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miR-1273g-3p participates in acute glucose fluctuation-induced autophagy, dysfunction, and proliferation attenuation in human umbilical vein endothelial cells

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1Department of Critical Care Medicine, West China Hospital; 2College of Life Science; 3State Key Laboratory of Biotherapy and Cancer Center, West China Hospital; 4College of Chemical Engineering; 5West China School of Preclinical and Forensic Medicine; and 6Anesthesia Laboratory, West China Hospital, Sichuan University, Chengdu, China

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Guo J, Sang Y, Yin T, Wang B, Yang W, Li X, Li H, Kang Y. miR-1273g-3p participates in acute glucose fluctuation-induced autophagy, dysfunction, and proliferation attenuation in human umbilical vein endothelial cells. Am J Physiol Endocrinol Metab 310: E734–E743, 2016. First published February 23, 2016; doi:10.1152/ajpendo.00444.2015.—Acute glucose fluctuations (AGF) often cause high mortality among critically ill patients, but the mechanisms induced by AGF are not clear. Recent studies suggest that endothelial dysfunction is a key factor that leads to high mortality among critically ill patients. Our goal is to evaluate the phenomenon and mechanisms of endothelial dysfunction induced by AGF. In this study, the functions of human umbilical vein endothelial cells (HUVECs) were compared after treatment with sustained high glucose (SHG), AGF in two groups (AGF1 fluctuations between 5 and 16 mM and AGF2 fluctuations between 5 and 25 mM), and normal glucose levels as a control group (CTR). The medium of the groups was changed every 4 h. The influence of AGF on wound healing was also tested on C57BL/6 mice. The results show that cell proliferation, angiogenesis, and migration functions were injured in the SHG and both AGF groups. AGF2 group shows the worse condition in vitro. In vivo, the wound healing was delayed after the AGF treatment. Furthermore, the markers of apoptosis and autophagy were analyzed. We observed that the autophagy changed in all treatment groups, but apoptosis showed no change. To get to know the mechanism of dysfunction and autophagy, we performed the microRNA chip assay and real-time PCR and found miR-1273g-3p remarkably changed in AGF2 group. After the mimic and inhibitor of miR-1273g-3p were transfected during the AGF2 treatment, we found that the dysfunction and autophagy were partially enhanced by miR-1273g-3p mimic and reversed by miR-1273g-3p inhibitor in AGF2 group. Thus, we conclude that AGF can induce more dysfunction and autophagy, and miR-1273g-3p is also an important factor that leads to the injury.

Increasing glycemic variability can greatly increase the risk of mortality among critically ill patients. Several previous studies suggest that the variability in glucose levels over time is an important determinant of mortality among critically ill patients (21, 22, 58). Alternatively, recent evidence shows that glucose fluctuations may influence the development of diabetic complications (4, 47) and may produce more serious injury in organ functions than sustained high blood glucose. Krinsley and Preiser (20) reported that if a blood glucose range of 70–140 mg/dl is maintained for >80% of the time, the chances of survival of nondiabetic critically ill adults will be greatly increased. Organ injury induced by glucose toxicity is commonly observed in both intensive care units and endocrine departments. The damage is also observed in in vitro cell culture and animal studies. Therefore, some authors have proposed that “glycemic variability” is a new therapeutic challenge in diabetes and the critical care setting (7).

The mechanism of the production of glucose fluctuations is variable in several studies (35–39, 57) in both preclinical and clinical research studies. However, this is not appropriate for the critically ill patients who suffer from more random and frequent glucose fluctuations. Therefore, it is important to conduct a study to adequately evaluate the effect of glucose fluctuations in critical care setting.

The endothelial cells are frequently attacked by various diseases, especially for critically ill patients and patients who suffer from diabetes, which may result in its activation and dysfunction (25). According to related researches, a variety of factors can influence the function of endothelial cells, and the mechanisms of this influence are apoptosis and autophagy (9, 12, 33). But so far, few researchers have done research on how endothelial cells are affected by glucose fluctuations. This study aimed at evaluating the injury of human umbilical vein endothelial cells (HUVECs) induced by sustained high glucose (SHG) and two levels of acute glucose fluctuations (AGF). Furthermore, we will offer a further evaluation of the impact of SHG and AGF on autophagy and apoptosis.

