Apelin-13 reduces high glucose-induced mitochondrial dysfunction in cochlear hair cells by inhibiting endoplasmic reticulum stress

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Abstract. The complex manifestation of diabetic hearing loss and the relative inaccessibility of the inner ear contribute to the lack of research. The present study aimed to reveal the role of Apelin-13, a critical regulator of lipid metabolism, in diabetes-induced hearing loss. Cochlear hair cells treated with high glucose (HG) were adopted as an *in vitro* research model, and the impacts of Apelin-13 on cellular oxidative stress, apoptosis, mitochondrial dysfunction and endoplasmic reticulum (ER) stress were determined. In addition, cells were treated with the ER stress agonist tunicamycin to further explore its potential role in the regulatory effects of Apelin-13. Apelin-13 inhibited oxidative stress and apoptosis in the HG-induced cells. Additionally, Apelin-13 elevated mitochondrial membrane potential and ATP production, whereas it reduced mitochondrial reactive oxygen species levels. The levels of ER stress-related proteins exhibited a downward trend in response to Apelin-13. By contrast, tunicamycin reversed the effects of Apelin-13 on the aforementioned aspects, suggesting the role of ER stress in the regulatory effects of Apelin-13. In conclusion, the present study elucidated the protective role of Apelin-13 in ameliorating HG-induced mitochondrial functional impairment in cochlear hair cells by inhibiting ER stress.

Introduction

The prevalence of diabetes is increasing worldwide, posing a significant threat to various body systems, including large blood vessels, microvessels and nerves (1,2), thereby resulting in a spectrum of diabetes-related complications, such as diabetic nephropathy, retinopathy and cardiomyopathy (3). However, the insidious and multifactorial nature of diabetic hearing loss often results in it being a clinically underdiagnosed complication of diabetes, as highlighted by the American Diabetes Association (4). A significant prevalence of hearing impairment among individuals with type II diabetes has been identified, ranging from 34.4 to 60.2% (5). Hearing loss and diabetes share some common risk factors since damage to the insulin system can lead to problems anywhere in the body. Insulin receptors, glucose transporters and components of insulin signaling are present in the sensory receptors and supporting cells of the cochlea, suggesting that hearing is susceptible to impairment by glucose utilization (6). Notably, comprehensive meta-analyses have confirmed the persistent correlation between diabetes and hearing impairment, irrespective of diabetes type, underscoring the need to identify the intricate mechanisms linking diabetes to auditory dysfunction (7). Oxidative stress induced by elevated glucose levels is recognized as a pivotal factor precipitating cellular apoptosis in diabetes (8). Central to cellular aerobic respiration, reactive oxygen species (ROS) regulation and various signaling pathways (9), mitochondria have emerged as a crucial focal point for understanding the disease mechanism and formulating innovative therapeutic interventions.

Apelins are primarily found in endocardial and vascular endothelial cells, suggesting that they may originate from these tissues. Central sources of apelin, such as from magnocellular neurons, also contribute to circulating Apelins. Apelins act in an autocrine/paracrine manner (10). In the context of regulating glucose levels, the role of Apelin-13, which is a critical regulator of lipid metabolism, has assumed significance. Recent research has highlighted the diverse protective roles of Apelin-13 in mitigating diabetic complications, such as promoting nitric oxide production and ameliorating renal tissue fibrosis in diabetic nephropathy (11). Moreover, Apelin-13 has been demonstrated to possess the potential to improve glucose and lipid metabolism in mice with gestational diabetes, and to reduce oxidative stress and inflammation via activation of the PI3K/AKT pathway. In addition, it confers a protective effect on pancreatic islets, thereby improving pregnancy outcomes (12). Diabetes is an independent risk factor for vascular calcification (13), and Apelin-13 may reduce high glucose (HG)-induced calcification of mouse aortic vascular smooth muscle cells by inhibiting ROS-mediated DNA damage (14). Notably, its potential in preventing noise-induced cochlear damage and hearing loss (15), as well as in enhancing the survival of hair cell-like cells against oxidative stress damage (16), is gaining attention. However, the precise role of Apelin-13 in diabetes-induced hearing loss remains to be elucidated, necessitating further exploration.

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Given that cochlear hair cells are highly sensitive to damage (17), they were adopted as an *in vitro* research model in the present study, and the hypothesis that Apelin-13 could play a protective role against HG in cochlear hair cells by maintaining mitochondrial function was proposed. The present study set the experimental foundation for the application of Apelin-13 as a potential treatment strategy for diabetic hearing loss.

