Sevoflurane Postconditioning Inhibits Pulmonary Apoptosis via PI3K/AKT in Dog Cardiopulmonary Bypass Model

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ABSTRACT

Aims: The study aimed to investigate the protective effects and regulatory mechanism of sevoflurane postconditioning (SPC) in pulmonary apoptosis induced by cardiopulmonary bypass (CPB).

Methods: Twenty-four healthy dogs were divided into a control (C group), ischemia/reperfusion (I/R group), sevoflurane postconditioning (S group), and wortmannin group (S+W group). At 10 min after the establishment of CPB, the left pulmonary artery was blocked. When the pulmonary artery was reopened, 2% sevoflurane was administered. Wortmannin was delivered 10 min before the pulmonary artery was open. Before thoracotomy was implemented (T1), when the artery was reopened (T2) and 2 h after CPB (T3), blood and the inferior lobe of the left lung were isolated and subjected to gas analysis, pathological examination, western blot, and TUNEL staining.

Results: No obvious changes were observed in the C group throughout the experiment. The conditions of all treated groups progressively deteriorated, and no difference could be found except in the number of apoptotic cells of T3 between the S+W and I/R groups. At T2, the treated groups showed similar conditions. At T3, the lung function and structure of the S group were improved in I/R and S+W groups. The S group showed the highest p-Akt expression, the lowest cleaved-caspase 3 expression, and apoptotic cell percentage.

Conclusions: Ischemia-reperfusion of the lung during CPB reduces lung function and injures the pulmonary structure via inducing lung apoptosis. Sevoflurane postconditioning preserves lung function and structure by alleviating apoptosis via activation of PI3K/Akt.

INTRODUCTION

Every year, more than one million patients worldwide undergo cardiac surgery [Denault 2010]. Cardiopulmonary bypass (CPB) facilitates the repair of cardiac lesions in adults and children. Pulmonary dysfunction is the most frequent complication in the early postoperative phase following cardiac surgery involving extracorporeal circulation, which greatly influences the outcome of patients [Kiessling 2014]. In addition, it was found that standard CPB and cardioplegic arrest-initiated apoptosis in pneumocytes and the cell loss might be responsible for severe organ dysfunction [Klass 2007; Yu 2013; Fischer 2000; Van Putte 2005]. Therefore, the inhibition of apoptosis of pneumocytes is beneficial to protect against lung injury induced by CPB. Nowadays, sevoflurane preconditioning as an antiapoptosis agent exerts a protective effect against ischemia-reperfusion damage in heart, brain, and liver [Rodríguez 2015; Wang 2016; Wang 2010; Li 2014]. Furthermore, sevoflurane postconditioning (SPC) was found to exert a protective effect in different models [Inamura 2010; Jeon 2013; Meybohm 2011]. However, it was unknown whether sevoflurane postconditioning exerts a protective effect on CPB-induced lung injury. Therefore, this study was designed to explore the effect of sevoflurane postconditioning on lung injury induced by CPB and whether antiapoptosis was involved in the protective mechanisms.

The PI3K/AKT pathway plays a key role in normal cellular functions including proliferation, adhesion, migration, invasion, energy metabolism, protein synthesis, and prosurvival. It has been found that the PI3K associated signaling pathway is involved in the cardioprotective pathway against ischemia/reperfusion (I/R) injury [Zhang 2014; Wei 2015; Yao 2014]. Moreover, researchers found that sevoflurane preconditioning is associated with its properties of antiapoptosis via activation of the PI3K/Akt pathway [Zhang 2015; Liu 2015]. Accordingly, we hypothesize that the PI3K/Akt pathway activation is involved in lung-protective signaling induced by sevoflurane postconditioning and that this activation inhibits apoptosis.

MATERIALS AND METHODS

Animals and experimental design: All animal studies were performed in accordance with Zunyi Medical College’s policy for the care and use of animals for scientific purposes.
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The occurrence of apoptosis was evaluated by using a gas analyzer. The respiratory index (RI), and oxygenation index (OI) were calculated using the following formula: OI = PaO2/FIO2; RI = P(A-a)O2/PaO2 = FiO2 × 713−PaCO2 − PaO2 (Fio2, fraction of inspiration O2; PaCO2, partial pressure of CO2 in arterial blood; PaO2, partial pressure of O2 in arterial blood).

Histological examination: To evaluate the morphological severity, the tissues were fixed in 10% formaldehyde, embedded in paraffin, and stained with hematoxylin and eosin (H&E).

