Diabetes impairs the protective effects of sevoflurane postconditioning in myocardium subjected to ischemia/reperfusion injury in rats: important role of Drp1

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Research article

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Abstract

Background

Sevoflurane postconditioning (SevP) is an effective way in relieving myocardial ischemia/reperfusion (IR) injury, which doesn't work well in diabetic myocardium unfortunately. Prior studies have noted the importance of increasing oxidative stress in diabetic tissues. Noteworthily, mitochondrial fission mediated by dynamin-related protein 1 (Drp1) is an upstream pathway of reactive oxygen production. Whether Drp1 dependent mitochondrial fission is associated with the ineffectiveness of SevP in diabetic myocardium remains unknown. The aim of this study was to explore the important role of Drp1 in diabetic myocardium and investigate whether Drp1 inhibition could restore the cardioprotective effect of SevP.

Methods

In the first part, adult male Sprague-Dawley(SD) rats were divided into 6 groups. Rats in diabetic groups were fed with high-fat and high-sugar for 8 weeks, and then received a injection of streptozotocin (35 mg/kg) intraperitoneally. Myocardial IR was induced by 30 min occlusion of left anterior descending branch of coronary artery followed by 120 min reperfusion. SevP was applied by continuous inhalation of 2.5% sevoflurane 1 min before reperfusion, which lasted for 10 min. In the second part, mdivi-1 was used to investigate whether Drp1 inhibition could restore the cardioprotective effects of SevP in diabetic myocardium against I/R injury. The myocardial infarct size, pathology, mitochondrial ultrastructure, cardiomyocyte apoptosis, total SOD activity, MDA content, and Drp1 expression were detected.

Results

The diabetic myocardium displayed severer injury with greater infarct size and apoptosis. Up-regulated Drp1 expression concomitant with increased mitochondrial fission and oxidative stress were observed in diabetic myocardium subjected to I/R. The deteriorated changes were alleviated in normal but not in diabetic rats. Importantly, mdivi-1 administration significantly suppressed mitochondrial fission and oxidative stress, and the beneficial effects of SevP were restored by mdivi-1.

Conclusions

The present study indicates a crucial role of Drp1 dependent mitochondrial fission in diabetic myocardium subjected to IR. Drp1 inhibition may be effective in restoring the effect of SevP in reducing diabetic myocardial IR injury.

Background
Myocardial ischemia/reperfusion (I/R) injury is a phenomenon that often occurs in coronary artery bypass surgery, cardiopulmonary resuscitation and organ transplantation, which has been a troubling problem of anesthesiologists.\textsuperscript{1} Sevoflurane application at the initiation of reperfusion has been reported to be effective in reducing myocardial I/R injury, which is termed as sevoflurane postconditioning (SevP).\textsuperscript{2} However, studies have discovered that SevP did not work well in diabetic myocardium, the exact mechanisms responsible for which have not been elucidated.\textsuperscript{3}

Recent researches have highlighted the importance of mitochondrial fission in apoptosis, which has also been proved as an upstream pathway of oxygen free radicals production.\textsuperscript{4, 5, 6} Mitochondria appear to be abnormal when fission process was intensified, thus causing the proapoptotic molecules within mitochondria leaking into cytoplasm to trigger apoptosis. Whereas integral mitochondrial membrane could prevent the proapoptotic molecules from leakage.\textsuperscript{7} Mitochondrial fission process is mediated by dynamin-related protein 1 (Drp1), which is located in cytoplasm.\textsuperscript{8} It has been revealed that increased expression of Drp1 was closely associated with insulin resistance and diabetes.\textsuperscript{9} Surprisingly, Drp1 inhibition was effective in preserving mitochondrial integrity and mitochondrial dysfunction.\textsuperscript{10} Leinninger et al reported that increased Drp1 expression, excessive mitochondrial fission, and increased reactive oxygen species were shown in spinal cord dorsal root ganglion cells under hyperglycemia condition.\textsuperscript{11} The above-mentioned suggested that Drp1 related mitochondrial fission and apoptosis directly participated in the pathology of diabetes and its complications. However, whether or not Drp1 expression was up-regulated in diabetic heart subjected to I/R remains unknown. As a result, the aim of our present study was to investigate the involvement of Drp1 as well as its downstream factors in weakening cardioprotective effects of SevP in diabetic hearts against IR injury.