Materials and methods

Cell culture and treatment. The mouse cochlear hair cell line HEI-OC1 (cat. no. M8-0401; OriCell, Guangzhou Cyagen Biotech Co. Ltd.) was cultured in DMEM supplemented with 5% FBS and 1% penicillin-streptomycin (all from Gibco; Thermo Fisher Scientific, Inc.) in an atmosphere containing 5% CO_2 at 37˚C. HEI-OC1 cells were starved without serum for 12 h to synchronize cell proliferation, after which, they were pretreated with Apelin-13 (0.01, 0.05 and 0.1 nM; Selleck Chemicals) for 24 h (18) and then cultured under HG (25 mM glucose) conditions. For mechanistic studies, HEI-OC1 cells were pretreated with the endoplasmic reticulum (ER) stress agonist tunicamycin (TM; 5 μg/ml; Selleck Chemicals) for 6 h.

Cell Counting Kit-8 (CCK-8). HEI-OC1 cells were seeded in a 96-well plate ($5x10^3$/well), pretreated with Apelin-13 (0.01, 0.05 and 0.1 nM) for 24 h and cultured with or without HG for 48 h. CCK-8 reagent (10 μl; Dalian Meilun Biology Technology Co., Ltd.) was then added to the wells and, after 2 h of co-culture at 37˚C, the absorbance at 450 nm was recorded using a microplate reader (Tecan Group, Ltd.). Cell viability was calculated based on a standard curve.

Oxidative stress indicators. Oxidative stress indicators, including malondialdehyde (MDA; cat. no. S0131; Beyotime Institute of Biotechnology), ROS (cat. no. HL10124.3; Haling), superoxide dismutase (SOD; cat. no. S0101) and glutathione peroxidase (GSH-Px; cat. no. S0056; both from Beyotime Institute of Biotechnology), were determined in HEI-OC1 cells using commercial assay kits according to the manufacturers' instructions. The cells were lysed in an ice bath, centrifuged at 10,000 x g for 10 min at 4˚C, and the supernatants were collected for subsequent determination. The samples were incubated with specific working solutions and the absorbance was recorded.

Flow cytometry. Apoptosis of HEI-OC1 cells was determined using flow cytometry (FACSCanto; BD Biosciences) with an Annexin V-FITC/PI assay kit (cat. no. KGA108; Nanjing KeyGen Biotech Co., Ltd.). The experiment was performed according to the kit manufacturer's instructions. HEI-OC1 cells were gathered and centrifuged at 300 x g for 5 min at 4˚C, the supernatant was discarded and the cells were washed once with PBS. After further centrifugation, the cells were suspended in 500 μl 1X Annexin V binding buffer, and were then treated with 5 μl Annexin V-FITC and 5 μl PI. HEI-OC1 cells were incubated in the dark for 15 min and apoptosis was analyzed using FlowJo 10.6.2 software (BD Biosciences).

Caspase3 activity. Routine cell lysis and supernatant collection following centrifugation were performed as aforementioned, followed by protein quantification. Subsequently, 45 μl Reaction buffer, 50 μl samples to be tested and 5 μl Ac-DEVD-pNA (cat. no. E-CK-A383; Elabscience Biotechnology, Inc.) were sequentially mixed. The absorbance was recorded at 405 nm after incubation at 37˚C for 1-2 h.

Mitochondrial membrane potential (MMP) and mitochondrial ROS (mtROS). JC-1 probe (HY-K0601) and MitoSOX Red probe (cat. no. HY-D1055; MedChemExpress) were separately used to identify MMP and mtROS. The probes were dissolved in DMSO and diluted to a 2 μM working solution with serum-free DMEM. HEI-OC1 cells were incubated with working solution for 20 min in the dark, and then washed twice with PBS. Images were then acquired under a fluorescence microscope (Olympus Corporation).

ATP level detection. ATP levels in HEI-OC1 cells were measured using an ATP detection kit (cat. no. S0026; Beyotime Institute of Biotechnology). The supernatant of the cell lysate was obtained as aforementioned. ATP levels were measured according to the kit's instructions, and the absorbance was recorded at 405 nm.

Western blotting. Proteins were extracted from HEI-OC1 cells after lysis using RIPA buffer (cat. no. BL504A; Biosharp; Labgic Technology Co., Ltd.) on ice, and the protein concentration was determined using the bicinchoninic acid method and normalized. Subsequently, SDS-polyacrylamide gel electrophoresis on 6 or 8% gels was performed to separate proteins (20 μg/lane). Protein samples were then transferred to PVDF membranes (MilliporeSigma) and the membranes were blocked with 5% BSA (Sigma-Aldrich; Merck KGaA) at room temperature for 1 h. Diluted primary antibodies against CHOP (cat. no. 15204-1-AP; 1:1,000; Proteintech Group, Inc.), GRP78 (cat. no. 11587-1-AP; 1:5,000; Proteintech Group, Inc.), XBP1 (cat. no. 24168-1-AP; 1:1,000; Proteintech Group, Inc.), phosphorylated (p)-PERK (cat. no. 3179; 1:1,000; Cell Signaling Technology, Inc.), PERK (3192; 1:1,000; Cell Signaling Technology, Inc.) and β-actin (cat. no. 81115-1-RR; 1:10,000; Proteintech Group, Inc.) were used to incubate the membranes at 4˚C overnight, followed by incubation with a HRP-conjugated secondary antibody (cat. no. SA00001-2; 1:5,000; Proteintech Group, Inc.) at 37˚C for 1 h. The ECL solution (Vazyme Biotech Co., Ltd.) was then added to evenly cover the membranes for 1 min, and densitometric analysis was performed using the Image-Pro Plus 7.0 software (Media Cybernetics, Inc.).

