, cells were subjected to the indicated treatments for indicated time. Afterwards, cell vitality was examined at 490 nm on a microplate reader (Synergy™ 2; BioTek Instruments, Inc.) or cell counter (Thermo Fisher).

2.9. Statistical analysis

All data were analyzed using GraphPad software (version: GraphPad Prism5). Data are presented as mean \pm SEM and the standard errors of the mean in this study were carried out in triplicates. Statistical comparisons between 2 groups were performed by t test. $P < 0.05$ was considered to be a statistically significant difference.

Results

3.1 Evaluation of transduce efficiency of Ad-Sphk1 and Ad-shSphk1

Sphk1 gene plays an important role in the regulation of autophagy in several cell types [17], and this gene was also reported to be associated with coronary artery disease [18]. Therefore, it is interesting to probe the effects of Sphk1 on autophagy in H9C2 cells. To better dissect the functions of Sphk1, the overexpression and knockdown adenovirus vectors including Ad-Sphk1 and Ad-shSphk1 were generated, respectively. To confirm the success of adenovirus vectors, the virus particles of Ad-Sphk1, Ad-Sphk1-ctrl, Ad-shSphk1, and Ad-shSphk1-ctrl were used to infect H9C2 cells, which showed potent fluorescence intensity in cells infected by all four vectors (**Figure 1A**). Since Ad-Sphk1 vector carries chimera cDNA encoding DDDDK, thus to further verify, DDDDK protein was measured using western blot method, which

indicated that Ad-Sphk1 adenovirus infected H9C2 cells expressed DDDDK protein compared with Ad-Sphk1-ctrl and negative control (NC) (**Figure 1B**).

3.2 Sphk1 tightly regulated autophagy in glucose supplemented H9C2 cell model

Since adenoviruses expressing Sphk1 gene and shSphk1 have been established, and then the effects of Sphk1 on autophagy were further probed. In addition, to check the regulation of Sphk1 on DCM, the glucose supplemented H9C2 cell model was established, in which 5.5 mM and 25 mM glucose were added in cell culture medium. Then, we firstly used Ad-Sphk1, Ad-Sphk1-ctrl, Ad-shSphk1, and Ad-shSphk1-ctrl to transduce glucose (5.5 mM and 25 mM) treated H9c2 cells, indicating high transduction efficiency measured by fluorescence microscope (Supplementary Figure 1A), qRT-PCR (Supplementary Figure 1B) and western blot (Figure 2A). Overexpression of Sphk1 was found to significantly increase the protein level of the autophagy-associated markers including Beclin 1 and LC3B in 25 mM glucose treated cells (Figure 2B), meaning that Sphk1 promoted autophagy.

To further verify, MDC staining was used, which indicated that gain of function of Sphk1 gene increased autophagy induction in both 5.5 and 25 mM glucose treated cells, and loss-of function of Sphk1 gene decreased autophagy induction in both 5.5 and 25 mM glucose treated cells measured using fluorescence microscope (Figure 3A). The similar results were observed by using flow cytometry (Figure 3B).

3.3 Silibinin increased autophagy in glucose supplemented H9C2 cell model

Silibinin is a key component of Silymarin that is an extract isolated from herb *Silybum marianum* (milk thistle), and this chemical exerts anti-oxidant and anti-inflammatory effects to protect many types of cells from injury caused by various risks [19]. To check the effects of autophagy on vitality of cardiomyocytes under high glucose condition, different concentrations of silibinin were used to treat lentivirus expressing both GFP and LC3B infected H9C2 cells, which indicated that silibinin enhanced autophagy in a dose dependent manner (**Figure 4A**). To further investigate, beclin1 and LC3B protein level were measured in Ad-Sphk1, Ad-shSphk1, glucose and silibinin co-treated cells using western blot. Interestingly, it was found that higher glucose (25 mM) decreased beclin1 and LC3B level irrespective of silibinin, Ad-Sphk1 and Ad-shSphk1 treatments compared with lower glucose (5.5 mM) group (**Figure 4B**). Notably, higher glucose increased cytotoxicity and decreased cell vitality, and inhibition of autophagy by autophagy inhibitors 3-MA and Z-DEVD-FMK increased cytotoxicity and decreased cell vitality (**Table 2**). Above results manifested that autophagy closely regulated cell vitality of cardiomyocytes under high glucose circumstance, silibinin was able to promote autophagy in cardiomyocytes, and might be a candidate therapy for DCM disease.