**Methods**

**Animals and ethics statement**

Adult male Sprague-Dawley (SD) rats, weighing 220 ~ 250 g, were provided by the Experimental Animal Center of Shanxi Medical University, China. All animals were housed and handled according to Shanxi Medical University Institutional Animal Care. All mouse experimental procedures were performed in accordance with the Regulations for the Administration of Affairs Concerning Experimental Animals approved by the State Council of People's Republic of China. Model of type 2 diabetic rats was induced according to references\textsuperscript{12}, which were given high-fat and high-sugar diet for 8 weeks and then received a intraperitoneal injection of streptozotocin (STZ) (Sigma, USA) at the dose of 35 mg/kg. The administration lasted for 7 consecutive days. Age-matched control rats were injected with equal volume of citrate buffer. Blood glucose levels were measured every day. Rats whose blood glucose levels were higher than 16.7 mmol/L were identified as type 2 diabetic rats. All rats continued to be fed for a month, during which insulin intervention and diet control were not given.

**Grouping**
The study was conducted under the guidance of international ethical standards. After chowing for one month, rats were randomly divided into six groups \( (n = 8 \text{ each}) \) (Fig. 1): (1) normal sham group (S); (2) normal ischemia/reperfusion group (I/R); (3) normal sevoflurane postconditioning group (Sev); (4) diabetic sham group (DS); (5) diabetic ischemia/reperfusion group (DI/R); (6) diabetic sevoflurane postconditioning group (DSev). Furthermore, to investigate whether or not inhibiting Drp1 could restore the cardioprotective effects of SevP in diabetic hearts against IR injury, the following grouping was performed in the second part of our study \( (n = 8 \text{ each}) \): (1) group DI/R; (2) group DSev; (3) group DI/R + Mdivi-1; (4) group DI/R + DMSO (5) group DSev + Mdivi-1; (6) group DSev + DMSO. Mdivi-1 (Sigma, USA; 25 mg/kg) was the specific inhibitor of Drp1, while DMSO was the vehicle (1% DMSO dissolved in 0.9% NaCl) as control. They were respectively intravenously given 30 min before ischemia with the same volume.

**Myocardial I/R model**

Rats were anesthetized with 25% urethane at the dose of 5 ml/kg. After intubation, they were ventilated artificially with small animal ventilator (ALC-V8; Shanghai Oort Biotechnology Co., Ltd.). During thoracotomy, the left anterior descending coronary artery (LAD) was occluded. Elevated ST-segment and towering T-wave indicated ischemic myocardium. Following 30 min ischemia, reperfusion was performed for 120 min. S groups only received thoracotomy and threading, without LAD ligation. I/R was induced by 30 min ischemia and 120 min reperfusion. SevP was accomplished by continuous inhalation of 2.5% sevoflurane1 min before reperfusion, which lasted for 10 min. Experimental groups and treatment were shown in Fig. 1.

As SD rats were under deep anaesthetic without consciousness during the procedure, when hearts removed from their bodies, the blood circulation would stop, and a straight line was shown on ECG, which signified they were dead in anaesthetic, without euthanasia.

**Measurement of myocardial infarct size and apoptosis**

After 120 min reperfusion, LAD was occluded again at the same position, then 3 ml Evans blue (Sigma, USA) dye of 0.5% concentration was injected into the aorta. The hearts of rats were rapidly removed and placed in a refrigerator at -20°C for 20 min. Subsequently, the left ventricle (LV) tissues were cut into 5 slices (2 ~ 3 mm thick) perpendicularly to the long axis of the heart. The slices were incubated for 15 minutes in 0.5% TTC (Sigma, USA) phosphoric acid buffer solution (pH 7.4) at 37 °C. Evans blue dying was used to distinguish the non-ischemic area from the ischemic area, which was also called area-at-risk (AAR). The viable ischemic myocardium could be dyed red by TTC, while the infarct myocardial area (IS) was not stained, which remained pale. These slices of LV tissue were dried by filter paper and weighed in the electronic balance respectively. Then the myocardium of IS and AAR were weighed and separated. The percentage of IS from the ischemic area (IS/AAR) and AAR from the left ventricle (AAR/LV) were calculated.