Statistical analysis. Data are presented as the mean ± SD of three independent experiments, and data analysis was performed using SPSS 25.0 software (IBM Corp.). Data were analyzed using one-way ANOVA followed by Tukey's post hoc test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Apelin-13 inhibits oxidative stress and apoptosis in HG-treated HEI-OC1 cells. The results of the CCK-8 assay exhibited that Apelin-13 at concentrations of 0.01, 0.05 and 0.1 nM had no

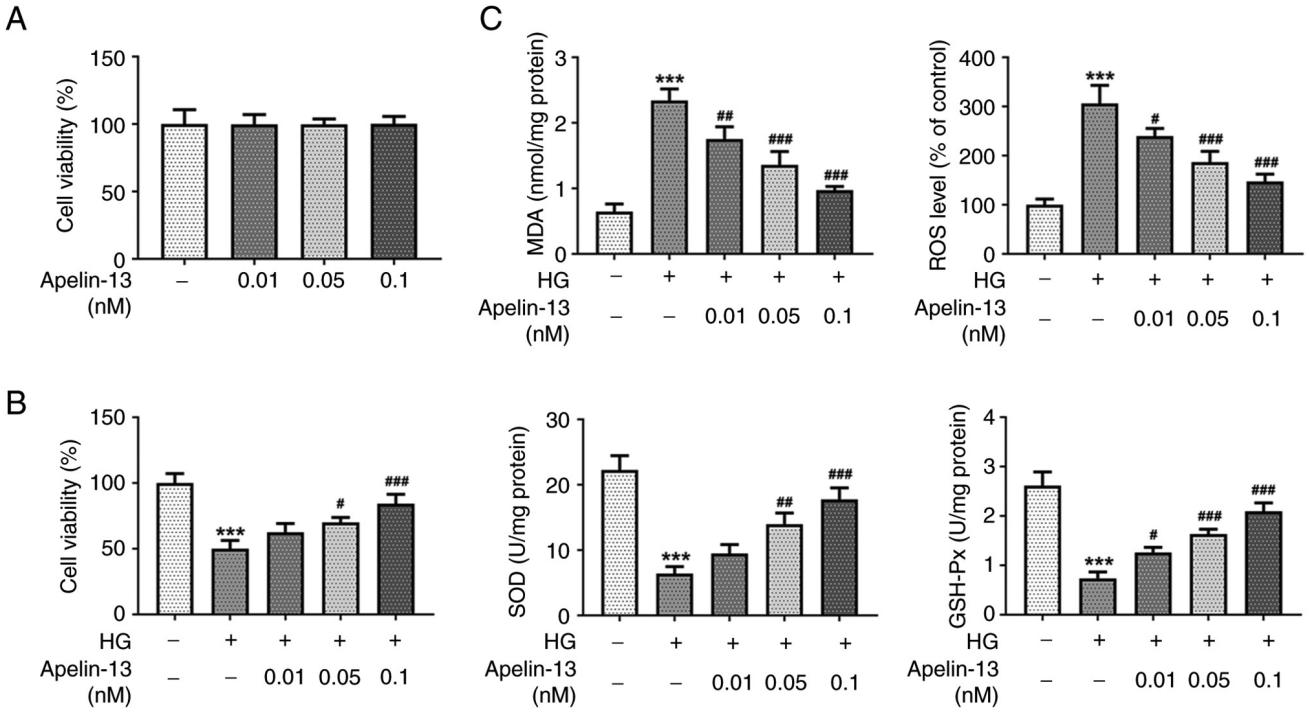

Figure 1. Apelin-13 inhibits oxidative stress in HG-treated HEI-OC1 cells. The effect of Apelin-13 (A) without or (B) with HG on HEI-OC1 cell viability was determined using the Cell Counting Kit 8 assay. (C) The level of oxidative stress in HEI-OC1 cells was assessed by measuring MDA and ROS levels, and SOD and GSH-Px activities. ***P<0.001 vs. control; $^{\#}$P<0.05, $^{\#\#}$P<0.01 and $^{\#\#\#}$P<0.001 vs. HG. HG, high glucose; MDA, malondialdehyde; ROS, reactive oxygen species; SOD, superoxide dismutase; GSH-Px, glutathione peroxidase.

