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Sevoflurane pretreatment inhibits the myocardial apoptosis caused by hypoxia reoxygenation through AMPK pathway: An experimental study

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ABSTRACT

Objective: To study whether sevoflurane pretreatment inhibits the myocardial apoptosis caused by hypoxia reoxygenation through AMPK pathway.
Methods: H9c2 myocardial cell lines were cultured and divided into control group (C group), hypoxia reoxygenation group (H/R group), sevoflurane pretreatment + hypoxia reoxygenation group (SP group) and sevoflurane combined with Compound C pretreatment + hypoxia reoxygenation group (ComC group), and the cell proliferation activity and apoptosis rate, myocardial enzyme levels in culture medium as well as the expression of apoptosis genes and p-AMPK in cells were determined.
Results: p-AMPK expression in cells of H/R group was significantly lower than that of C group, SP group was significantly higher than that of H/R group; cell proliferation activity value and Bcl-2 expression in cells of H/R group were significantly lower than those of C group, SP group were significantly higher than those of H/R group, ComC group were significantly lower than those of SP group; apoptosis rate, LDH, CK and AST levels as well as the Bax and Caspase-3 expression in cells of H/R group were significantly higher than those of C group, SP group were significantly lower than those of H/R group, ComC group were significantly higher than those of SP group.
Conclusions: Sevoflurane pretreatment can activate AMPK signaling pathway to inhibit the myocardial apoptosis caused by hypoxia reoxygenation.

1. Introduction

Acute myocardial infarction is a common disease of cardiovascular system, and with the development of thrombolysis therapy, interventional therapy and other reperfusion therapy, patients can obtain blood reperfusion in time after acute myocardial infarction [1,2]. During reperfusion therapy for patients with myocardial infarction, myocardial ischemia-reperfusion injury is the important cause of acute heart failure and treatment failure, and reducing myocardial ischemia-reperfusion injury is the important target to improve the effect of reperfusion treatment for myocardial infarction [3,4]. Sevoflurane is the inhalation anesthetic with antioxidant, anti-inflammatory, anti-apoptotic and other effects, existing experimental studies have confirmed that sevoflurane can alleviate myocardial ischemia-reperfusion injury in rats [5,6], but the specific molecular mechanism for sevoflurane to alleviate myocardial ischemia-reperfusion injury is not clear. AMP-activated protein kinase (AMPK) is considered as the central regulatory molecule of cardiac energy metabolism, and after activated by phosphorylation, the molecule can improve the myocardial cell energy metabolism and reduce myocardial cell damage [7,8]. *In vitro* cultured myocardial cell hypoxia reoxygenation can simulate the pathological physiological process of ischemia reperfusion injury during perfusion treatment, and is the ideal model to study the molecular mechanism and treatment of myocardial ischemia-reperfusion injury. In the following study, whether sevoflurane pretreatment inhibited myocardial apoptosis caused by hypoxia reoxygenation through AMPK pathway was analyzed.

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2. Materials and methods

2.1. Experimental materials

H9c2 myocardial cell lines were bought from Shanghai cell bank of Chinese Academy of Sciences, DMEM, fetal bovine serum and 0.125% trypsin were bought from Gibco Company, sevoflurane and AMPK inhibitor Compound C were purchased from Sigma Company, p-AMPK, AMPK, Bcl-2, Bax and Caspase-3 monoclonal antibodies were bought from Abcam Company, MTS cell viability detection kits were purchased from Promega Company, and the LDH, CK and AST enzyme-linked immunosorbent assay kits AnnexinV-FITC apoptosis detection kits were bought from Shanghai Beyotime Company.

2.2. Experimental methods

2.2.1. Cell grouping and treatment methods

After subculture, H9c2 myocardial cells with logarithmic phase were collected and divided into control group (C group), hypoxia reoxygenation group (H/R group), sevoflurane pretreatment + hypoxia reoxygenation group (SP group) and sevoflurane combined with Compound C pretreatment + hypoxia reoxygenation group (ComC group). C group were cultured in 95%O_2+5%CO_2 incubator; H/R group were placed in 95%N_2+5%CO_2 incubator for 14 h of hypoxic culture and then transferred into 95%O_2+5%CO_2 incubator for 3 h of reoxygenation; SP group were pretreated with final concentration 2.0 mmol/L of sevoflurane for three times, 15 min for each time, and then received hypoxia reoxygenation treatment according to the method of H/R group; ComC group were pretreated with final concentration 2.0 mmol/L of sevoflurane as well as final concentration 20 μmol/L of Compound C for three times, 15 min for each time, and then received hypoxia reoxygenation treatment according to the method of H/R group.