MicroRNAs (miRNAs) play a critical gene regulatory role by mediating posttranscriptional gene regulation and RNA silencing. They are small RNA molecules (~23 nt), and they are expressed endogenously. The first miRNAs were discovered in C. elegans, but the regulations of this kind commonly exist in animals and plants (3). Saito et al. (43a) discovered that miR-200c and miR141 are important factors that cause rats’ cardiomyocyte damage treated by glucose fluctuations. It has rarely been studied whether miRNAs involved in glucose fluctuations; endothelial dysfunction; wound healing; autophagy; miR-1273g-3p
fluctuation induce injury in HUVECs. In this study, we focus on the role of upregulated miRNAs on HUVECs injury. Owing to our research, we will be able to get a better understanding of the injury mechanism induced by AGF.

**MATERIALS AND METHODS**

**Cell culture.** The HUVECs were purchased from the Cell Resource Center, Shanghai Institutes for Biological Sciences (Shanghai, China), and maintained in Dulbecco’s modified Eagle’s medium (Gibco; Thermo Fisher Scientific, Waltham, MA) containing 10% fetal bovine serum (Gibco), 100 U/ml penicillin, and 100 μg/ml streptomycin. The primary cell culture medium was changed 24 h after seeding. When the cells reached 80% confluence they were subcultured using 0.01% trypsin-EDTA, which was inactivated by dilution.

HUVECs were seeded at equal density (1.0 × 10^5 cells/ml) into gelatin-coated dishes or wells and allowed to attach overnight. Then they were treated the following experimental conditions for 5 days. The cells were grouped as follows: 1) control (CTR), constant normal glucose medium (5 mM), with fresh medium being changed every 4 h; 2) SHG, sustaining high glucose medium (25 mM), with fresh medium being changed every 4 h; 3) AGF group 1 (AGF1), alternating between 5 and 16 mM every 4 h; and 4) AGF group 2 (AGF2), alternating between 5 and 25 mM every 4 h. This program was chosen from the common glucose fluctuations observed in the critical care department along with fluctuations used during basic research situations. (Fig. 1A).

For autophagy flux analysis, the CTR and AGF2 groups were given DMSO or bafilomycin A1 (10 nM; Sigma) when medium was refreshed simultaneously.

**Cell viability assay.** HUVECs were planted in a 24-well plate (2 × 10^4 cells/well) according to the design. After digestion with trypsin every 24 h, the cells were subjected to cell count calculation using a Countstar automated cell analyzer (Shanghai Ruiyu Biotech, Shanghai, China) according to the manufacturer’s instructions. The data obtained was to investigate the influence of AGF on the HUVECs’ proliferation. All of the experiments were performed in triplicate.

**Matrigel tube formation assay.** This assay was performed as recommended by earlier research (24, 50). Briefly, each well of the 96-well plates was precoated with 50 μl of Matrigel (BD Biosciences, Bedford, MA) and allowed to polymerize for 30 min at 37°C. After treatment as shown in Fig. 1A, the HUVECs (2 × 10⁴ cells/well) were seeded into the Matrigel-coated wells in endothelial cell medium containing 2% FBS at 37°C. Cells started to form capillary-like structures at 4 h and reached their optimum growth 8 h later. Three random tube images were photographed at 4 and 8 h with a digital camera attached to an Olympus inverted digital camera in low power fields (×100). The length of the total tube in each well was measured and calculated using National Institutes of Health (NIH) ImageJ software.

**Endothelial cell migration assay.** The HUVECs wound healing assay was conducted following the method recommended by earlier research (24, 29), with few modifications. The treated HUVECs were seeded in 12-well plates and cultured until the cells reached confluence. The cell monolayer in each well was scraped with a sterile 200-μl pipette tip three times to form parallel lines, followed by washing with sterile PBS 24 h later, the cells along the scraped lines were photographed at the same position in low power fields (×100), and NIH ImageJ software was used for quantification of cell migration assay.