Western blotting analysis: The tissue was homogenized with protein lysis buffer. The homogenate was centrifuged (15 000g, 4°C) for 15 min. The supernatants were assayed for protein concentration using a Bicinchoninic Acid (BCA) Protein Assay Kit (Beyotime Institute of Biotechnology, Jiangsu, China) and stored at −80°C. All protein samples were electrophoresed on sodium dodecyl sulfate (SDS)–polyacrylamide gels (60 μg protein/sample was loaded for each immunoblot lane) and transferred to polyvinylidene fluoride membranes. Membranes were blocked in phosphate buffered saline (PBS) buffer containing 5% powdered milk for 1.5–2 h, washed, and incubated with primary antibody overnight at 4°C [p-Akt: 1:700 (Cell Signaling Technology, USA); cleaved-caspase3: 1:600 (Abcam, UK); β-actin: 1:800 (Bioss, China)]. After incubation, samples were washed five times with PBST (phosphate buffered saline with 0.1% Tween 20) and incubated with near-infrared-tag monoclonal antibody (1:6000) for 1.5 h. Membranes were imaged using a LiCor Odyssey scanner (LiCor, Lincoln, NE, USA) and values were obtained with Odyssey 3.0 analytical software (LiCor, Lincoln, NE, USA).

TUNEL staining: The occurrence of apoptosis was assessed using TUNEL assay. In brief, the sections were treated with 20 μg/ml proteinase K for 15–30 min at room temperature and at 37°C for 15 min. Subsequently, the sections were washed with PBS. After the addition of 50 μl TUNEL reaction mixture, the sections were incubated for 60 min at 37°C, rinsed with PBS, and visualized using converter-POD for 30 min at 37°C for 15 min. Then, the sections were washed with PBS and incubated with 50–100 μl 0.05 % 3,3’-diaminobenzidine (DAB) as a chromogen for 10 min at room temperature. Under the light microscope, images were then obtained at 400 x magnification following the random selection of five non-overlapping regions. The number of apoptotic cells and total cells were counted, and then the cell apoptosis rate was determined by the following equation: cell apoptosis rate = the number of apoptotic cells/total cells × 100%.

Statistical analysis: Statistical analyses were carried out using SPSS 17.0 (IBM SPSS Statistics for Windows, IBM Corp., Armonk, NY, USA). Data were presented as mean±SD. Comparisons between groups for statistical significance were performed with Student’s t test and analysis of variance. Comparisons of data in the same group were performed with repeated measures analysis of variance (ANOVA), and Mauchly’s test of sphericity was used (the epsilon correction was used when necessary). P < 0.05 was considered statistically significant.

RESULTS

Baseline characteristics: No significant differences were observed among the four dog groups with regard to body weight, body area surface, CPB time, after CPB time, the lowest nose temperature, and volume of liquid (P > 0.05). (Table 1)
Sevoflurane postconditioning improves lung function following I/R. Table 2 shows the changes in pulmonary function parameters. (Table 2) There was no difference among the four groups at T1 ($P > 0.05$). In the C group, signs of reduced lung function could not be found ($P > 0.05$). As the CPB went on, the lung function in the I/R, S, and S+W groups deteriorated indicated by growing OI and decreasing RI. At T2, no difference could be found among I/R, S, and S+W groups ($P > 0.05$). However, at T3, the lung function of the S group was better than both the I/R and S+W groups ($P < 0.05$). There was no significant difference between the I/R group and the S+W group ($P > 0.05$). These results show that sevoflurane postconditioning protects the lung function and the effect could be inhibited by wortmannin.

**Sevoflurane postconditioning alleviates pulmonary ultrastructural disorder:** H&E staining results showed that the alveolar structure at T1 was complete and had no abnormal changes, such as effusions of inflammatory cells and widened pulmonary interstitial space in all groups. At T2, no significant changes could be found in the C group. (Figure 1) In the other three groups (I/R, S, and S+W groups), some alveolar walls broke and some alveolar intervals widened, few inflammation cells and angiotelectasis could be found. At 2 h after CPB, in the C group, the alveolar walls were still smooth and intact, rare inflammation cells could be found; in I/R and S+W groups, the most serious and obvious changes were found, including ruptured walls, collapsed alveolar cavity with lots of red cells and inflammation cells; in S group, parts of walls ruptured with few inflammation cells and red cells. These results indicated that during CPB, ischemia-reperfusion was one of the main reasons of pulmonary damage and sevoflurane postconditioning could preserve lung structure and the protective effect could be abolished by the inhibitor of PI3K.

**Sevoflurane postconditioning reduces the apoptotic index of lung:** At T1, the rate of apoptosis in lung tissues was not significantly different among all groups ($P > 0.05$). In the C group, no difference could be found at all three time points. In the other three groups, the number of apoptotic cells steadily increased with time after CPB was processed. At T2, the rate of apoptosis in the I/R, S, and S+W groups was no different ($P > 0.05$). At T3, the number of TUNEL-positive cells was significantly higher in the I/R group compared with the other three groups ($P < 0.05$), sevoflurane postconditioning reduced the number significantly and PI3K inhibitor alleviated but did not abolish the effect of sevoflurane.