2.2.2. Cell proliferation and apoptosis detection methods

To detect cell proliferation activity, the cells were inoculated in 96-well plate, three parallel wells were made for each bath of cells and each condition, 5 batches of cells were repeated, 20 μL MTS test fluid was added in the culture medium after different conditions of treatment, the cell plate was put back in the incubator, continued to be incubated for 4 h, then taken out and oscillated for 15 min, the absorbance at 490 nm wavelength was measured in microplate reader, the absorbance was used as cell proliferation activity value, the mean proliferation activity value of each batch of cells in C group was set to 1 to calculate the relative cell proliferation activity of other groups; to detect apoptosis rate, the cells were inoculated in 6-well cell plate and treated with different conditions, then the culture medium was abandoned, 5 μL Annexin V and 5 μL PI were added in turn for 10 min of incubation away from light, and the apoptosis rate of various groups of cells were determined in flow cytometer.

2.2.3. Detection methods of myocardial enzyme levels in cell culture medium

To detect myocardial enzyme levels in the cell culture medium, the cells were inoculated in 12-well cell plate and treated with different conditions to collect culture medium and cells, enzyme-linked immunosorbent assay kits were used to detect LDH, CK and AST levels in culture medium, the BCA kits were used to detect the total protein content in the cells, and the LDH, CK and AST levels per mg total protein were calculated.

2.2.4. Detection methods of apoptosis gene expression in cells

To detect apoptosis gene expression in cells, the cells were inoculated in 12-well cell plate and treated with different conditions, the culture medium was abandoned, the cells were collected, added in RIPA lysis buffer and fully broken with cell scraper, the broken cells were centrifuged at 4 °C and 12 000 r/min for 20 min to separate protein suspension for Western blot, p-AMPK, AMPK, Bcl-2, Bax, Caspase-3 and β-actin antibodies were incubated after electrophoresis and closure, the protein bands were obtained at last after development, AMPK was used as reference to calculate the expression of p-AMPK, and β-actin was used as reference to calculate the Bcl-2, Bax and Caspase-3 expression.

2.3. Statistical methods

SPSS20.0 software was used to input and analyze data, measurement data among groups was by variance analysis and LSD-t test and $P < 0.05$ indicated to have statistical significant difference.

3. Results

3.1. p-AMPK expression in cells with hypoxia reoxygenation and sevoflurane pretreatment

p-AMPK expression in cells of C group, H/R group and SP group was as follows: p-AMPK expression in cells of C group was (1.00 ± 0.14), p-AMPK expression in cells of H/R group was (0.38 ± 0.05) and p-AMPK expression in cells of SP group was (1.28 ± 0.18). Variance analysis and LSD-t test pair-wise comparison was as follows: p-AMPK expression in cells of H/R group was significantly lower than that of C group, p-AMPK expression in cells of SP group was significantly higher than that of H/R group, and differences in pair-wise comparison of p-AMPK expression were statistically significant among three groups of cells ($P < 0.05$).

3.2. Effect of sevoflurane pretreatment and AMPK inhibitor pretreatment on myocardial cell proliferation activity and apoptosis rate under hypoxia reoxygenation conditions

Analysis of myocardial cell proliferation activity and apoptosis rate among C group, H/R group, SP group and ComC group was as follows: cell proliferation activity value of H/R group was significantly lower than that of C group, and the apoptosis rate was significantly higher than that of C group; cell proliferation activity value of SP group was significantly higher than that of H/R group, and the apoptosis rate was significantly lower than that of H/R group; cell proliferation activity value of ComC group was significantly lower than that of SP group, and the apoptosis rate was significantly higher than that of SP group. As shown in Table 1, differences in pair-wise comparison of cell proliferation activity value and apoptosis rate were statistically significant among four groups of cells ($P < 0.05$).

Table 1
Myocardial cell proliferation activity and apoptosis rate, myocardial enzyme levels in myocardial cell culture medium, and apoptosis gene expression in myocardial cells.