**Apoptosis assay.** Cells were washed, resuspended and examined with the apoptosis detection kit (Beyotime Institute of Biotechnology) according to the manufacturer’s instructions. Stained cells were analyzed by fluorescence-activated cell sorting (FACSCalibur; BD Biosciences)

![Fig. 1. Effect of high glucose on proliferation and migration in human umbilical vein endothelial cells (HUVECs).](http://ajpendo.physiology.org/)

**Fig. 1.** Effect of high glucose on proliferation and migration in human umbilical vein endothelial cells (HUVECs). A: HUVEC treatment design. HUVECs were treated in the experimental condition for 5 days. They were grouped as follows: 1) constant normal glucose medium, alternating fresh medium every 4 h (CTR; 5 mM); 2) sustained high-glucose media, alternating fresh medium every 4 h (SHG; 25 mM); 3) alternating normal- and high-glucose media every 4 h (acute glucose fluctuation group 1 (AGF1); 5–16 mM); and 4) alternating normal- and higher-glucose medium every 4 h (AGF2; 5–25 mM). B: the trend of cell proliferation during 5 days of therapy was evaluated by counting cell nos. every 24 h. C and D: HUVEC matrigel tube formation assay. C: after all groups were cultured on matrigels for 4 or 8 h, tube formation was recorded by phase contrast microscopy. Representative photomicrographs show the formation of tube-like structures. D: the length of tubes formed in the assays was quantified. The total tube length from each of the 5 randomly chosen fields was quantified using ImageJ software. E and F: HUVEC cell migration assay. E: a cell-free area was introduced with a pipette tip, and micrographs were taken at 0 and 24 h after scratch wounding. F: quantification of the results of cell migration assays in E. Representative data in C and E are from one of 3 independent experiments. Representative fields (magnification, ×100). Scale bar, 200 μm. Results are presented as means ± SE from 3 independent experiments. **P < 0.001. NS, no significant difference.
**Transmission electron microscopy analysis.** The HUVECs were fixed with 3% glutaraldehyde at room temperature and postfixed in 1% osmium tetroxide for 50 min. Samples were sent into the electron microscope chamber (West China School of Medicine, Sichuan University) to produce sections. The ultrastructure of cells was studied under a transmission electron microscope (Hitachi, Tokyo, Japan).

**Western blot analysis.** The cells were lysed in RIPA lysis buffer (Beyotime, Beijing, China) supplemented with protease inhibitor cocktail (Roche, Mannheim, Germany), and the protein concentration of the cell lysates was measured by the BCA method (Pierce). Equal amounts of protein were loaded and separated via SDS-PAGE and then transferred onto PVDF membranes (Millipore, Bedford, MA), and the blots were probed with related antibodies as primary antibodies. Membranes were further incubated with secondary antibodies conjugated to horseradish peroxidase, which were detected by an ECL reagent. Quantification of band intensity was carried out using ImageJ software. The results are expressed as the means ± SD.

**MicroRNA microarray and real-time PCR of HUVECs.** Total RNA was prepared from the treatment of cells using TRIzol Reagent (Invitrogen) according to the manufacturer’s instructions. The concentrations and purity of total RNA were determined spectrophotometrically using a NanoDrop 2000C Spectrophotometer (Thermo). RNA integrity was assessed by an Agilent 2100 bioanalyzer (Agilent Technologies). Microarray assay was performed using a service provider (LC Sciences, Hangzhou, China). The assay was performed using μParaflo microfluidic chip. Fluorescence images were collected using a laser scanner (GenePix 4000B; Molecular Device) and digitized using Array-Pro image analysis software (Media Cybernetics). Data were analyzed by first subtracting the background and then normalizing the signals using a LOWESS filter (locally weighted regression). The alteration in the expression levels of miRNAs was defined as those with greater than twofold changes.

Stem-loop qRT-PCR for miRNA was done on a CFX96 Touch Real-Time PCR Detection System (Bio-Rad). RNA integrity was monitored by electrophoresis on a denaturing agarose gel. A first-strand cDNA was prepared from total RNA from each sample using a PrimeScript RT reagent Kit with gDNA Eraser (Perfect Real Time; Takara Biotechnology, Dalian, China) according to the protocols of the manufacturer. The RT reaction was performed using treated total RNA and the RT primer. The cDNA was then used for real-time PCR of microRNAs. U6 was used as endogenous control. Real-time quantitative PCR was performed using the standard SYBR Green PCR protocol (SYBR Premix Ex Taq II (TliRnaseHPlus), Takara, catalogue no. RR820A), and each sample was analyzed in triplicate. The level of miRNA expression was measured using the quantification cycle value. The primers for miRNAs and the control gene were purchased from Ribobio (Guangzhou, China).