The expression of cleaved caspase 3, one of the important markers of apoptosis, was detected via Western blot. (Figure 3) At T1, there was no significant difference among all the groups ($P > 0.05$). As the extension of CPB, the expression of cleaved-caspase 3 in the C group was no different, the levels of cleaved-caspase 3 increased continually in the other three groups.

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**Table 1. The general statistics of each group during CPB (N = 6)**

<table>
<thead>
<tr>
<th>Variables</th>
<th>Control group</th>
<th>I/R group</th>
<th>S group</th>
<th>S+W group</th>
<th>$P$-values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (kg)</td>
<td>12.4±1.14</td>
<td>12.08±1.53</td>
<td>11.75±1.33</td>
<td>11.92±1.16</td>
<td>$P &gt; 0.05$</td>
</tr>
<tr>
<td>Body surface area (m$^2$)</td>
<td>0.58±0.04</td>
<td>0.57±0.05</td>
<td>0.56±0.04</td>
<td>0.56±0.04</td>
<td>$P &gt; 0.05$</td>
</tr>
<tr>
<td>CPB time (min)</td>
<td>104.83±1.47</td>
<td>105.67±2.88</td>
<td>106.17±3.31</td>
<td>105.50±2.74</td>
<td>$P &gt; 0.05$</td>
</tr>
<tr>
<td>After CPB time (min)</td>
<td>123.83±1.72</td>
<td>124.50±1.87</td>
<td>124.50±1.64</td>
<td>124.00±2.00</td>
<td>$P &gt; 0.05$</td>
</tr>
<tr>
<td>Lowest nose temperature (℃)</td>
<td>31.68±0.41</td>
<td>31.83±0.50</td>
<td>31.76±0.48</td>
<td>31.51±0.43</td>
<td>$P &gt; 0.05$</td>
</tr>
<tr>
<td>Volume of liquid (ml)</td>
<td>359.67±8.40</td>
<td>350.00±7.07</td>
<td>356.00±5.89</td>
<td>354.17±10.68</td>
<td>$P &gt; 0.05$</td>
</tr>
</tbody>
</table>

Data were presented as mean ± SD. $P > 0.05$ for comparison of four dog groups

**Table 2. Pulmonary function parameters**

<table>
<thead>
<tr>
<th>Group</th>
<th>T1</th>
<th>T2</th>
<th>T3</th>
<th>T1</th>
<th>T2</th>
<th>T3</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>0.40±0.17</td>
<td>0.44±0.19</td>
<td>0.56±0.024</td>
<td>450.67±25.70</td>
<td>438.17±23.59</td>
<td>431.50±19.81</td>
</tr>
<tr>
<td>I/R</td>
<td>0.41±0.22</td>
<td>1.37±0.25*‡</td>
<td>3.72±0.64*†‡</td>
<td>456.50±22.69</td>
<td>373.33±21.98*† ‡</td>
<td>226.67±24.76*† ‡</td>
</tr>
<tr>
<td>S</td>
<td>0.49±0.17</td>
<td>1.24±0.14*‡</td>
<td>2.03±0.29**†‡ §</td>
<td>466.33±20.12</td>
<td>376.33±17.64*‡</td>
<td>325.33±19.02*†‡ §</td>
</tr>
<tr>
<td>S+W</td>
<td>0.44±0.22</td>
<td>1.28±0.21*‡</td>
<td>3.23±0.52*†∥</td>
<td>448.50±22.58</td>
<td>369.00±14.10*†</td>
<td>250.67±41.82*†∥</td>
</tr>
</tbody>
</table>

Data were expressed with mean ± SD. *$P < 0.05$, compared with T1 in the same group; †$P < 0.05$, compared with T2 in the same group; ‡$P < 0.05$, compared with the C group at the same time point; §$P < 0.05$, compared with the I/R group at the same time point; ∥$P < 0.05$, compared with the S group at the same time point.
Figure 1. Pathological examination of lung in different groups using H&E staining. At T2, no significant changes could be found in the C group, whereas the other three groups showed broken alveolar walls and some extent of inflammation and angioatelectasis. At T3, no significant changes could be found in the C group; I/R and S+W groups showed serious inflammation. Ruptured walls collapsed alveolar cavity with numerous red cells and inflammation cells were observed. In contrast, the S group showed medium extent of inflammation, suggesting the protective effects of sevoflurane postconditioning.