The myocardial apoptosis was determined by terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) assay. After reperfusion, the ischemic myocardial tissues were collected...
for apoptosis determination. They were fixed in paraformaldehyde and made into paraffin slice with 4 µm thickness, which were measured by an TUNEL kit (Wuhan Boster biological Engineering Co, Ltd, China). After being scanned by digital pathology scanner (Aperio, USA), five horizons were randomly selected at each slice. The conductors were unaware of the information of this study. Nuclei of apoptotic cells displayed brown, while normal nuclei blue. Apoptosis index (AI) was calculated as the percentage of positive nuclei to the total number of nuclei.

**Measurement of creatine kinase-MB(CK-MB) and troponin I (TnI) content**

After reperfusion, blood samples were obtained for measurement of CK-MB and LDH content. According to the instructions, CK-MB was measured by enzyme immunoassay using an assay kit (Wuhan Boster biological Engineering Co, Ltd, China), as well as TnI was detected by an Ultra Troponin I determination kit (Nanjing Jiancheng Bioengineering Institute, China).

**Determination of malondialdehyde (MDA) and Superoxide dismutase(SOD) levels**

After reperfusion, the ischemic myocardial tissue were collected for measurement of MDA and SOD levels. SOD is one of the antioxidant enzymes, whose total vitality is determined by Xanthine oxidase method using a nassay kit (Nanjing Jiancheng Bioengineering Institute, China). MDA is the end product of lipid peroxidation, whose content is determined by thiobarbituric acid method using an assay kit (Nanjing Jiancheng Bioengineering Institute, China).

**Myocardial histopathology and mitochondrial ultrastructure**

After reperfusion, the ischemic myocardial tissues were collected for HE staining. They were fixed in paraformaldehyde and then embedded in paraffin, made into slice with 4 µm thickness. Hematoxylin and eosin (HE) staining was performed to observe the histopathological change of myocardium. Photo micrographs were taken with digital pathology scanner. The operators were unaware of the experimental grouping and other information.

After reperfusion, the ischemic myocardial tissues were collected. And then they were rinsed and homogenized into grain tissue of 1mm³ size, which were subsequently fixed in glutaraldehyde phosphate buffer and osmium tetroxide. After being rinsed, dehydrated, embedded and sliced, uranyl acetate and lead citrate double staining was performed. Prepared samples were examined with an transmission electron microscope (JEM-2100, Japan). Mitochondrial ultrastructural changes were evaluated by an investigator blind to the experimental grouping and other information.

**Determination of Drp1 expression**

To evaluate the location and expression of Drp1 in ischemic myocardial tissue, immunohistochemical staining was carried out. Paraformaldehyde-fixed myocardial tissues were made into slices with 4 µm
thickness and underwent antigen retrieval and endogenous peroxidase inactivation subsequently. After being incubated, the slices were stained with a rabbit anti-mouse polyclonal antibody against Drp1 (1:250; Beijing Biosynthesis Biotechnology Co., Ltd.) at 4°C. After incubation for 24 h, goat anti-rabbit IgG secondary antibody was added. These slices were colored with DAB and stained by hematoxylin, and then were scanned with Digital pathology scanner. Positive expression of Drp1 displayed brown granules cytoplasm, and BI-2000 medical image analysis system was used to examine the mean optical density (OD) of Drp1.

PT-PCR was performed to examine the expression of Drp1 mRNA. After reperfusion, ischemic myocardial tissue was obtained to be frozen in liquid nitrogen and then transferred to refrigerator with a temperature of -80°C. According to the instructions of total RNA extraction kit (Shanghai biological Technology Co, Ltd), RNA was transcribed reversely to complimentary DNA(cDNA) using TaqMan Reverse Transcription Reagents. Then Drp1 gene was amplified. Primer sequence and amplified fragment length were shown as followed: mouse Drp1 forward: 5'-ATGCCTGTGGGCTAATGAAC-3'; reverse: 5'-CTCCAATTCGACCACCACCATCT-3; product length: 168 bp; mouse β-Actin forward: 5'-GTCAGGTCATCACTATCGGCAAT-3', reverse: 5'-AGAGGTCTTTACGGATGCAAGCT-3'; product length: 147 bp. The reaction started with a initial denaturation (94°C, 10 min, enzyme activation Tag), followed by 45 cycles of denaturation (94°C, 15 s), annealing (60°C, 20 s) and extension (72°C, 15 s). Using β-Actin as control, relative expression of Drp1 mRNA was calculated by the method of $2^{-\Delta\Delta CT}$.