**Transfection with miRNA mimics and inhibitor.** The miR-1273g-3p mimics (mciRNA miRNA mimic) and inhibitor (microF miRNA inhibitor) were obtained from Ribobio. After 24 h of being cultured in serum-free medium, cells (3 × 10^5/well, 24-well plates) were transfected with the mimics or inhibitors by using C10511-05 riboFect (C10511-05 riboFect CP Transfection Kit) according to the manufacturer’s instructions. The alternating nonnormal and high concentration glucose (5 mM/25 mM) treatment continued until the end of the test (5 days), and the mimics and inhibitors were transfected at 0 and 72 h each time for 6 h. At last, the cells were harvested for next detections. All of the experiments were performed in triplicate.

**Wound healing in vivo.** Specific pathogen-free, 12- to 14-wk-old male C57BL/6 mice were obtained from a local breeding facility at the Sichuan University. The mice were randomly divided into two groups, sham (CTR; n = 5) and AGF-injected (AGF; n = 5). An excisional splinting model was generated as recommended by earlier research (53), with few modifications. Briefly, all mice were anesthetized through intraperitoneal injection of 10% chloral hydrate (10 g/100 ml). Hair was removed from the dorsal surface before two 6-mm, full-thickness excision skin wounds were created on each side of the midline, using a surgical punch. Different glucose concentration fluid was injected intradermally around the wound every 4 h to produce a glucose fluctuation of wound for 72 h. The glucose was diluted with normal saline to the concentration of 6 and 25 mM. The CTR group was injected intradermally 1 mm away from the wound edge with 6 mM (50 μl/wound) every 4 h (close to normal blood glucose levels of mice). The AGF group was injected with 6 or 25 mM alternatively during the same time points. Both two groups were injected for 72 h, as shown in Fig. 4A. Images were photographed on day 0, 4, 7, 10, and 14 using the same camera and mode. NIH ImageJ software was used for quantification of the wound area. All mouse experiments were carried out in strict accordance with the regulations of and approved by the Animal Care and Use Committee of Sichuan University.

**Statistical analysis.** All statistical analyses were done with GraphPad Prism version 6.0 (GraphPad Software, San Diego, CA). Data are presented as means ± SD. Statistical analysis was performed by Student’s t-test for comparing two groups. The differences among more than three groups were determined using one-way analysis of variance with Bonferroni’s multiple comparison tests to compare each of the two groups. A probability of P < 0.05 indicated statistical significance.

**RESULTS**

**The effect of high glucose (sustained or fluctuating) on the proliferation of HUVECs.** The cells were grouped as follows (Fig. 1A): 1) CTR, constant normal glucose medium (5 mM), with fresh medium being changed every 4 h; 2) SHG medium (25 mM), with fresh medium being changed every 4 h; 3) AGF 1, alternating between 5 and 16 mM every 4 h; and 4) AGF2, alternating between 5 and 25 mM every 4 h.

Whether AGF influenced the proliferation of HUVECs, the Countstar automated cell analyzer was used to calculate the cells number. (Fig. 1B). Among the high-glucose concentration groups (SHG, AGF1, and AGF2), proliferation was significantly decreased compared with the normal glucose group. AGF2 cells showed significantly lower proliferation than the other groups (P < 0.001). However, there is no significant difference in HUVEC proliferation between the SHG and AGF1 groups (P > 0.05).

Distinct differences among the four groups were observed from day 3 to day 5 of treatment. This result proved that high glucose could seriously inhibit HUVEC proliferation, and this effect was enhanced when the cells were exposed to intermittent higher glucose concentrations (AGF2).

**The effect of high glucose (sustained or fluctuating) on inducing dysfunction of HUVECs.** The ability to form a tubule of and approved by the Animal Care and Use Committee of Sichuan University. The effect of high glucose (sustained or fluctuating) on the proliferation of HUVECs. The cells were grouped as follows (Fig. 1A): 1) CTR, constant normal glucose medium (5 mM), with fresh medium being changed every 4 h; 2) SHG medium (25 mM), with fresh medium being changed every 4 h; 3) AGF 1, alternating between 5 and 16 mM every 4 h; and 4) AGF2, alternating between 5 and 25 mM every 4 h.

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**The effect of high glucose (sustained or fluctuating) on inducing dysfunction of HUVECs.** The ability to form a tubular-like network (Fig. 1, C and D) and cell migration was used to characterize the function of HUVECs (Fig. 1, E and F).

We investigated the effects of high glucose on endothelial cell angiogenic potentials by using the Matrigel matrix tube formation assay. The assay is a well-established system for examining angiogenesis under in vitro conditions. All treated HUVEC groups cultured in Matrigel began to fuse into
tubular networks 4 h later, and the tubular networks reached their maximum at 8 h (Fig. 1C). The HUVECs cultured in CTR medium formed a more extensive tube network than others did. By contrast, the HUVECs cultured in AGF2 medium formed only separate clusters or smaller networks. There is no significant difference between SHG and AGF1 (Fig. 1D).

