Effect of Propofol on Expression of PKC mRNA During Pulmonary Ischemia-reperfusion Injury

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Abstract

Aim: The objectives of the present work was to study the effect of propofol on the expression of protein kinase C(PKC)mRNA during pulmonary ischemia-reperfusion injury (PIRI) in the rabbits.

Methods: Single lung ischemia-reperfusion model was administrated in vivo. Twenty-seven experimental rabbits were randomly divided into three groups(n=9):sham-operated group(Sham), PIR group(I/R) and PIR+propofol group(PPF). Changes of several parameters, including malondialdehyde (MDA), superoxide dismutase (SOD), lung wet/dry weight (W/D) and the index of quantitative assessment (IQA) of histologic lung injury were measured at 60 minutes after reperfusion. Meanwhile the location and expression of PKC mRNA were observed. Lung tissues were examined under the optical microscope and the electron microscope.

Results: As compared with group I/R, PKC mRNA notably expressed in intima and extima of small pulmonary artery as well as thin-wall vessels(mostly small pulmonary veins)in PPF group. The average optical density values of PKC-α, δ and θ mRNA in small pulmonary veins in group PPF had significance (all P<0.01) increased. SOD increased ,while MDA,W/D and IQA decreased at 60 minutes after reperfusion in lung tissue (P<0.01 and P<0.05). Abnormal changes of the lung tissue in morphologically were lessen markedly in group PPF.

Conclusion: our studies demonstrated that propofol possessed significantly protective effects on PIRI in rabbits by activating PKC-α δ and θmRNA expression in lung tissue, raising NO level, reducing OFR level and decreasing lipid peroxidation.

Keywords: Ischemia-reperfusion injury, Lung, protein kinase C, Oxygen free radicals, Nitric oxide, Propofol

Abbreviations

PKC: protein kinase C; PIRI: pulmonary ischemia-reperfusion injury; PIR: pulmonary ischemia-reperfusion; Sham: sham-operated group; I/R: ischemia-reperfusion injury group; PPF: pulmonary ischemia-reperfusion + propofol group; MDA: malondialdehyde; SOD: superoxide dismutase; W/D: wet/dry weight; IQA: the index of quantitative assessment.

INTRODUCTION

In recent years, With the rapid development of medical science, the novel medical methods like lung-transplantation, heart-lung transplantation and lung thrombolytic therapy have been continually establishing and developing.[1,2]. However, Lung ischemia-reperfusion injury always serves as an important element affecting prognosis after thrombolysis and transplantation. Therefore, it is

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urgent to search methods of antagonistic PIRI [3,4]. In this study, based on the PIRI model of experimental rabbits, we observed the expression orientation of protein kinase C(PKC) mRNA in lung tissue, the activity of superoxide dismutase(SOD), concentration of malondialdehyde(MDA), content of nitric oxide (NO), wet to dry ratio(W/D) of tissue, index of quantitative assessment (IQA) of histological lung injury and the change of lung histomorphology, finding out the influence of propofol (PPF) on them. In order to investigate the mechanism of it anti-PIRI, which provided theoretical basis for improving the protection of lung during perioperative period.

MATERIALS AND METHODS

Model

We used 27 male Japanese big-ear rabbits, (2.0-2.5)kg, intravenous with ethyl carbamate (1.0g/kg). After trachea incision and intubation, inhale pure oxygen with animal broad-spectrum antibiotics. Ventilation frequency was (30 ~ 40 )times / min. Tidal volume of two-lung ventilations was (15 ~ 20ml) / kg, while tidal volume of one-lung ventilations was (8 ~ 10ml) / kg. And respiratory ratio was 1:1.25. Then unilateral external jugular vein were separated to intubate, continuous static drops with physiological saline (0.5 ~ 1.5)ml/min. Open the left chest between the 4th and the 5th ribs, and inject intravenously of heparin for anticoagulant (1.0mg/kg *BW). Sekido’s method was used to establish rabbit's PIRI in vivo pattern, that is using occlusion method to block left lung vessels and bronchus completely, after certain time, recovering ventilation and perfusion.