**Statistical analysis**

All data were expressed as mean ± SD. SPSS 13.0 statistical software was used for analysis. Differences between groups were compared by one-way ANOVA test. Student-Newman-Keuls test was used to compare differences between two groups. Values of $P<0.05$ (two-tailed) were declared as statistically significant.

**Results**

**Type 2 diabetic rats model was successfully established**

Rats in diabetic groups showed characteristic symptoms of diabetes including hyperglycemia, polydipsia, diuresis, polyphagia and low body weight. As shown in Table 1, the concentration of blood glucose in diabetic rats was significantly higher than that of normal rats ($P<0.05$). The body weights were lower in diabetic rats compared to normal rats ($P<0.05$).
Table 1
Changes in plasma glucose and body weight of normal and diabetic rats at different times

| Parameters                  | Normal groups | Diabetic groups |
|-----------------------------|---------------|----------------|
|                             | base 1w 2w 3w 4w | base 1w 2w 3w 4w |
| Plasma glucose (mmol/L)     | 6.0 ± 0.8 5.8 ± 0.9 6.0 ± 0.7 6.1 ± 1.0 | 6.0 ± 0.7 26.3 ± 1.4a 25.7 ± 1.2a 26.9 ± 1.3a |
| Body weight (g)             | 248 ± 9 249 ± 10 256 ± 10 268 ± 9 274 ± 7 | 250 ± 10 241 ± 10a 225 ± 8a 220 ± 11a 215 ± 10a |

All values are expressed as mean ± S.D. Plasma glucose and body weight were measured every day before the establishment of myocardial I/R model. *P* < 0.05 versus normal rats at the time of 1, 2, 3, 4 weeks after STZ injection.

Cardioprotective effects of SevP is attenuated in type 2 diabetic rats

To evaluate whether SevP could attenuate myocardial I/R injury in normal and diabetic rats, the myocardial infarct size and apoptosis were measured, and hematoxylin and eosin staining was performed. Pathological changes of ischemic myocardial tissue were shown in Fig. 2A. Cardiomyocytes were observed under a light microscope. Irregular fiber arrangement, swelling cells, broken nuclear membrane and dissolved nucleus were exhibited in groups which subjected to myocardial I/R injury. As compared with I/R group, I/R injury was ameliorated in the Sev group. The myocardial structural injury could be markedly alleviated in normal rats but not in diabetic rats. Furthermore, data of infarct size and apoptosis are shown in Fig. 2B. No infarct and ischemic area were detected in the two sham groups. There was no significant difference in the AAR/LV among other four groups (*P* > 0.05). The I/R groups exhibited more severe myocardial injury with increased IS/AAR ratio and apoptosis index (AI) (*P* < 0.05), and TUNEL-positive cardiomyocytes with brown nucleis were more obvious in diabetic rats as shown in Fig. 2C. SevP significantly alleviated all these changes in normal rats but not in diabetic rats (*P* < 0.05), showing the values of IS/AAR and AI in DI/R and DSev groups had no statistical differences (*P* > 0.05).

SevP could reduce myocardial oxidative stress in normal rats but not in diabetic rats

Data were shown in Fig. 3. Similarly, myocardial oxidative stress induced by I/R was more obvious in diabetic rats, showing decreased levels of SOD and increased levels of MDA (*P* < 0.05). Notably, SevP could remarkably reduce the myocardial oxidative stress in normal rats compared with group I/R (*P* < 0.05), while the levels of SOD and MDA in group DI/R and DSev had no statistical significance (*P* > 0.05). Thus it can be concluded that SevP could not preserve the diabetic heart from oxidative stress injury.