Discussion

Along life quality improvement, DCM disease becomes a commonplace problem all over the world, and is bringing a severe burden for the public health. Thus the development of novel and effective medicines or therapies is warranted to defend the problematic disease. In the present study, we generated gain and loss-of functions of Sphk1 gene adenovirus vectors to transduce H9C2 cells. Sphk1 was confirmed to promote autophagy, high glucose remarkably decreased autophagy and cell vitality. Silibinin enhanced autophagy in H9c2 cells under high glucose condition, and might be a promising medicine to treat DCM.

Sphk1 gene plays an important role in a variety of diseases including diabetes mellitus and vascular complications [20, 21], although the underlying mechanism remains poorly clear. This gene has been reported to be tightly associated to autophagy process in several cell types. The previous study has been demonstrated that Sphk1 was capable of promoting autophagy in human peritoneal mesothelial cells (HPMCs), and this process aided the adhesion and invasion of HPMCs [17]. Wang and co-workers demonstrated that microRNA-506-3p was able to inhibit autophagy through targeting Sphk1 in osteosarcoma cells [22]. In another study, authors demonstrated that Sphk1/ERK/p-ERK pathway involved in the induction of autophagy in colon cancer cells [23]. Inconsistently, it was reported that the inhibition of Sphk1 expression could induce autophagy in head and neck squamous cell carcinoma cells [24]. The conflict might be due to that the physiological variation exists in different cell types. In the present study, we confirmed that activation of Sphk1 was able to induce autophagy and silence of the gene was capable of inhibiting autophagy in myocardial cells at high glucose condition (Figure 2 and Figure 3).

Importantly, the present study found that autophagy had capacity to ameliorate cytotoxicity (Table 2). Autophagy is a cellular non-specific, bulk degradation process, which is in charge of defending against nutrition environmental stress via removing damaged macromolecules and organelles [15]. Thus autophagy is closely related to the cell survival and growth. In dead, many studies have been found that autophagy supports cell proliferation and growth. For examples, a mostly recent study performed by Yang and co-authors indicated that autophagy was capable of protecting nucleus pulposus cells from the apoptosis induced cyclic mechanical tension [25]. Autophagy is also reported to potently increase human hepatocellular carcinoma HepG2 cell proliferation, by using this notion, Okubo *et al*/ screened approximately 130 kinds of crude drugs to discovery effective crude drugs supporting cell growth via regulating autophagy process[26].

Emerging evidence indicate that diabetes mellitus is linked to cardiovascular disease. To dissect the pathogenesis and develop effective therapies against diabetes induced cardiovascular disease. A glucose supplemented myocardial cell model was used in the present study. Importantly, our study confirmed high glucose inhibited autophagy and promoted cytotoxicity in myocardial cells (Figure 2 and Table 2). Sphk1 was demonstrated to induce autophagy in high glucose treated cells. These results are consistent with previous studies, in which, authors stated that Sphk1 was a modulator to closely regulate angiotensin II-induced vascular dysfunction in vascular tissues [27].

Importantly, our study confirmed that silibinin exerted induction of autophagy in myocardial cells (**Figure 4**). Interestingly, a previous study found the similar results, in which authors demonstrated that silibinin exerted protective effect on pancreatic β -cells from inflammation factors including TNF α - or IL-1 β -induced cell death [27]. Consistently, Rezabakhsh *et al*/reported glucose promoted the death of human endothelial cells, while silibinin was able to protect human endothelial cells from high glucose-induced cytotoxicity by inducing autophagy [28]. In another study, authors observed the similar phenomenon that silybin exerted cytoprotective effects on human, rat, chicken hepatoma cells and rat myoblasts, although the protection effect was not related to the autophagy process [29]. Similarly, silibinin was stated to reduce arsenic-induced oxidative stress mediated cardiotoxicity and dyslipidemia in rats [30], and alleviate autophagic cytotoxicity caused by oxidative stress in cortical neurons and cerebral ischemia-reperfusion injury [31]. Thus according to the results obtained from the present study and other studies, silibinin might potentially promote cell survival in glucose induced cell injury via enhancing autophagy activity in myocardial cells.