To further examine the effect of high glucose on endothelial cell migration, we performed a wound-healing assay to characterize the cell migration response in HUVECs. Wound scratch was introduced according to design conditions (shown in Fig. 1A), and the wound area was observed 24 h later. Compared with the CTR group, high glucose medium inhibited the wound-healing ability of the HUVECs (Fig. 1E). AGF2 showed more serious inhibition than the AGF1 and SHG, but there was no statistically significant difference between AGF1 and SHG (Fig. 1F). These studies indicate that high glucose can induce dysfunction of HUVECs, and AGF2 causes the most serious dysfunction.

The mechanism of inhibition of HUVEC proliferation in AGF2 is autophagy but not apoptosis. We investigated whether the dysfunction induced by AGF2 was related to apoptosis or autophagy. The expression of apoptosis-associated gene was measured by Western blot (Fig. 2, A and B). High glucose (SHG, AGF1, and AGF2) does not significantly alter the cleaved caspase 3 protein level compared with CTR group. After being labeled by annexin V and propidium iodide, cell apoptosis was analyzed using flow cytometry (Fig. 2, E and F). The apoptotic (annexin V positive) rate was calculated (Fig. 2F). There was no significant difference among the four groups. Apoptotic cell rate analysis conforms to the expression trends of apoptosis-associated proteins (caspase 3 and cleaved caspase 3).

LC3-II is a key protein marker for autophagic activity (18). The autophagy-associated gene (LC3 and P62) expression was detected by Western blot analysis and densitometry (Fig. 2, A and B). Compared with CTR, the amount of LC3-II in SHG or AGF2 is dramatically increased. The expression of LC3-II is also upregulated in AGF1, but the increment is lower than AGF2. LC3-II and P62 do not influence the expression of P62 compared with CTR. In contrast, the P62 expression in SHG and AGF2 is lower than CTR. The expression of P62 induced by AGF2 is significantly decreased compared with AGF1 and CTR.

The mechanism of LC3-II increment lies in the fact that AGF2 either promotes or blocks the basal autophagy. lysosomal protease inhibitors are needed to clarify the mechanism. After bafilomycin A1 (an inhibitor of the lysosome acidification) blocked autophagic flux, levels of LC3-II and P62 are increased in AGF2 (Fig. 2, C and D). This result indicates that the increment of LC3-II and decrement of P62 in AGF2 are caused by enhancement of autophagic flux, not inhibition of autophagic degradation. These results suggest that autophagy may play a crucial role in AGF2. Based on the data in Fig. 1, AGF2 causes the most serious dysfunction. So, we chose AGF2 as the main research group.

To verify the Western blot and flow cytometry results, transmission electron microscopy (TEM) analysis was performed. Because the AGF2 group autophagy maker was more significant, we did the test only in the CTR, SHG, and AGF2 groups (Fig. 2G). As shown in the TEM photos, massive vacuolization (indicated by arrows in Fig. 2G) in the cytoplasm and autolysosome formation (indicated by arrowheads in Fig. 2G) are observed in both SHG- and AGF2-treated HUVEC cells. There are more autophagic vacuoles per cell in AGF2 group (Fig. 2G, right). In contrast, these features of autophagy were rarely observed in the CTR-treated cells (Fig. 2G, left).

The results demonstrate that AGF2 induces autophagy in HUVECs, not apoptosis. AGF1 failed to induce the same serious autophagy.

Acute glucose fluctuations induced dysfunction and autophagy of endothelial cells via miR-1273g-3p. MicroRNAs play an important role in gene posttranscriptional repression. The regulatory roles are prevalent in animals and plants (2). We performed miRNA array analysis for AGF2- or CTR-treated HUVECs.

The miRNA expression array indicated that a total of 13 miRNAs are differentially expressed (fold change >2) between the AGF2 and CTR groups. A heat map of these 13 changed miRNAs was generated based on miRNAs expression in AGF2-treated HUVECs compared with the CTR group (Fig. 3A). To confirm the expression levels of miR-1273g-3p, real-time PCR was performed. The data of the real-time PCR is consistent with the microarray data (Fig. 3B). Those results indicate that miR-1273g-3p expression is increased in the AGF2 group.