Excessive mitochondrial fission and increased Drp1 expression were shown in diabetic rats
To explore whether diabetes alters myocardial mitochondrial morphology and dynamics, we examined the morphological changes of mitochondria by electron microscopy. The results showed that compared with the two sham groups, the number of mitochondria in myocardium subjected to I/R was increased, with more severe ultrastructural damage. SevP could reduce mitochondrial ultrastructural damage in normal rats, exhibiting intact membrane and structure, intensive and orderly arranged cristae. While mitochondria in diabetic myocardium subjected to I/R exhibited aggravated injury, with fragmented structure, fractured membrane, destructed cristae and blurred Z line. SevP failed to preserve mitochondria from ultrastructural damage in diabetic rats, which showed swelling and dissolving mitochondria.

To further investigate the mechanisms responsible for mitochondrial fission, we assessed Drp1 expression by immunohistochemistry and quantitative real-time PCR from protein and mRNA levels, which is the main mediator of mitochondrial fission. As shown in Fig. 4, the expression of Drp1 in other groups was significantly higher than that in sham groups ($P < 0.05$), and sevP could significantly reduce the expression of Drp1 ($P < 0.05$). However, Drp1 expression was intensified by diabetes, and no significant difference was identified in group DI/R and DSev ($P > 0.05$). These results suggested increased mitochondrial fission in diabetic rats, which may subsequently impair mitochondrial morphology and cell function.

**Cardioprotective effect of sevP was restored by Drp1 inhibitior mdivi-1 in diabetic rats**

Since Drp1 plays an important role in maintaining normal mitochondrial morphology and cell function, we next aimed to investigate whether inhibition of Drp1 causes changes in mitochondrial structure and restores cardioprotective effect of sevP in diabetic rats by pharmacological blockade of Drp1 with mdivi-1, a specific inhibitor of Drp1. Importantly, mdivi-1 significantly suppressed mitochondrial fission and protected mitochondrial ultrastructure from diabetes-induced deleterious effects. What’s more, the values of IS/AAR in DSev + mdivi-1 group were significantly decreased compared with the DSev group ($P < 0.05$). The cardiac enzymes CK-MB and TnI, which were markers of myocardial cellular injury, were also significantly decreased in the DSev + mdivi-1 group ($P < 0.05$). To validate mitochondrial fission acts as an upstream pathway of oxidative stress in diabetic conditions, we next determined the levels of SOD and MDA. As shown in Fig. 5, mdivi-1 administration markedly reduced oxidative stress in DSev + mdivi-1 group compared with DSev group ($P < 0.05$), manifested as increased levels of SOD and decreased levels of MDA. These data suggested that Drp1 is a likely upstream signaling molecule that controls oxidative stress and apoptosis under diabetic conditions. Inhibiting Drp1 is an effective way in restoring cardioprotective effect of SevP.

**Discussion**

Diabetes is one of the most relevant risk factors for cardiovascular disease, especially coronary heart disease. Some promising measures including pre-, post-, remote ischemic or pharmacological conditioning seems ineffective in alleviating diabetic myocardial I/R injury. In the present study, we
proved that sevP failed to protect the diabetic heart from I/R injury, showing an increased myocardial infarct size and apoptosis in diabetic rats. Additionally, more fragmented and damaged mitochondria were shown in diabetic rats, which was probably the main reason responsible for the loss of sevP cardioprotection. And inhibiting Drp1, which was a direct regulator of mitochondrial fission, could restore cardioprotective effects of sevP.

Increased oxidative stress is closely associated with diabetes and its complications, manifested as excessive production of reactive oxide species (ROS) and depleted antioxidant defense enzymes. Excessive ROS could damage bilayer membranes of cell and mitochondria, causing lipid peroxidative reaction of membranes, which was considered to be a primary mechanism responsible for mitochondrial and cell dysfunction, especially apoptosis. It has been revealed that exaggerated oxidative stress under diabetic conditions was a causing factor that accentuated myocardial injury and rendered the heart less sensitive to the cardioprotection of isoflurane, which could be restored by N-acetylcysteine, a scavenger for ROS. In the present study, we measured SOD enzyme activity and MDA levels that reflected antioxidant systems and lipid peroxidation degree respectively. As a result, our data demonstrated a decline of SOD activity together with an increase of MDA levels in diabetic myocardium subjected to I/R. SevP significantly increased the activity of SOD and decreased the levels of MDA in normal hearts, whereas the above-mentioned effects were absent in diabetic rats. These results suggested that modulating ways of ROS production may be promising in relieving myocardial I/R injury by SevP strategy in diabetic rats.