In summary, our study was first to find that Sphk1 gene involved in regulation of high glucose induced cytotoxicity in myocardial cells. Of note, a broadly used medicine, silibinin exerted promotion effects on autophagy in high glucose treated myocardial cells. Thus, our study shall aid efforts to figuring out pathogenesis and development of novel therapies against DCM diseases.

Declarations

Acknowledgements

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Author contributions

W.A., P.X., L.L., and Y.G. performed the experiment; L.W., H.F., and Y.C. contributed to the scientific discussion, facilities, and manuscript editing; W.A. and Y.C. conceived the project and wrote the manuscript.

Compliance with ethical standards

Conflicts of Interest

We have declared that no competing interests exist. The authors alone are responsible for the content and writing of the paper.

Ethical approval

The study was approved by the Ethics Committee of Shenzhen Nanshan People's Hospital and The six affiliated Hospital of Shenzhen University Health Center.

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Figure Legends

Figure 1. Assessment of adenovirus vectors including Ad-Sphk1, Ad-Sphk1-ctrl, Ad-shSphk1 and Ad-shSphk1-ctrl. (A) The fluorescence intensity and cell morphology after adenovirus infection (48 h) measured by fluorescence microscope. (B) Western blot results confirmed the successful overexpression of Sphk1 in H9c2 cells.

Figure 2. Sphk1 involved in promotion of autophagy process in high glucose treated H9c2 cells measured by qRT-PCR and western blot assays. (A) The effects of Ad-Sphk1 and Ad-shSphk1 on mRNA expression of Beclin1 tested by qRT-PCR assay. (B) The effects of Ad-Sphk1 and Ad-shSphk1 on Sphk1, Beclin1 and LC3B protein level examined by western blot method. The results are expressed as the means \pm SEM (n=3 for each group). * P<0.05 compared with the control group.

Figure 3. The effects of Sphk1 gene on autophagy in H9c2 cells under high glucose conditions. (A) Fluorescence density measured by fluorescence microscope after MDC staining following adenovirus transduction under high glucose condition in H9c2 cells (original magnification, x20). (B) Flow cytometry analysis on H9c2 cells after MDC staining following adenovirus transduction under high glucose condition.

Figure 4. Silibinin promoted autophagy process in H9c2 cells. (A) Silibinin enhanced autophagy activity in a dose-dependent manner. (B) The effects of co-treatments of adenovirus vectors, silibinin and glucose.