Owing to the observed increment of miR-1273g-3p in the AGF2 group, we hypothesize that miR-1273g-3p may play a role in the injury process. To test this hypothesis, we transfected miR1273g-3p mimic or inhibitor within the AGF2 treatment program. The miR-1273g-3p mimic significantly enhances the inhibition of proliferation in AGF2, but the miR-1273g-3p inhibitor partially reverses the inhibitory effect (Fig. 3C). The trends of mimic and inhibitor in wound-healing assay are consistent with proliferation analysis (Fig. 3, D and E). The inhibitor reverses the inhibition on migration induced by AGF2 in HUVECs. However, neither the mimic nor the inhibitor affected the tube formation assay compared with AGF2 (data not shown).

To define affection of miR-1273g-3p on autophagy induced by AGF2, we analyzed the autophagy maker (LC3-II) expression by Western blot analysis and densitometry (Fig. 3, F and G). The mimic remarkably enhances the LC3-II expression, and the inhibitor reverses the LC3-II expression compared with AGF2. These results indicate that miR-1273g-3p partially participates in regulating autophagy induced by AGF2, migration attenuation, and proliferation inhibition in HUVECs.

AGF delays wound healing in vivo. We demonstrated that AGF2 induced dysfunction in HUVECs in vitro. Therefore, we hypothesized that AGF might inhibit cutaneous wound angiogenesis and delay wound healing in vivo. After the induction of a full-thickness cutaneous wound (6 mm in diameter), a different concentration of glucose injection was injected into the skin around wound edge for 72 h. The mice were grouped as AGF and CTR. During 72 h of treatment the CTR mice were treated with 6 mM glucose (closely normal mice blood glucose), with repeated injection every 4 h (Fig. 4A). The AGF mice were treated with glucose fluctuated between 6 and 25 mM and alternated every 4 h (Fig. 4A). Figure 4B shows the initial phase of clotting and crust formation in both groups. About 10 days later, CTR wounds were almost healed, but AGF wounds were still covered with a crust. There is a significant difference between two groups from day 4 to day 10.
Fig. 2. AGF2 suppress HUVEC proliferation through autophagy, not apoptosis. A–D: autophagy-related proteins (LC3, P62) and apoptosis-related protein (caspase 3) expression. A: After HUVECs were treated by different glucose conditions (CTR, SHG, AGF1, and AGF2), cell lysates were analyzed by Western blotting with different antibodies. β-Actin was used as a loading control. B: quantification of Western blot data in A from 3 separate experiments. C: AGF2 was treated with bafilomycin A1 (BA) while the glucose fluctuated. After treatment, the cell lysates of 3 groups (AGF2, CTR, and AGF2 + BA) were analyzed for expression of P62 and LC3-II. D: quantification of Western blot data in C from 3 separate experiments. The amount of each protein was normalized against the amount of β-actin. E and F: apoptotic cells were analyzed by flow cytometry after high-glucose treatment. E: HUVECs were treated as Fig. 1A and then stained with annexin V and propidium iodide. The apoptotic cells (the annexin V-positive cells) are indicated as the percentage of gated cells. F: quantification apoptotic cells from 3 separate tests. G: autophagosome is observed under transmission electron microscopy. N, cell nucleus; arrow, autophagic vacuoles; arrowhead, autolysosome. Scale bars, 2 (3 left images, magnification = 5,000) and 1 μm (far right image, magnification = 10,000). Results are presented as means ± SE of 3 independent experiments. Representative data is from one of 3 independent experiments. *P < 0.05; **P < 0.001.
miR-1273g-3p ATTENDS IN AGF-INDUCED DYSFUNCTION IN HUVECs