Figure 2. Sevoflurane postconditioning reduces the apoptotic index of lung. Representative images and quantification of TUNEL stainings at T1, T2, and T3. Data were expressed with mean ± SD. *P < 0.05, compared with T1 in the same group; †P < 0.05, compared with T2 in the same group; ‡P < 0.05, compared with the C group at the same time point; §P < 0.05 compared with the I/R group at the same time point; &P < 0.05, compared with the S group at the same time point.
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The same result that sevoflurane exerts protective effects against cerebral and ischemia leads to activation of PI3K/AKT [Inamura 2010; Li 2012]. So, we believe that the active AKT is an important endogenous protective mechanism. Nowadays, it was found that sevoflurane exerts protective effects against cerebral and intestinal ischemia-reperfusion damage via the PI3K/Akt pathway [Zhang 2015; Liu 2015; Ye 2015]. The same result could be found in this study. It could be seen that sevoflurane postconditioning preserved lung function and alleviated changes of structure, which were abolished by wortmannin.

DISCUSSION

In the present study, 2.0% sevoflurane was administered for 30 min since the onset of reperfusion following ischemia. We found that SPC significantly improved lung function and lung structure and decreased the apoptotic level. The novel finding of this study is that SPC increases the phosphorylation of AKT. Administration of wortmannin before SPC abolished the protective effect against lung injury. Our study demonstrated a direct link between PI3K/AKT pathway activation and the attenuation of lung apoptosis induced by SPC.

Most studies of SPC are on the heart and brain and indicate that SPC is an effective method to reduce I/R injury [Lee 2015; Li 2013; Zhang 2015]. Few studies found that SPC was effective against lung injury induced by lipopolysaccharide [Zhao 2014; Steurer 2009]. The results of this study indicate that SPC exerts a protective effect against lung injury during CPB. Therefore, these studies support the idea that SPC serves as a protective strategy.

In this study, the expression of p-Akt increased during the ischemic period in all groups except the C group. This result is similar to Li et al. [Li 2015]. In their study, the expression of p-Akt in the IRI group was higher than in the control group, but with no significant difference. We think the difference between our studies was caused by different models. In their study, the pulmonary hilum was occluded with a non-crushing microvascular clamp without the CPB process. Therefore, the damage was not as harmful as our model. However, our results are similar to the findings of Zhang and R. So, we believe that the active AKT is an important endogenous protective mechanism. Nowadays, it was found that sevoflurane exerts protective effects against cerebral and intestinal ischemia-reperfusion damage via the PI3K/Akt pathway [Zhang 2015; Liu 2015; Ye 2015]. The same result could be found in this study. It could be seen that sevoflurane postconditioning preserved lung function and alleviated changes of structure, which were abolished by wortmannin.

Researchers found that antiapoptosis was involved in the protective mechanisms of sevoflurane [Inamura 2010; Zhang 2015]. In addition, PI3K/Akt is an important signal transduction pathway that involves many cellular physiological activities. There is consensus that activation of Akt, which is known to be pro-survival signaling, plays an important role in myocardial protection [Tsai 2015]. So, we think the apoptosis effect of SPC was associated with PI3K/Akt signal pathway. The notion was verified by the results of this study. A recent study indicated that the level of miR-320 was positively associated with RI, but negatively correlated with OI [Yang 2015]. Furthermore, it was found that miR-320 can promote apoptosis [Ren 2009]. In this study, RI and OI in the C group were with no difference in the extension of CPB. However, in the other three groups that underwent IR, both greatly changed. The changes in lung function were similar to cleaved-caspase-3 which is the key enzyme required in the mitochondria-caused apoptosis. Combining with the results of TUNEL staining, it can be concluded that IR is one of the main reasons for pulmonary apoptosis during CPB. This conclusion is accordant with previous research [Yang 2015; Slottosch 2014]. In this study, all apoptosis-related indexes were relieved by SPC and all were reversed by wortmannin. So, this study provided the direct information to support our notion that antiapoptosis via the PI3K/Akt pathway against...
pulmonary injury induced by IR is involved in the protective mechanisms of SPC during CPB. Furthermore, the apoptotic cells of S+W were less than the I/R group, indicating that the antiapoptotic effect of SPC could be totally reversed by PI3K inhibitor. Therefore, we inferred that other mechanisms of antiapoptosis were involved in SPC.

CONCLUSION

In conclusion, 2% sevoflurane delivered at the onset of reperfusion for 30 min is beneficial to those who receive CPB treatment exerting protective effect against pulmonary apoptosis. This study provides direct data for the notion that the PI3K/Akt pathway plays an important role in the antiapoptosis induced by sevoflurane postconditioning on lung injury caused by CPB.

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REFERENCES