Tables

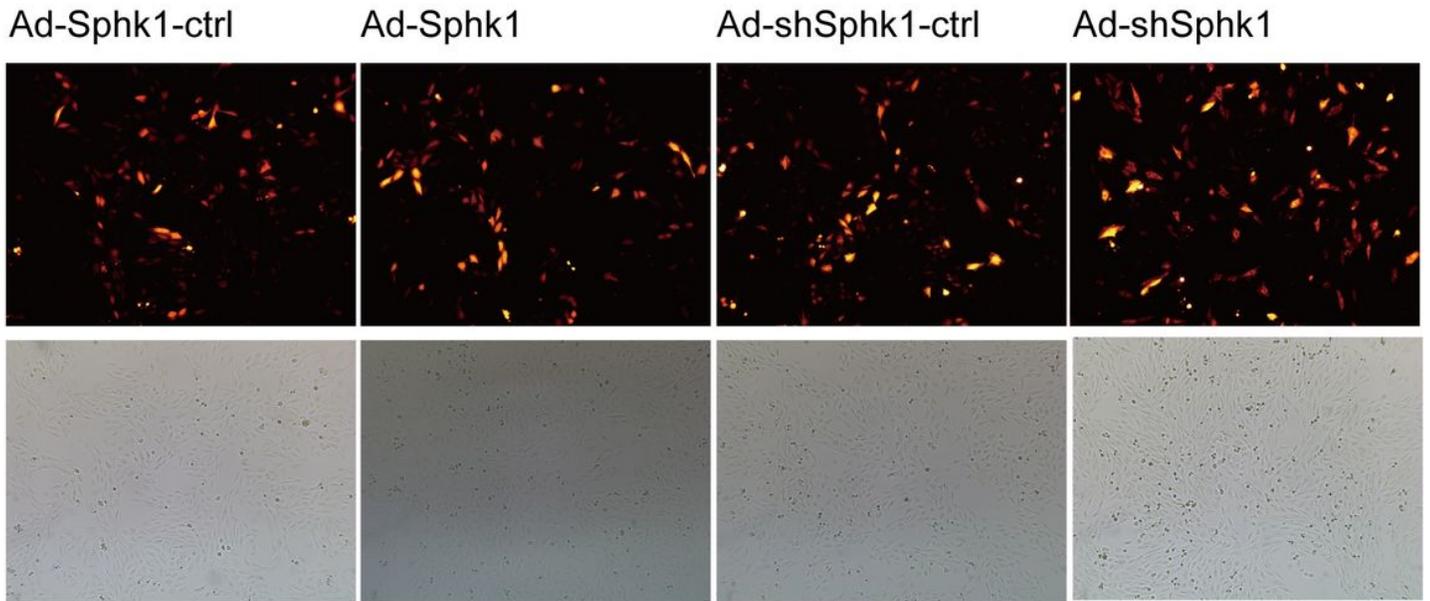
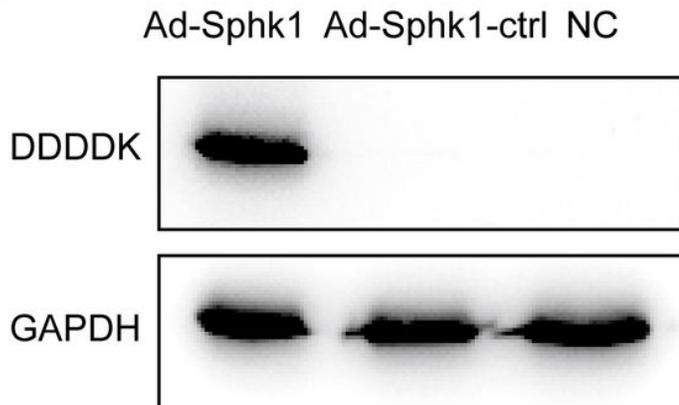
Table 1. primers for qRT-PCR analysis used in the study.

Gene	Species	Type	Sequence
GAPDH	Rat	Sense	TATGACTCTACCCACGGCAAG
		Anti-sense	TGAAGACGCCAGTAGACTCC
Sphk1	Rat	Sense	CCTTTAAACTGATGCTCACCGAAC
		Anti-sense	TCGTGCATCAGACCGTCACC
Beclin 1	Rat	Sense	TTCCATATCTGGCACAGCGGACA
		Anti-sense	CCATAGGGAACAAGTCGGTACCTC