Experimental Groups

We divided the rabbits into three groups randomly. And each group consisted of 9 rabbits.
1. The sham-operation group (Sham): Dissociate left hilum, and block it. Then, Observe for 150 minutes.
2. The pulmonary ischemia-reperfusion group (I-R): After blocking left hilum, observe for 30 minutes. And then block the hilum of left lung for 60 minutes and restore the supply of blood and oxygen for another 60 minutes.
3. The lung ischemia-reperfusion + propofol group (PPF): Be maintained with propofol continuous infusion through the external jugular vein with a micro-infusion pump until the operation finished, 20mg • kg-1 • h-1 (Sweden AstraZeneca Co, 200mg • 20ml-1 • Support -1). Other procedures were similar to I-R group.

Lung Tissue PKC-A, Α Ø and in Situ Hybridization Analysis

Specimens Processing of Lung Tissue

After fixation, the tissues were dehydrated with ethanol(75%, 85%, 95% and 100%) for 30min each, and embedded in paraffin. Next, cut them into 5μm pieces, and mounted on polylysine coated glass slides.

PKC Isoenzyme Oligonucleotide Probe Sequence

PKC-α:
5’--CAGGA CGTGG CCAAC CGCTT CGCCC GCAAA--3’
5’--GAATG ACTTC ATGGG ATCCC TTTCC TTTGG--3’
5’--CCACG CTCTG CGGAA TGGA T CACAC TGAGA--3’

PKC-δ:
Steps of in Situ Hybridization

1. Tissue sections were baked at 60°C overnight, and were treated with xylene 15min*2 times, 100% alcohol 2 times, and 95% and 80% alcohol 5min*1 time for each to dewax.

2. Treat with 3% H2O2-methanol at room temperature for 10min to inactivate endogenous peroxidase. Then, washing with water 5min*2times ,which was disposed by DEPC.

3. The sections were added 3%pepsin that diluted with citric acid freshly, digesting for10min,to expose fragments of mRNA nucleic acids. Next, wash with 0.5mmol/L PBS(pH=7.4) 5min*3times and wash with water for one time.

4. Sections were added with 20μL pre-hybridization-solution at 37℃ for 3h. Then, soak up excess liquid without washing.

5. Add 20μL hybridization- solution containing PKC isoenzymes oligonucleotide probes on them. And covered with in situ hybridization dedicated cover slip at 42°C overnight/about 17h). Uncover the cover slip, and wash with 2*SSC 5min*2times,0.5*SSC 15min*2times,0.2*SSC 15min*1time. The negative control group were added hybridization-solution without probes.

6. Add 5%BSA dilute normal horse serum to block nonspecific antibody at 37°C for 30min. Soak up excess liquid without washing.

7. Add biotinylated mouse-anti-digoxin at 37°C for 60min, and wash with 0.5mmol/L PBS 5min*4times.

8. Add SABC fluid at 37°C for 20min,and wash with 0.5 mmol/L PBS 5min*3times.

9. Add biotinylated peroxidase at 37°C for 20min,and wash with 0.5 mmol/L PBS 5min*3times.

10. DAB reagent chromogenic reaction were lasted for 20min.Then wash sufficiently. Hematoxylin stain and wash sufficiently. Next, 75%,85% and 95% alcohol 5min for each ,and 100% alcohol 5min*2times to dewax. Use Xylene to make it transparent 5min*3 times. Close with Clearmout.

Judging criteria

Lung tissues which were colorated of yellow brown were mRNA positive expression of PKC isozymes.

Absorbance analysis

Using absorbance analysis software which developed in East China University of Science and Technology to read the absorbance values. We made the nonspecific staining of extra vascular connective tissue as negative cells background. The nonspecific staining background were removed from the measured blood vesse. Then, read the absorbance values.

Analyze 5-10 thin-walled small blood vessels (mainly small pulmonary vein) in each film. Repeat 3 times, and take the average.
Measures of MDA Content, SOD Activity, NO Level and W / D Value of Lung Tissues

The animals were sacrificed after reperfusion for 60min., and about 100mg left lung tissues were cut for making into homogenate. It were used to measure MDA, SOD and metabolite of NO (NO2-/NO3-) levels with methods of TBA, xanthine oxidase and nitricreductase. The 3 kits were purchased from the Institute of Nanjing Jiancheng Bioengineering. Another 2g left lung tissue was weighted as wet weight, and then weight it after baking 24h in the electro-temperature drying oven as dry weight. The ratio of them was W/D.