Groups	Myocardial cell		Myocardial enzyme levels (U/mg)			Apoptosis gene expression		
	Proliferation activity	Apoptosis rate	LDH	CK	AST	Bcl-2	Bax	Caspase-3
C group	1.00 ± 0.13	5.29 ± 0.76	11.38 ± 1.48	8.62 ± 0.94	18.67 ± 2.24	1.00 ± 0.12	1.00 ± 0.11	1.00 ± 0.13
H/R group	0.32 ± 0.05[a]	22.46 ± 2.83[a]	47.65 ± 5.61[a]	27.61 ± 3.63[a]	74.14 ± 9.05[a]	0.33 ± 0.06[a]	2.76 ± 0.36[a]	3.06 ± 0.45[a]
SP group	0.84 ± 0.10[b]	8.72 ± 0.93[b]	18.87 ± 2.25[b]	11.49 ± 1.37[b]	27.64 ± 3.31[b]	0.79 ± 0.09[b]	1.45 ± 0.17[b]	1.52 ± 0.18[b]
ComC group	0.41 ± 0.06[c]	17.65 ± 1.82[c]	38.97 ± 5.16[c]	22.14 ± 3.05[c]	58.85 ± 7.51[c]	0.42 ± 0.05[c]	2.35 ± 0.31[c]	2.44 ± 0.34[c]

Data are expressed as mean ± SD; $n = 5$.
[a] $P < 0.05$ compared with C group. [b] $P < 0.05$ compared with H/R group. [c] $P < 0.05$ compared with SP group.

3.3. Effect of sevoflurane pretreatment and AMPK inhibitor pretreatment on myocardial enzyme levels in myocardial cell culture medium under hypoxia reoxygenation conditions

Analysis of myocardial enzyme LDH, CK and AST levels in myocardial cell culture medium among C group, H/R group, SP group and ComC group was as follows: LDH, CK and AST levels in cell culture medium of H/R group were significantly higher than those of C group; LDH, CK and AST levels in cell culture medium of SP group were significantly lower than those of H/R group; LDH, CK and AST levels in cell culture medium of ComC group were significantly higher than those of SP group. As shown in Table 1, differences in pair-wise comparison of LDH, CK and AST levels in cell culture medium were statistically significant among four groups of cells ($P < 0.05$).

3.4. Effect of sevoflurane pretreatment and AMPK inhibitor pretreatment on apoptosis gene expression in myocardial cells under hypoxia reoxygenation conditions

Analysis of apoptosis genes Bcl-2, Bax and Caspase-3 expression in myocardial cells among C group, H/R group, SP group and ComC group was as follows: Bcl-2 expression in cells of H/R group was significantly lower than that of C group while Bax and Caspase-3 expression were significantly higher than those of C group; Bcl-2 expression in cells of SP group was significantly higher than that of H/R group while Bax and Caspase-3 expression were significantly lower than those of H/R group; Bcl-2 expression in cells of ComC group was significantly lower than that of SP group while Bax and Caspase-3 expression were significantly higher than those of SP group. As shown in Table 1, differences in pair-wise comparison of Bcl-2, Bax and Caspase-3 expression in cells were statistically significant among four groups of cells ($P < 0.05$).

4. Discussion

Ischemia-reperfusion injury is the pathophysiological process that involves cell apoptosis, oxidative stress, inflammation and other complex changes, and the ischemia-reperfusion injury in ischemic myocardium after reperfusion therapy can cause acute heart failure and influence the effect of reperfusion therapy [9,10]. Prevention and treatment of myocardial ischemia-reperfusion injury has been the research hotspot in the cardiovascular field, inhalation anesthetic sevoflurane has anti-oxidant, anti-inflammatory and anti-apoptotic effect, and a number of animal studies have confirmed that sevoflurane can relieve myocardial ischemia-reperfusion injury in rats [11–13]. In the study, hypoxia reoxygenation of myocardial cells cultured *in vitro* was adopted to simulate the process of myocardial ischemia-reperfusion injury, and in order to define the effect of sevoflurane on myocardial cell injury caused by hypoxia reoxygenation, *in vitro* cultured myocardial cell proliferation activity and apoptosis rate were analyzed, cell proliferation activity value of H/R group was significantly lower than that of C group, and the apoptosis rate was significantly higher than that of C group, cell proliferation activity value of SP group was significantly higher than that of H/R group, and the apoptosis rate was significantly lower than that of H/R group. This means that the treatment conditions of hypoxia for 14 h and reoxygenation for 3 h can cause myocardial cell damage, the cell proliferation activity significantly reduces and the apoptosis rate increases significantly after hypoxia reoxygenation treatment; sevoflurane pretreatment can reduce the myocardial cell injury caused by hypoxia reoxygenation, and sevoflurane pretreatment can significantly increase the cell proliferation activity and reduce the apoptosis rate after hypoxia reoxygenation.