Mitochondria are major organelles producing ROS. What's more noteworthy, mitochondrial fission act as an upstream pathway of ROS production. Excessive mitochondrial fission leads to fragmented mitochondrial membranes and impaired respiratory chain, thus resulting in the opening of mitochondrial permeability transition pore (mPTP) and ATP production, consequently increasing ROS production as well as cell apoptosis. It has been indicated that excessive mitochondrial fission regulated by Drp1 was associated with apoptosis of various types of cells under diabetic condition, including dorsal root ganglion neurons, endothelial cells, islet cells, et al. Given that mitochondrial fission is aggravated in tissues influenced by diabetes, we hypothesized that Drp1 dependent mitochondrial fission played an important role in the insensitivity of diabetic myocardium to the cardioprotective effects of sevP. Furthermore we explored whether mitochondrial morphology and Drp1 expression were altered in diabetic myocardium in rats. As a result, our findings were consistent with previous studies, which demonstrated increased Drp1 expression, smaller and fragmented mitochondria with serious structural damage in diabetic myocardium. On the contrary, sevP could preserve the mitochondria from I/R injury in normal rats but not in diabetic rats.

We next targeted the role of Drp1 in diabetic myocardium subjected to I/R injury by using its inhibitor mdivi-1. As a result, the absence of sevP protection in diabetic hearts was reversed by Drp1 inhibition, evidenced as decreased levels of CK-MB and TnI in diabetic rats. In addition, mdivi-1 administration improved mitochondrial morphology presented as intact membrane and cristae structure, and the
oxidative stress level was also depleted. Furthermore, the combinative use of mdivi-1 and sevP could significantly decrease myocardial I/R injury in diabetic rats, implying the crucial role of Drp1 inhibition for sevP to confer cardioprotection. Of note, however, the up-regulated expression of Drp1 is not the main reason responsible for the loss of sevP protection in diabetic myocardium. Mitochondrial fission mediated by Drp1 is regulated by variety of post-translational ways, including ubiquitination, phosphorylation, S-nitrosylation, and so on.\textsuperscript{20,21,22} Whether there is a direct link between mitochondrial fission and these signaling pathway remains unknow, which needs further study.

**Conclusions**

In summary, our study demonstrated the ineffectiveness of sevP to relieve myocardial I/R injury in diabetic rats, which was possibly associated with increased Drp1 expression and mitochondrial fission in diabetic myocardium, whereas the cardioprotective effects of sevP could be restored by Drp1 inhibition. The results provided novel insights into the crucial role of mitochondrial fission in explaining the insensitivity of diabetic myocardium to sevP, which added to a promising alternative in the prevention and treatment of diabetic myocardium subjected to I/R.

**Abbreviations**

SevP: Sevoflurane postconditioning; IR: ischemia/reperfusion; Drp1: dynamin-related protein 1; SD: Sprague-Dawley; STZ: streptozotocin; LV: left ventricle; AAR: area-at-risk; IS: myocardial area; TUNEL: transferase-mediated dUTP-biotin nick end labeling; AI: Apoptosis index; CK-MB: creatine kinase-MB; TnI: troponin I; MDA: malondialdehyde; SOD: Superoxide dismutase; HE: Hematoxylin and eosin; OD: optical density; ROS: reactive oxide species; mPTP: mitochondrial permeability transition pore.

**Declarations**

-Ethics approval and consent to participate

All mouse experimental procedures were performed in accordance with the Regulations for the Administration of Affairs Concerning Experimental Animals approved by the State Council of People's Republic of China.

-Consent for publication

Not applicable.

-Availability of data and material

All data and materials are fully available without restriction. They were all presented on the table and figure part. And the data source files were all presented on the part of supplementary material.

-Competing interests
The authors declare that they have no competing interest.

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**Authors’ contributions**

JY, JDH and CFH designed the study. JY, WQY, XW, GXS, YLD and HW carried out the experimental work. JY and JDH collected the research data and drafted the manuscript, which was finally checked by CFH. All authors approved the final manuscript.

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