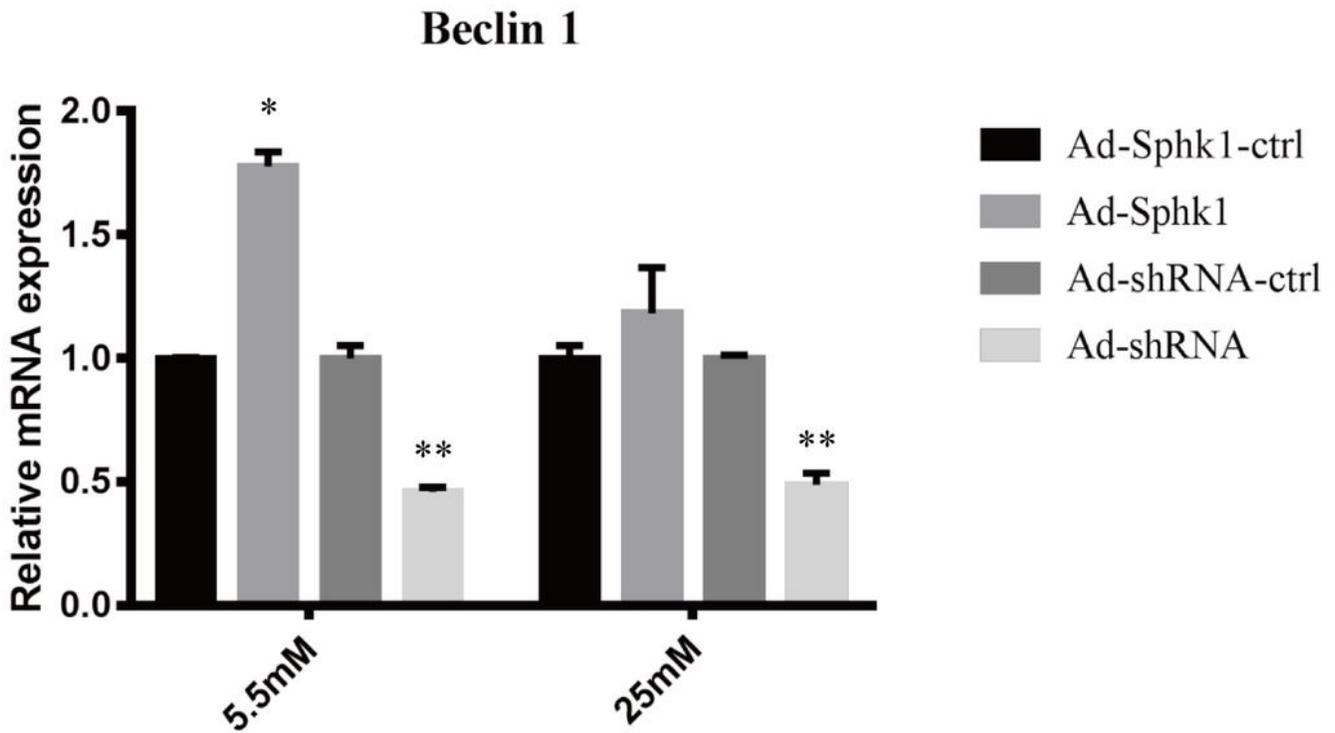
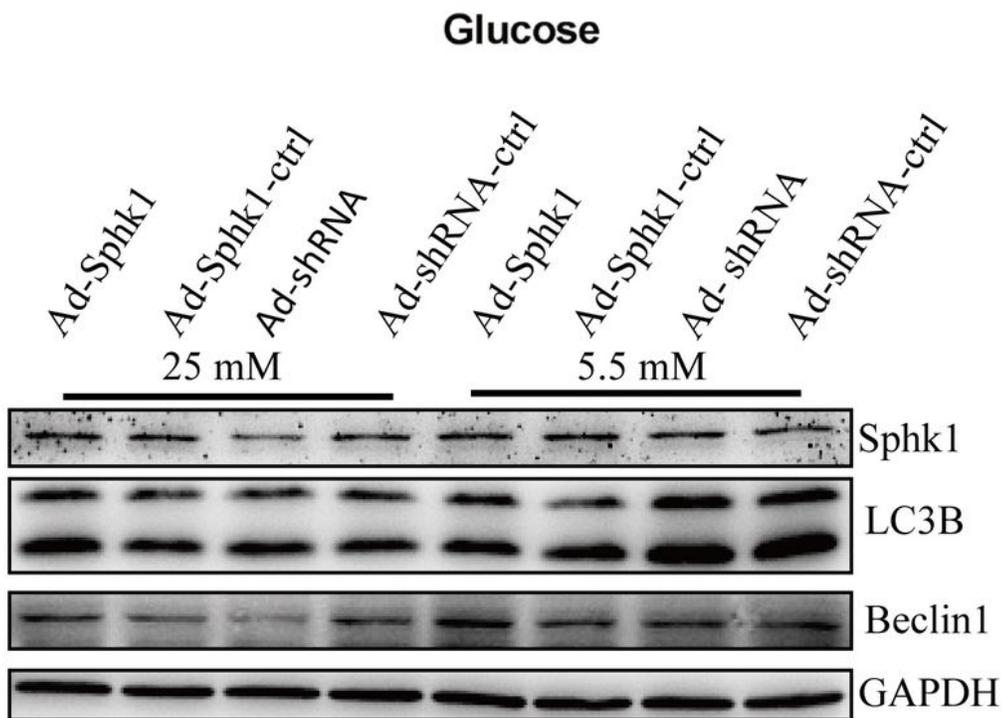
Table 2. Effect of co-treatment of autophagy inhibitor (3-methyladenine and Z-DEVD), glucose, and silibinin on cell vitality and cytotoxicity.