Examination with Light Microscope and Determinations of IQA

Take left lung lower lobe tissue about 1mm*1mm*1mm. Then, it was fixed with paraformaldehyde, paraffin embedded, sectioned and stained with HE. Observe the changes of configuration under optics microscope. According to the method introduced by Murata[5], observe 10 visions continuously from the 200 times fields of view. Next, calculatie the number of alveolar damage accounts for the percentage of the total number of alveolar [the number of damage alveolar, which containing more than 2 red blood cells (RBC) and / or neutrophils (PMN), accounted for counting the percentage of the total number of alveolar ]. That is, the ratio of the number of alveolar damage, lung damage as a quantitative evaluation index (IQA).

Electron Microscopy Examination of Lung Tissue

There are 4 cases in each groups. Take the left hilar tissue, about the 1cm3, fixed with 2.5% glutaraldehyde, 1% osmium tetroxide, after fixed, ethanol - acetone series gradient dehydration after Epon812 embedded, LKB-V-thin slices Slicing machine, H-600-transmission electron microscopy observation.

Data Analysis

All the datas were expressed with mean ± standard deviation (x ± s) and that were analyzed by variance. Using Pearson to research the relationship among the parameters of MDA, SOD, W / D, IQA and PKC Isoenzyme.

RESULTS

The Changes of the Absorbance Value of PKC Isozyme in Situ Hybridization Pulmonary Venules Absorbance Values Among 3 Groups Rabbits During PIRI

Figure 1. Comparison of mean ISH OA values of PKC Isoenzymes in pulmonary small veins among three groups in rabbits (x±s, n=9). # P<0.05, ##P<0.01 vs. Sham; & P<0.05, && P<0.01 vs. I-R

The variance analysis showed that the expression of pulmonary venules PKC-α, δ and θ mRNA in IR
group were increased. However, there was no significant difference with the S group (P> 0.05). The expression of pulmonary venules PKC-α, δ and θ mRNA in PPF group was significantly higher than those in Sham group (P <0.05 and P <0.01), and the expression of PKC-α and δ mRNA was higher than those in IR group (P <0.05 and P <0.01). (Figure 1)

Changes of Lung Tissues SOD Activity, MDA Content and the Level of NO Among 3 Groups Rabbits During PIRI

The concentration of MDA in IR group was significantly higher than that in Sham group, while the activity of SOD was remarkably lower than that in Sham group (P<0.01); the concentration of MDA in PPF group was significantly lower than that in IR group, while SOD activity and NO2-/NO3-levels were significantly higher than those in IR group (P<0.01). However, SOD and MDA in PPF group were not significantly different from those in Sham group (P> 0.05), while the level of NO was significantly higher than that in Sham group (P<0.01). (Figure 2)

![Figure 2. Comparison of SOD activity, MDA content and NO level in lung tissue among three groups in rabbits (x±s, n=9). ##P<0.01 vs. sham group; &&P<0.01 vs. I-R group](image)

Changes of Lung W/D and the Value of IQA Among 3 Groups Rabbits During PIRI

Using the analysis of variance, after reperfusion 60min, the lung W/D and IQA value in IR group were significantly higher than those in Sham group (P<0.01). And the lung W/D and IQA value in PPF group were significantly lower than those in IR group (P<0.05 and P<0.01). There was no significant difference between Sham group and PPF group about W/D value (P> 0.05). (Figure 3)

![Figure 3. Comparison of W/D and IQA values in lung tissue among three groups in rabbits (x±s, n=9). ##P<0.01 vs. Sham; & P<0.05, && P<0.01 vs. I-R](image)
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The Relationship Among the Parameters of Lung Tissue MDA, SOD, W/D, IQA and PKC Isoenzyme

Linear correlation analysis showed that there was a negative correlation between MDA and PKC-α, δ and θ mRNA in situ hybridization absorbance values, and the r (correlation coefficient) were -0.701, -0.651 and -0.626, P <0.01 respectively. While there was a positive correlation between SOD and PKC-α, δ and θ mRNA in situ hybridization absorbance values, and r were 0.574,0.620 and 0.435, P <0.01, 0.01 and 0.05 respectively. There was a negative correlation between W/D and PKCα, δ, and θ mRNA in situ hybridization absorbance values, and r were -0.721, -0.698 and -0.478, P <0.01, 0.01 and 0.05 respectively. There was also a negative correlation between IQA and PKCα, δ, and θ mRNA in situ hybridization absorbance values, and r were -0.695, -0.743 and -0.494, P <0.01, 0.01 and 0.05 respectively.