significant effect on HEI-OC1 cell viability (Fig. 1A). However, in HG-treated cells, HG caused a decrease in cell viability. As the concentration of Apelin-13 treatment increased, the cell viability increased in a concentration-dependent manner (Fig. 1B). The levels of oxidative stress in HEI-OC1 cells were assessed by determining changes in indicators. Compared with those in the control group, the MDA and ROS levels in the HG group were significantly increased, while the activities of SOD and GSH-Px were significantly decreased. Apelin-13 treatment could reduce MDA and ROS levels, and increase SOD and GSH-Px activities in HG-treated cells in a concentration-dependent manner (Fig. 1C). Flow cytometric analysis revealed that the proportion of apoptotic cells increased in the HG group, and Apelin-13 treatment could significantly reduce cell apoptosis (Fig. 2A and B). Meanwhile, caspase3 activity in HEI-OC1 cells displayed an upward trend under the influence of HG, and Apelin-13 reduced the increase in caspase3 activity in a concentration-dependent manner (Fig. 2C).

Apelin-13 reduces mitochondrial dysfunction in HG-treated HEI-OC1 cells. The JC-1 probe detected a decrease in aggregates in HEI-OC1 cells in the HG group, accompanied by an increase in monomers, indicating a decrease in MMP. Apelin-13 treatment maintained MMP, and the group treated with a concentration of 0.1 nM had the best effect (Fig. 3A). In addition, HG induced an increase in mtROS production and Apelin-13 decreased the amount of mtROS in a concentration-dependent manner (Fig. 3B). Notably, ATP production of HEI-OC1 cells in the HG group was impaired and lower ATP levels were detected than those in the control group. As the concentration of Apelin-13 treatment increased, ATP production levels were restored in a concentration-dependent manner (Fig. 3C). Western blotting revealed that HG induced ER stress, and the expression levels of related proteins CHOP, GRP78, XBP1 and p-PERK/PERK in HEI-OC1 cells were significantly increased. However, their levels were also affected by Apelin-13 treatment and demonstrated a downward trend compared with those in the HG group (Fig. 3D).

ER stress agonist reverses the effects of Apelin-13. To further explore the potential role of ER stress in the regulatory effects of Apelin-13, HEI-OC1 cells were pretreated with TM to stimulate ER stress. Compared with in the HG + Apelin-13 group, additional TM treatment significantly increased MDA and ROS levels, while SOD and GSH-Px activities were decreased (Fig. 4A). The results of flow cytometry (Fig. 4B and C) and the caspase3 kit (Fig. 4D) revealed that TM elevated the proportion of apoptotic cells and intracellular caspase3 activity. These results indicated that TM destroyed the inhibitory effect of Apelin-13 on cellular oxidative stress and apoptosis. Subsequently, MMP was re-evaluated to confirm the effect of TM on mitochondrial function. Additional TM treatment lowered the MMP (Fig. 5A), with a concomitant increase in mtROS, as reflected using the MitoSOX Red probe (Fig. 5B). Similarly, the ATP levels increased by Apelin-13 were affected by TM, resulting in a significant decrease in cellular ATP production (Fig. 5C).

Discussion

The gradual onset and complex manifestation of diabetic hearing loss contribute to the lack of research on it,