3-MA	1 mM	1 mM	0	0	0	0
Z-DEVD-FMK	0	0	50 μ M	50 μ M	0	0
Glucose	5.5 mM	25 mM	5.5 mM	25 mM	5.5 mM	2.5 mM
Silibinin	15 μ M					
Cell vitality	93.0%	91.0%	92.0%	87.0%	96.0%	94.0%
Cytotoxicity	7.0%	9.0%	8.0%	13.0%	4.0%	6.0%

Figures

A**B****Figure 1**

Assessment of adenovirus vectors including Ad-Sphk1, Ad-Sphk1-ctrl, Ad-shSphk1 and Ad-shSphk1-ctrl. (A) The fluorescence intensity and cell morphology after adenovirus infection (48 h) measured by fluorescence microscope. (B) Western blot results confirmed the successful overexpression of Sphk1 in H9c2 cells.

A**B****Figure 2**

Sphk1 involved in promotion of autophagy process in high glucose treated H9c2 cells measured by qRT-PCR and western blot assays. (A) The effects of Ad-Sphk1 and Ad-shSphk1 on mRNA expression of Beclin1 tested by qRT-PCR assay. (B) The effects of Ad-Sphk1 and Ad-shSphk1 on Sphk1, Beclin1 and LC3B protein level examined by western blot method. The results are expressed as the means \pm SEM (n=3 for each group). * P<0.05 compared with the control group.