Myocardial enzymes LDH, CK and AST are the common clinical biochemical indicators to evaluate the degree of myocardial injury. In physiological conditions, the myocardial enzymes exist in the cytoplasm of myocardial cells, and the extracellular myocardial enzyme levels are extremely low; in the case of ischemia-reperfusion injury in myocardial cells, the cells rupture and the myocardial enzymes are massively released into the blood circulation, characterized by the elevated serum myocardial enzyme levels. In the myocardial cells cultured *in vitro*, the cell injury caused by hypoxia reoxygenation can cause the release of myocardial enzymes in cells into the culture medium, the myocardial enzyme levels in culture medium were analyzed to reflect the damage degree of myocardial cells cultured *in vitro*, and the results showed that LDH, CK and AST levels in cell culture medium of H/R group were significantly higher than those of C group; LDH, CK and AST levels in cell culture medium of SP group were significantly lower than those of H/R group. The change trend of above myocardial enzymes in culture medium is consistent with that of cell proliferation activity and apoptosis rate, it indicates that hypoxia reoxygenation can cause myocardial cell injury, and sevoflurane pretreatment can reduce the myocardial cell injury caused by hypoxia reoxygenation. Mitochondrial apoptosis is the important mechanism that leads to the myocardial cell hypoxia reoxygenation damage, and hypoxia reoxygenation stimulation can affect the cell energy metabolism, damage mitochondrial function and cause the cytochrome C release from mitochondria into the cytoplasm, which activates Caspase-3 and induces apoptosis [14,15]. Bax/

Bcl-2 are the important molecules that regulate mitochondrial membrane permeability to cytochrome C, Bax can increase mitochondrial membrane permeability to cytochrome C and promote cell apoptosis, and Bcl-2 can antagonize the Bax function and suppress apoptosis [16,17]. In the study, analysis of above mitochondrial apoptosis gene expression showed that Bcl-2 expression in cells of H/R group was significantly lower than that of C group while Bax and Caspase-3 expression were significantly higher than those of C group; Bcl-2 expression in cells of SP group was significantly higher than that of H/R group while Bax and Caspase-3 expression were significantly lower than those of H/R group. This means that hypoxia reoxygenation can regulate the expression of Bax/Bcl-2 to activate Caspase-3 and induce apoptosis; sevoflurane pretreatment can inhibit the Caspase-3 activation and cell apoptosis.

At present, it is still not completely clear about the molecular mechanism for sevoflurane to alleviate myocardial ischemia-reperfusion injury in rats and *in vitro* cultured myocardial cell hypoxia reoxygenation injury. AMPK is the serine/threonine protein kinase that regulates a variety of biological processes in the myocardial cells, and after phosphorylated activation, it can adjust the myocardial cell energy metabolism and protect the myocardial cells [18–20]. In order to define the role of AMPK in the process that sevoflurane pretreatment alleviated the hypoxia reoxygenation injury of myocardial cells, the p-AMPK expression was analyzed in the study, and the result showed that p-AMPK expression in cells of H/R group was significantly lower than that of C group, and p-AMPK expression in cells of SP group was significantly higher than that of H/R group. This means that hypoxia reoxygenation conditions can inhibit AMPK phosphorylation process and make the myocardial cells lose the protection from AMPK and be damaged; sevoflurane pretreatment could increase the AMPK phosphorylation and protect the myocardial cells with hypoxia reoxygenation through AMPK. After the effect of hypoxia reoxygenation and sevoflurane pretreatment on AMPK phosphorylation was confirmed, AMPK inhibitors combined with sevoflurane treatment was adopted in the study to determine whether sevoflurane alleviated the myocardial cell injury caused by hypoxia reoxygenation through by AMPK. The results showed that AMPK inhibitor combined with sevoflurane treatment could reverse the effect of sevoflurane on inhibiting myocardial cell apoptosis, reducing myocardial enzyme release and adjusting the Bax/Bcl-2 expression. This means that the effect of sevoflurane on relieving the myocardial cell injury caused by hypoxia reoxygenation is achieved through activating AMPK phosphorylation.

To sum up, it is believed that hypoxia reoxygenation can inhibit AMPK phosphorylation to cause myocardial cell apoptosis, and sevoflurane pretreatment can activate AMPK phosphorylation to relieve the myocardial cell injury caused by hypoxia reoxygenation.

Conflict of interest statement

We declare that we have no conflict of interest.

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