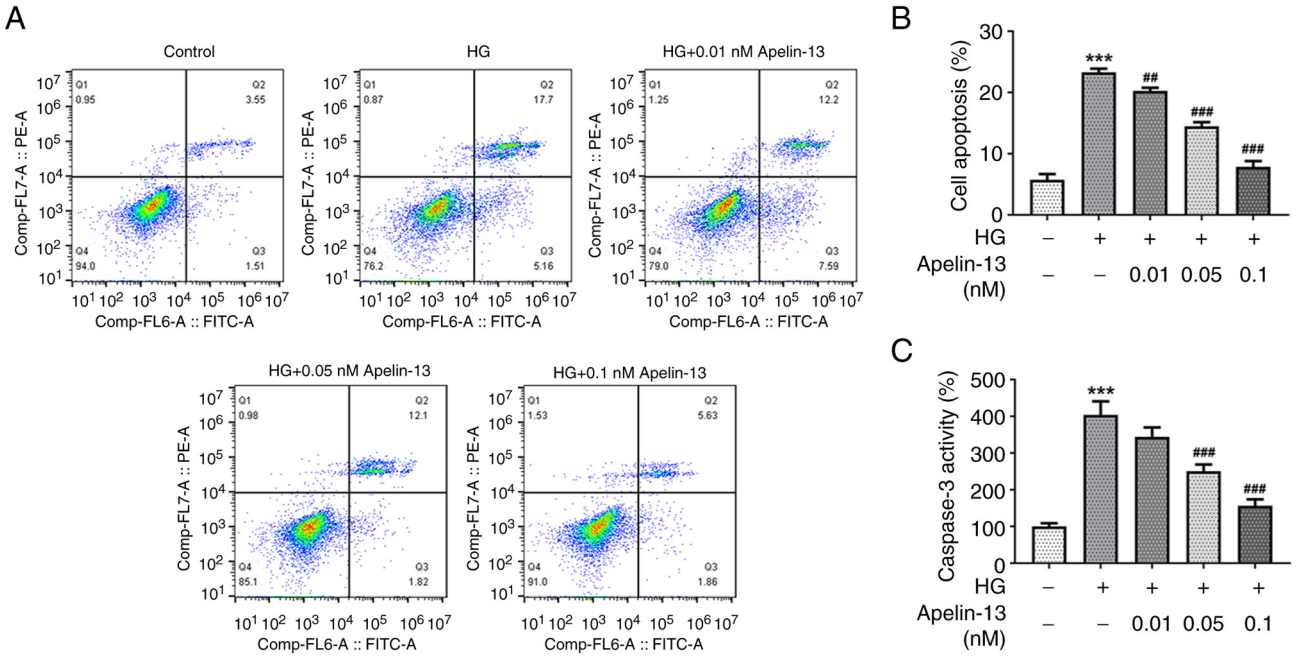

Figure 2. Apelin-13 inhibits apoptosis in HG-treated HEI-OC1 cells. (A) Flow cytometric analysis revealed the proportion of apoptotic cells upon HG and Apelin-13 treatment. (B) Quantitative results of flow cytometry. (C) Caspase3 activity upon HG and Apelin-13 treatment. ***P<0.001 vs. control; $^{\#\#}$P<0.01 and $^{\#\#\#}$P<0.001 vs. HG. HG, high glucose.

compounded by the intricate structure and relative inaccessibility of the inner ear (19). Consistent with hearing loss caused by ototoxicity, noise overstimulation, or aging, hair cells in the cochlea are preferentially affected (20). Hearing is not just a passive response to stimuli, the human auditory system is enhanced by active processes in the cochlear hair cells that amplify sound signals hundreds of times (21). Cochlear hair cell damage is the major cause of hearing loss and cells cannot regenerate spontaneously after damage (22). Therefore, the present study focused on cochlear hair cells. Elucidating the pathogenesis of diabetic hearing loss based on hair cells is necessary to offer insights crucial for early diagnosis, prevention and effective management strategies. Existing research has highlighted the detrimental impact of heightened glucose or fat levels on diabetes, resulting in increased mitochondrial load and the accumulation of damaged mitochondria within cells (23-25). The present study, employing JC-1 and MitoSOX Red probes combined with ATP detection, successfully demonstrated the adverse effects of HG intervention, including diminished MMP, reduced ATP production and an increase in mtROS within HEI-OC1 cells. These results indicated that mitochondrial damage increases upon HG treatment, which is consistent with the conclusions of previous studies. Notably, the therapeutic administration of Apelin-13 alleviated these aberrations. Additionally, the decrease in MMP can promote the further activation of procaspase3 by apoptotic bodies, thereby activating the caspase apoptotic pathway. In the present study, it was found that caspase3 activity was decreased after Apelin-13 treatment, and flow cytometry results revealed that cell apoptosis was also decreased. These findings indicated that Apelin-13 had potential in preserving mitochondrial function and impeding the progression of apoptotic signaling pathways within the cochlear hair cells. A recent study has also revealed that diabetic hearing loss is accompanied by mitochondrial structural or functional damage and activation of endogenous apoptotic pathways (26), which further supports that the inhibition of mitochondrial damage and apoptosis by Apelin-13 found in this research is beneficial to alleviating hearing loss.

Furthermore, the current findings elucidated the regulatory impact of Apelin-13 on ER stress-related proteins, reinforcing its role in mediating ER stress. Apelin-13 exhibits therapeutic potential in diabetic cardiovascular complication by reducing the unfolded protein response and endothelial dysfunction induced by the glycolytic metabolite methylglyoxal (27). It can be concluded that this finding is similarly consistent with the conclusion of the present study because the disease background is also a complication of diabetes and can affect ER-related functions. Notably, the observed parallels between the protective effects of Apelin-13 on the survival of dopaminergic neurons in Parkinson's disease mouse models via ER stress (28) underscore the multifaceted nature of its regulatory mechanisms. Furthermore, variations in serum Apelin-12 levels observed between patients with diabetes and controls accentuate the significance of Apelin as clinical markers (29). However, to date, there are a lack of studies elucidating the specific role of the isoform Apelin-13 in clinical diabetes. Hence, further research is warranted to reveal the clinical significance of Apelin-13.