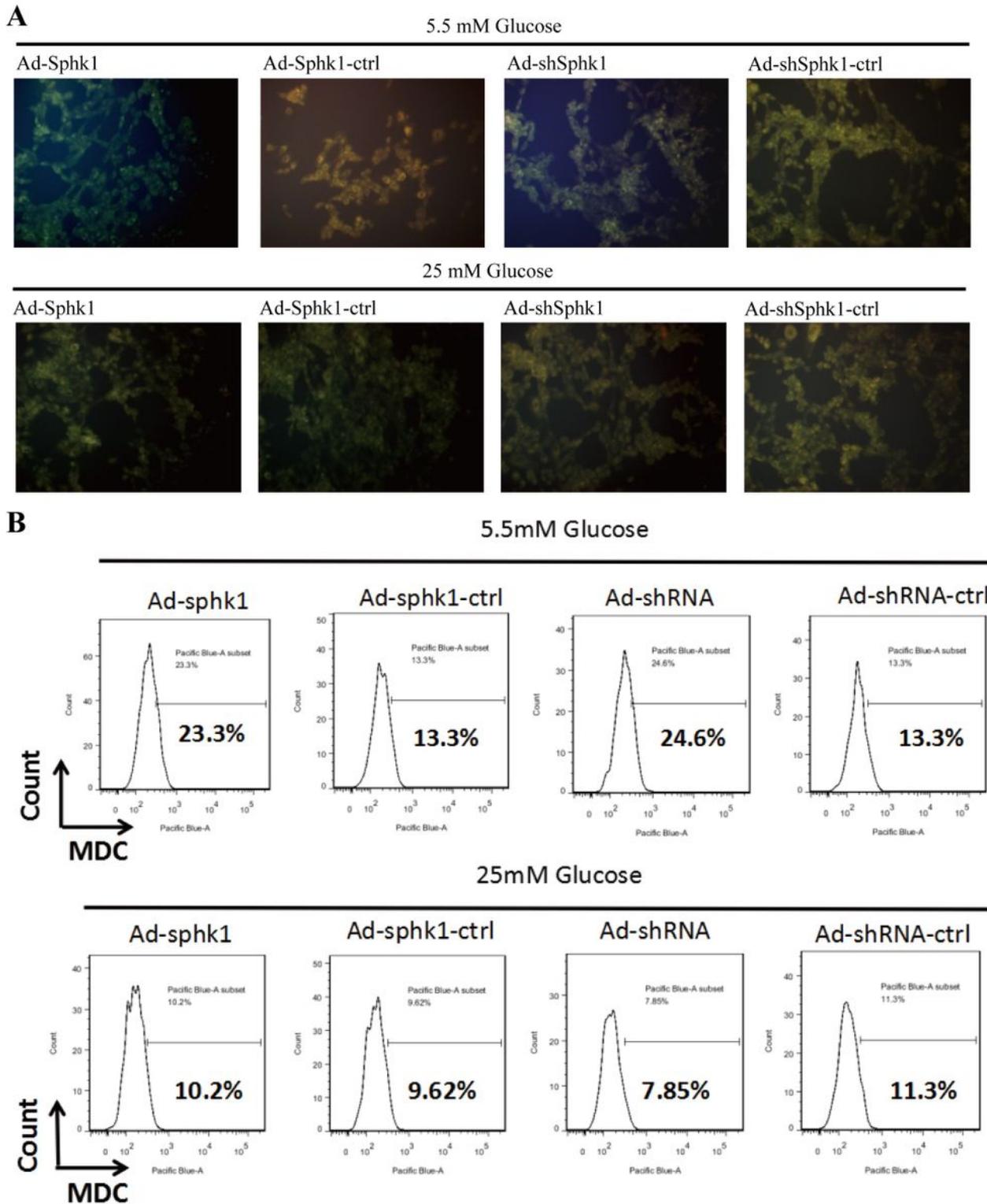


Figure 3

The effects of Sphk1 gene on autophagy in H9c2 cells under high glucose conditions. (A) Fluorescence density measured by fluorescence microscope after MDC staining following adenovirus transduction under high glucose condition in H9c2 cells (original magnification, x20). (B) Flow cytometry analysis on H9c2 cells after MDC staining following adenovirus transduction under high glucose condition.