Morphological Changes in Lung Tissue

Light microscope: Pulmonary interstitial and alveolar of S group were relatively complete and had not been seen the infiltration of inflammatory cells. IR group expressed as atelectasis with emphysema, and lung parenchyma widened and edema, infiltration of inflammatory cells, and there are a lot of blood component effusion in alveolar. The injury was obviously. In PPF group, the lung injury was decreased, and there were fewer infiltration of inflammatory cells, and the integrity of the alveolar was well.

Electron microscope: In IR group, cytoplasm pinocytosis vesicles in pulmonary tiny artery endothelial cells were increased, and edema of mitochondria, even vacuolization, were obvious. The subintimal basement membranes were edematous. Vacuolar degeneration was appeared. The endothelial cells were levitating. And capillary cavity blocked with PMN, and mitochondria of type II pneumonocytes were swelling, and the lamellar body reduced (Figure 4). In PPF group, the structure of small pulmonary artery endothelial was normal, and basement membrane of outside endothelial were integral, and endothelial cells had a dense connection. Type II pneumonocytes in morphology were normal, microvilli without falling, with no obvious alveolar septum of the PMN infiltration (Figure 5).

Figure4                          Figure5

Figure4. Ultra structure change of left lung in I-R group(TEM×15000)
Figure5. Ultra structure change of left lung in I-R+PPF group(TEM×15000)
DISCUSSION

PKC is a group of serine/threonine protein kinase, with a single peptide chain structure, which has 12 kinds of subtype. It is distributed widely in the body's tissues and cells, playing an important role in the secretion of cells, hormone releasing, muscle contraction, DNA and protein synthesis, cell proliferation and differentiation, etc. Lots of substances like adenosine, bradykinin, norepinephrine can activate PKC by different ways. And Activated PKC acted on "end-effector", such as endogenous antioxidant enzymes, induced nitric oxide synthase, heat shock protein, ATP-sensitive potassium channels, etc, to play a protective action. Former study proved [5,6] that PKC can activate and induce fibroblastic growth factor and reduce apoptosis of vascular endothelial cells after radiation injury. Tanigaki[7] found that PKC inhibitors H-7 can induced acute lung injury. This research found that PKC-α, δ, and θm RNA and the sensitive index of the degree of lung tissue damage showed significantly negative correlation, indicating that PKC isozyme mediated ischemia-reperfusion injury of lung cells, which play a protective role[8].

The results of this study showed that W/D and IQA value of lung tissues in IR group could be remarkably increased, and the morphology structure were gradually abnormal. After treating with propofol, W/D and IQA value were only slightly increased, and significantly lower than the corresponding values of IR group, while the abnormal morphology structure were alleviated. This indicates that propofol had protective effects on lung cells ischemia-reperfusion injury. From the results obtained, it is evident that the expression of PKC-α, δ, and θm RNA in PPF group were significantly higher than those in Sham group and IR group, and MDA concentration in PPF group was significantly lower than that in IR group, and SOD activity and NO levels in PPF group were significantly higher than those in IR group. Furthermore, MDA, SOD, NO and PKC-α, δ, and θ mRNA expression presented a significant straight-line relationship. This suggests that propofol may make PKC be translocated and activated, and protein substrates be phosphorylated though some signal transduction, arousing the activity of endogenous antioxidant and inducible nitric oxide synthase enhanced. During this period, endogenous medium like NO which played a protective effect on cells and dilated blood vessels were increasing. Thus it reduced PIRI. Kahraman, et.al.[9] thought that propofol could effectively prevent PIRI by reducing the level of oxygen free radicals and alleviating lipid peroxidation. At the same time, propofol could react with free radicals directly and generate 2,6-diisopropylphenoxy groups to inactivate free radicals. Murphy, et.al.[10] believed that propofol mainly interfered with hydrogen abstraction process of lipid peroxidation, and the formed phenolic groups and lipid peroxidation furtherly came into being a more stable inactive product, interrupting the chain reaction of lipid peroxidation. In addition, the good fat-soluble of propofol also made it easier to accumulate in the double lipid membrane. Thereby, it enhanced the cells' ability of anti-oxidative damage [11]. Of course, propofol can improve the NO levels of body to inhibit indirectly the adhesion and aggregation of PMN [12], or to inhibit indirectly xanthine oxidase and to decrease the production of superoxide anion [13], so that the lung tissues could be prevented from the damage of oxygen free radicals to reduce the PIRI effectively. It might also reduce calcium overload by reducing the lung tissues calcium content [14,15] to prevent and treat PIRI effectively.

CONFLICT OF INTERESTS

None

REFERENCES

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