In addition, earlier research has pointed to the complexities in understanding the interplay between hyperglycemia and hearing impairment, with some evidence indicating that blood glucose control, rather than diabetes *per se*, may be the primary determinant of hearing loss (6). It was considered by the authors that no conclusion can be made at present. Notably, the administration of exogenous Apelin-13 has demonstrated the potential to enhance glucose metabolism (30). In normal and diabetic mice, glucose utilization is improved in response to Apelin-13, which results in increased hypothalamic nitric

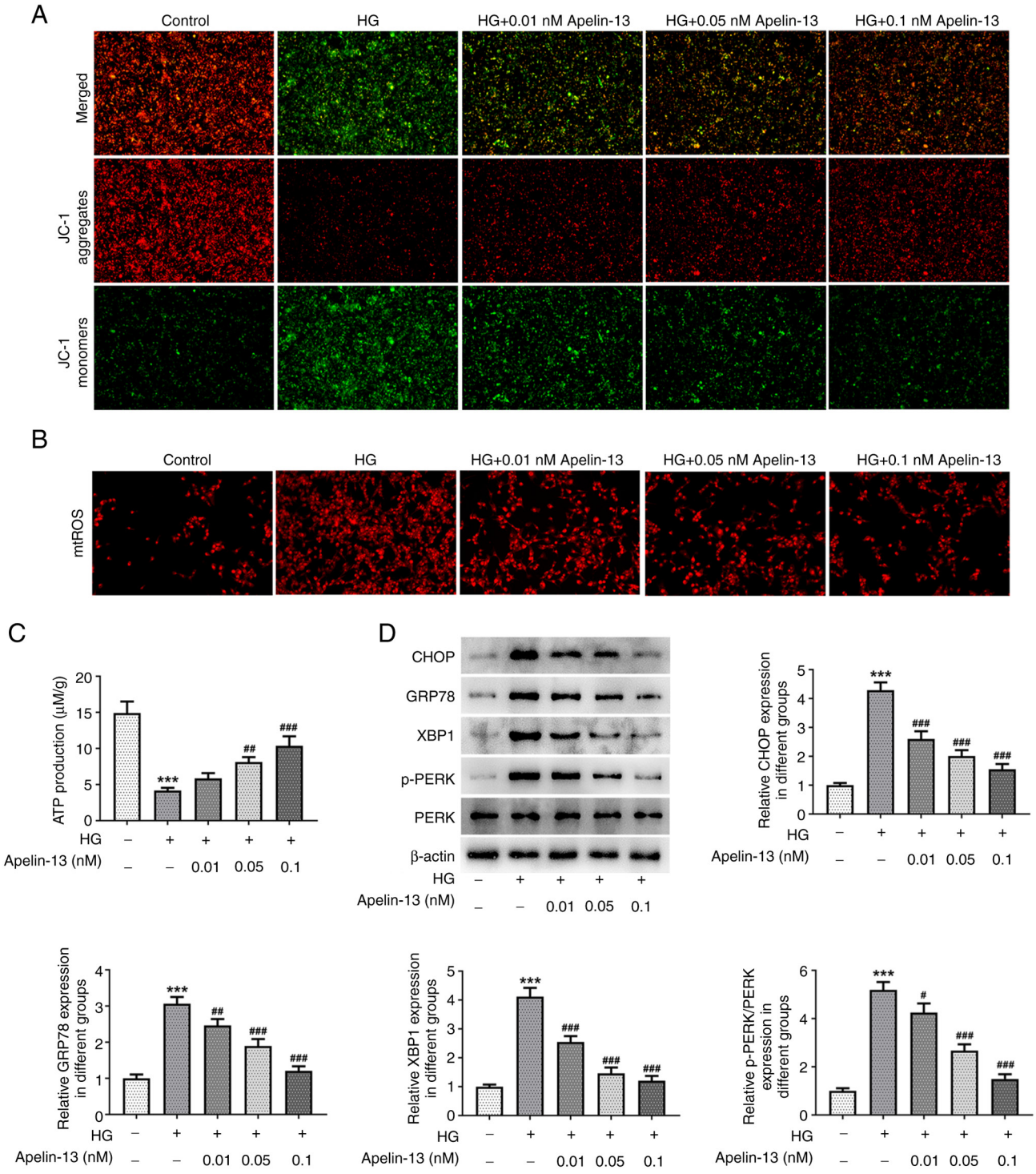

Figure 3. Apelin-13 reduces mitochondrial dysfunction in HG-treated HEI-OC1 cells. (A) JC-1 probes reflected the mitochondrial membrane potential (magnification, x200). (B) MitoSOX Red probes reflected mtROS production (magnification, x200). (C) ATP production upon HG and Apelin-13 treatment. (D) Western blotting revealed the expression levels of ER stress-related proteins in HEI-OC1 cells. ***P<0.001 vs. control; $^{\#}$P<0.05, $^{\#\#}$P<0.01 and $^{\#\#\#}$P<0.001 vs. HG. HG, high glucose; mtROS, mitochondrial reactive oxygen species; ER, endoplasmic reticulum.