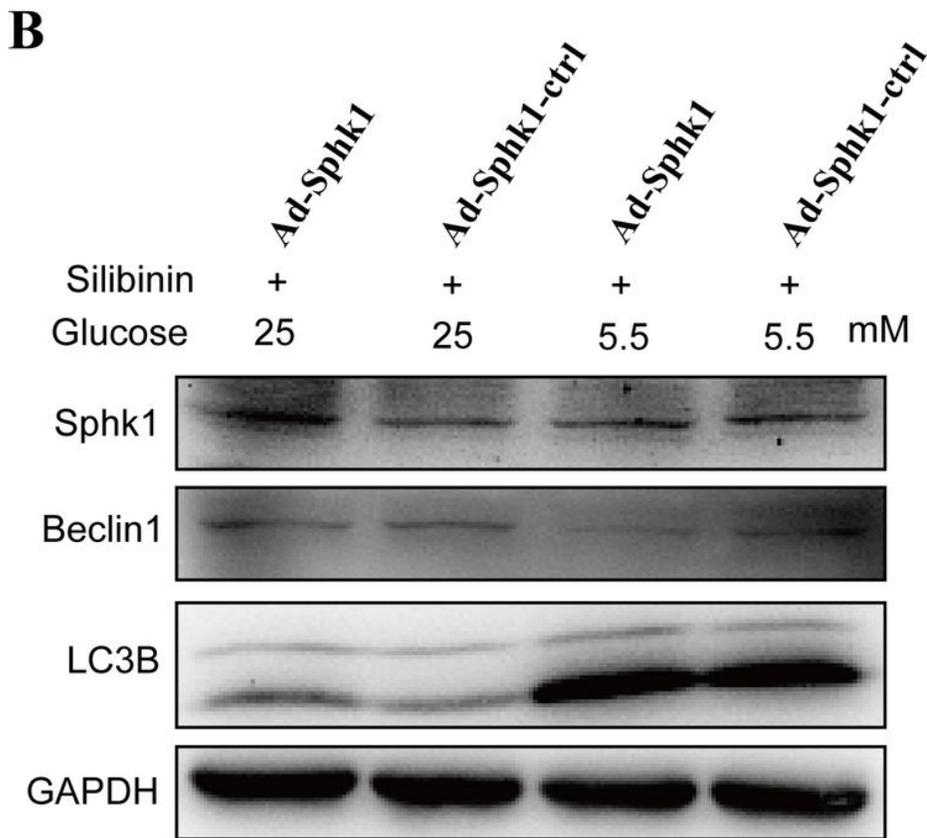
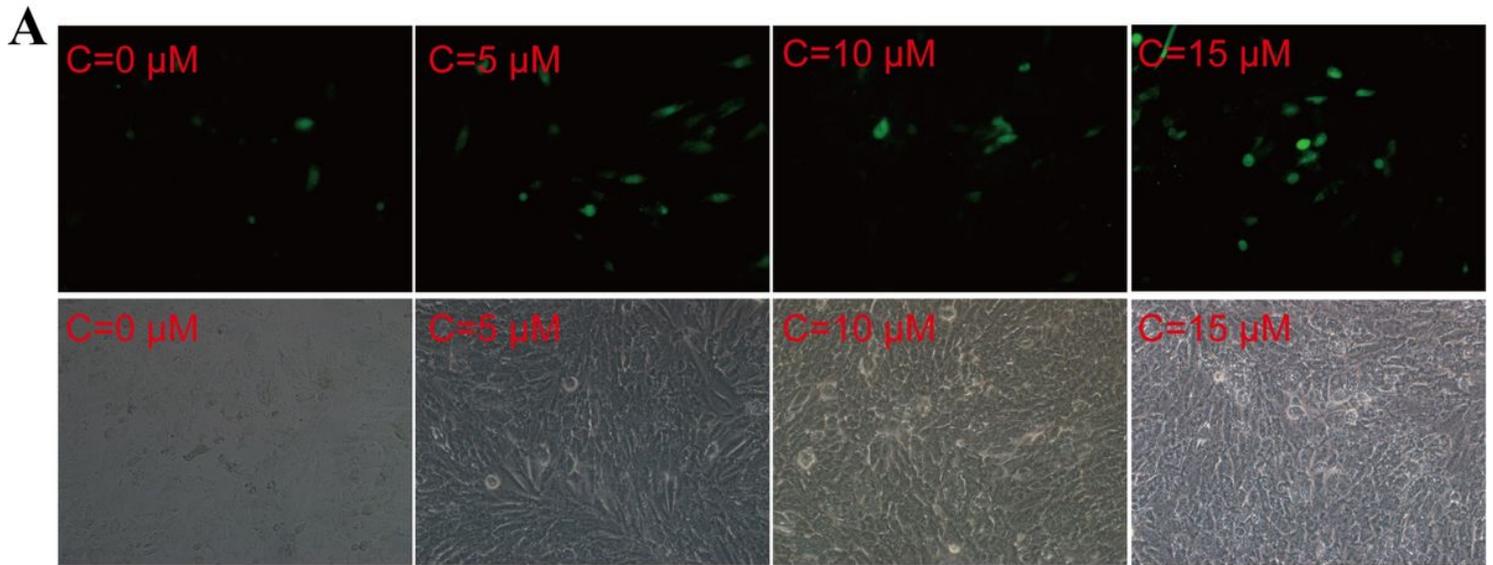


Figure 4

Silibinin promoted autophagy process in H9c2 cells. (A) Silibinin enhanced autophagy activity in a dose-dependent manner. (B) The effects of co-treatments of adenovirus vectors, silibinin and glucose.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [SupplementaryFigure.pdf](#)