Fig. 3. miR-1273g-3p contributes to a part of the influence of AGF2 on HUVECs. A: miRNAs are associated with alternate higher-glucose treated HUVECs by applying a significant threshold of 2-fold change. B: real-time PCR analysis of miR-1273g-3p expression in AGF2 and CTR. HUVECs were treated according to design plan (Fig. 1A) in 24-well plates. The AGF2 group was transfected with miR-1273g-3p mimic or inhibitor at the day 0 and day 3 time points (AGF2 + mimic, AGF2 + inhibitor). After 5 days of continuous treatment, the HUVECs were analyzed further. C: cell proliferation was measured by counting cell nos. after 5 days of treatment. D and E: HUVEC cell migration assay. After 5 days treatment, the transfected HUVECs (AGF2 + mimic, AGF2 + inhibitor) or parallel HUVECs (CTR, AGF2) were harvested and analyzed (see Fig. 1E). D: micrographs were taken at 0 and 24 h after scratch wounding. Representative fields (magnification, ×100); scale bar, 200 μm. E: quantification of the results of cell migration assays in D, F and G: autophagy-related protein (LC3-II) expression. F: after being treated with different glucose conditions or transfected miRNA mimic/inhibitor (*CTR; aAGF2; bAGF2 + miR-1273g-3p mimic; cAGF2 + miR-1273g-3p inhibitor), cell lysates were analyzed by Western blotting with LC3 antibody. β-Actin was used as a loading control. G: densitometric quantification of the Western blotting gel data in F using ImageJ software. D and F: these data are representative of 3 experiments. Results are presented as means ± SE from 3 independent experiments. *P < 0.05; **P < 0.001.
These data show that AGF could delay wound healing in vivo.

DISCUSSION

Lots of research shows that glucose fluctuations are more dangerous than sustained high blood glucose (4, 21), especially for critically ill patients and diabetes patients, but the exact mechanism is still not clear. Previous studies on glucose fluctuations have focused mainly on cell cultures with different glucose concentrations, which were often changed once a day (35–37, 39, 57); a few changed concentrations every 12 h, but not all of those fluctuations were fit for the critically ill patients whose glucose fluctuations were random and rapid (1, 10, 19). In addition, as a novel cell metabolism pathway, autophagy is more sensitive to energy changes. Research has shown that SHG could influence the function of endothelial progenitor cells through autophagy (16). Therefore, glucose fluctuations on endothelium cells can, in theory, influence the cell metabolism and function through autophagy. However, there is no related research about glucose fluctuations and endothelium cell autophagy.

According to previous studies, glucose fluctuations could induce more serious HUVEC dysfunction, which may be related to apoptosis (35, 38, 39) or senescence (28). But there is no research on the expression of autophagy, and the interval time of glucose fluctuations is always longer. Therefore, we assume that, compared with SHG, AGF can cause more serious injury and that the injury mechanism can be associated with autophagy. For this purpose, this study has successfully established an AGF cell model by intermittently replacing high-concentration glucose medium, which is of great importance to the study of the cell injury mechanism.

Our experiments show that compared with SHG and AGF1, AGF2 induced more serious injury, such as cell proliferation, tube formation, and migration. What’s more is that the injury induced by AGF was also observed in mice vivo. Compared with the designs of previous studies, our designs are closer to the blood glucose fluctuations observed in critically ill patients, and we did not compare these results with those of other research designs in which researchers might make cells fluctuate one time every day or every half-day.

Programmed cell death has been classified as apoptosis, necrosis, and autophagy. The main apoptotic pathways converge on aspartate-specific cysteine proteases (caspases; initiating 8, 9, and 10 and executioner 3, 6, and 7) (46). These caspases regulated the cleaved and activated downstream apoptotic proteins to regulate cell death. As one of the lysosomal degradation pathways, autophagy was essential to cell survival, differentiation, development, and homeostasis and played an adaptive role in protecting organisms against various pathologies such as infections, neurodegeneration, and aging, etc. The double-edged sword functions of autophagy could lead to either cell survival or cell death according to different stressors or the cellular environment (34, 55). In anti-cancer treatments, both autophagic inhibitor (44) and inducer have been considered as therapeutic means (8) and have often been applied to clinical practice. However, it remains unknown whether autophagy and apoptosis can be induced by this kind of AGF. If autophagy and apoptosis can be induced by AGF, how they come into being is still not very clear. Thus, investigating those phenomena and mechanisms is vital for the development of novel preventive and therapeutic approaches for glucose toxicity.
Interestingly, in our experiment, we did not observe extensive cell apoptosis by flow cytometry and Western blot after treatment by AGF for 5 days, and the results differed from those of earlier studies. We infer that different results may be caused by different culture conditions, such as higher glucose concentration and longer duration of SHG and AGF. Furthermore, autophagy was detected by electronic microscope and special protein markers such as LC3-II and P62 (Fig. 2A). LC3 protein was considered to be a key autophagy marker. In autophagy, LC3 localizes on autophagosomes and transforms from LC3-I to LC3-II during autophagosome formation (42). LC3 may also control the size of autophagosomes (52). P62 is a crucial mediator for the ubiquitinated clearance in autophagosome and may be degraded by itself once autophagy occurs (30). In addition, several biological processes such as cell signaling and differentiation processes occurred, especially with the removal of toxic protein aggregation (32). Degradation of P62 is associated with selective autophagy (17), but LC3 is involved in nonselective autophagy (51). Research shows that the amount of LC3-II is better than the ratio of LC3-II/LC3-I or LC3-II/(LC3-I + LC3-II) in terms of the process of autophagy (31). Therefore, we chose the amount of LC3-II for further research. Compared with control group, both the AGF2 and SHG groups had a significantly higher LC3-II along with a decreased P62 level. It is interesting that the result of p62 is not consistent with LC3-II in AGF1. We propose that glucose oscillation of AGF1 (5–16 mM) is too small to achieve the threshold triggering alteration of LC3-II and P62. Further research about AGF1 needs to be done. We chose AGF2 as the main research group because AGF2 causes the most serious dysfunction by glucose fluctuation compared with SHG and AGF1. (Fig. 1). What’s more is that with electronic microscopy we observed lots of autophagic vacuoles in the treatment group. We conclude that AGF2 also induces cell autophagy.