oxide release. Data suggest the identification of oral Apelin-13 administration as a new potential target for the treatment of metabolic disorders (31). Therefore, whether stemming from diabetes or elevated blood glucose levels, Apelin-13 appears to possess the capacity to address both factors. It should not be ignored that Apelin is found to have a short half-life in the human body, and rapid receptor desensitization can be achieved by coupling β-arrestins (32). Apelin analogues with enhanced biological activity and resistance to degradation have now been developed, compared with the endogenous peptide. For example, the apelin-13 analog MM07 increases forearm blood flow in human volunteers and is considered an excellent vasodilator (33). The development of analogs can avoid peptide degradation and circumvent the side effects of β-arrestin signaling pathway. Additionally, small molecule apelin agonists are also under development (34), and these

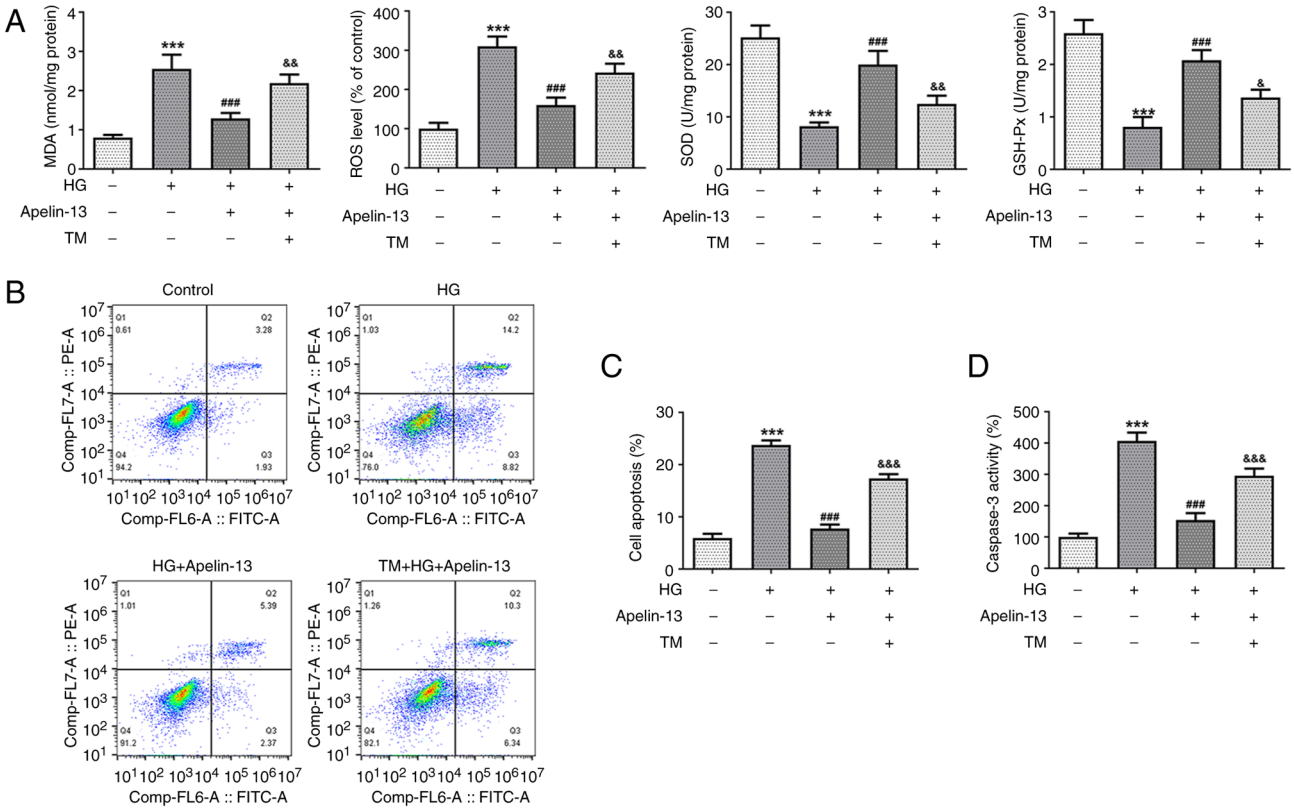

Figure 4. ER stress agonist reverses the effects of Apelin-13 on oxidative stress and apoptosis. HEI-OC1 cells were pretreated with TM to stimulate ER stress, and (A) the impacts of TM on oxidative stress were determined by measuring indicators. (B) Flow cytometry revealed the proportion of apoptotic cells. (C) Quantitative results of flow cytometry. (D) Caspase3 kit revealed intracellular caspase3 activity. ***P<0.001 vs. control; $^{###}$P<0.001 vs. HG; $^{\&}$P<0.05, $^{\&\&}$P<0.01 and $^{\&\&\&}$P<0.001 vs. HG + Apelin-13. ER, endoplasmic reticulum; TM, tunicamycin; HG, high glucose.