MicroRNAs (miRNAs, 20–26 nucleotides in length) are a family of highly conserved noncoding single-stranded RNAs, and they can regulate gene expression as the key posttranscriptional regulators in many physiological and pathological processes (14), including apoptosis (11), cell cycle, proliferation, and development, etc. (45). Several miRNAs have played a vital role in endothelial cell pathophysiology, such as cellular senescence, angiogenesis, and vascular inflammation (48). Some miRNAs can either activate or inhibit in the process of autophagy and hence, influence the cells’ functions (48, 49, 56). In a previous experiment, Li et al. (26) found that high-glucose-induced suppression of migration might be associated with miR-221 in HUVECs. However, which miRNAs will be changed in the process of AGF? If some miRNAs are changed in the injury process, which mechanism caused their change? The answers to these questions are not clear.

To study the relationship between injury and miRNAs, we chose the most seriously injured group and the control group for the miRNA chip assay. According to our miRNA chip and our reconfirmed results by real-time PCR, miR1273g-3p in HUVECs is significantly upregulated, which has been discovered in this study for the first time. miR-1273g-3p has rarely been studied. Ivashchenko et al. (15) reported the predicted target genes of miR-1273g-3p, and Hou et al. (13) found that miR-1273g-3p can promote A549 cell migration by targeting CNR1. According to the present research results, we postulate that miR1273g-3p should be partially responsible for HUVEC functions and injury induced by AGF. Therefore, the transfection by miRNA mimic and inhibitor of miR-1273g-3p was conducted. miRNA mimic and inhibitor also changed the proliferation and migration of HUVECs induced by AGF. This might be a new therapy target in prohibiting the injury induced by AGF. There are still some miRNAs altered in AGF2 groups. Those miRNAs need to be further studied in the further research work. In this article, we focus on miR1273g-3p.

In previous research (6, 40, 43), the results showed that oxidative stress and inflammation are two important mechanisms in glucose fluctuations that induce diabetes-associated cardiovascular damage. All of those results are observed in patients. Some scientists (5, 23) discover that oxidative stress can be induced by high glucose. We did evaluate the inducible nitric oxide synthase and reactive oxygen species (data not shown), but we did not achieve desirable results. We propose that the mechanisms of glycemic variability inducing cardiovascular damage in patients are different from HUVEC damage induced by AGF. Therefore, we have focused on the autophagy and miRNAs in this study.

Because evidence about the AGF model in animals has been lacking until now, our main task is to study the influence of AGF on endothelial cells in this study. To study in vivo, we referred to the study methods of Rossiter et al. (41), who observed the drug’s influence through local treatment on animals. Although we observed a wounding-curing barrier in AGF group, we believe that if we can control the animal’s fluctuations of blood glucose systematically, the results should be more desirable. Additionally, the in vivo mechanism needs further study.

In conclusion, our study shows that AGF indeed causes the dysfunction of HUVECs. Furthermore, the upregulation of autophagy and miR-1273g-3p was confirmed for the first time in this culture environment. miR-1273g-3p might be a novel regulator in cell proliferation and migration function in the treatment by AGF. In the future, the relationship between autophagy and the miRNA requires in-depth research. Therefore, our research offers a novel therapeutic strategy to deal with the injury induced by AGF.

DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

REFERENCES