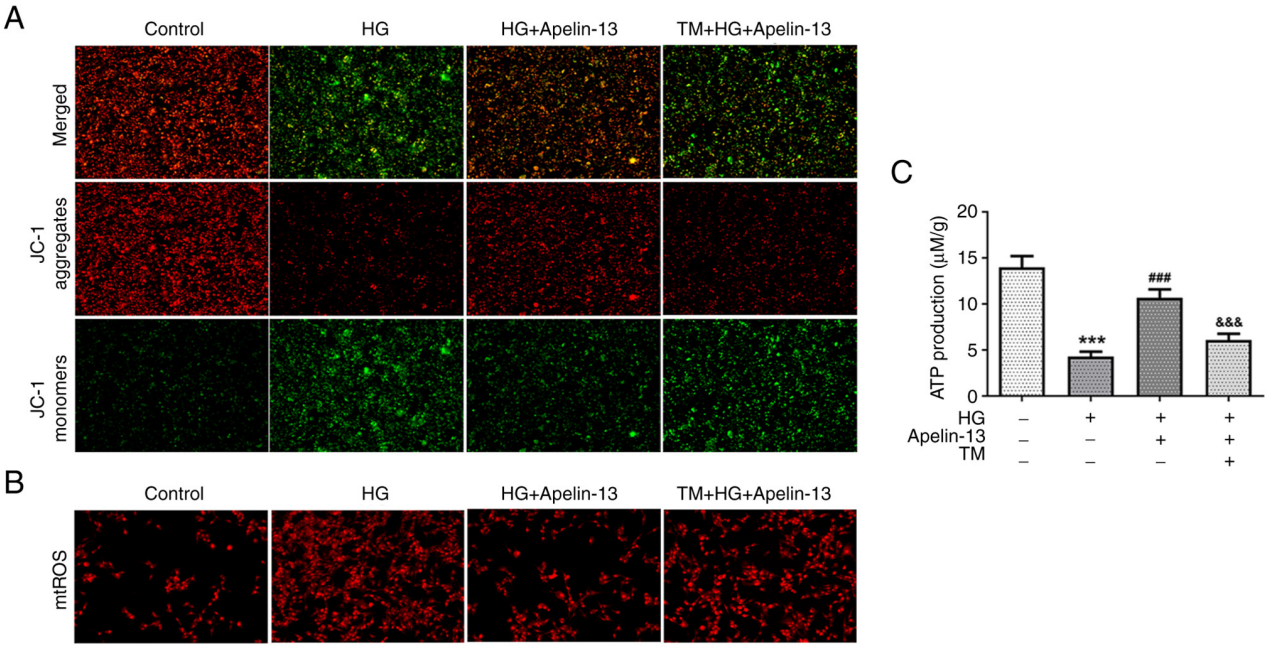

Figure 5. ER stress agonist reverses the effects of Apelin-13 on mitochondrial dysfunction. (A) The impacts of TM on mitochondrial membrane potential as reflected by JC-1 probes (magnification, x200. (B) The impacts of TM on mtROS production as reflected by MitoSOX Red probes (magnification, x200). (C) The impacts of TM on ATP production. ***P<0.001 vs. control; $^{###}$P<0.001 vs. HG; $^{\&\&\&}$P<0.001 vs. HG + Apelin-13. ER, endoplasmic reticulum; TM, tunicamycin; mtROS, mitochondrial reactive oxygen species; HG, high glucose.

studies suggest different apelin-based therapeutic strategies. Currently, Apelin-13 or its analogs are not used to treat various forms of hearing loss because clinical research on Apelin is in its infancy. Nevertheless, according to the results in the present

study, it is considered by the authors it has great potential in hearing loss. Diabetes often involves insulin resistance or impaired insulin signaling, which can lead to mitochondrial dysfunction. The current results may indicate that Apelin-13 acts independently of the insulin signaling pathway. Additionally, elevated glucose levels lead to increased oxidative stress, damaging cellular components including mitochondria. The ability of Apelin-13 to reduce mitochondrial dysfunction suggests its potential role in reducing oxidative stress within cochlear hair cells, which may contribute to maintain their function and vitality under diabetic conditions.

In conclusion, the present study elucidated the protective role of Apelin-13 in ameliorating HG-induced mitochondrial functional impairment in cochlear hair cells by inhibiting ER stress. This research paves the way for harnessing the Apelin-13 signaling system as a promising therapeutic strategy for combating diabetic hearing loss. While existing studies have underscored the physiological role of Apelin-13 in glucose metabolism, further research is warranted to unravel the complex nuances of the Apelin system (35), encompassing novel regulatory ligands and other Apelin isoforms. These are the limitations to the present study, which also include the use of single mouse-derived cells, and the exploration of the role of Apelin-13 and its analogues on glucose metabolism and diabetic hearing loss in the *in vivo* environment.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

ZH and JG contributed to study design, implementing methods and drafting the manuscript. JG and TH contributed to methods and data analysis, and confirm the authenticity of all the raw data. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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