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Original Article

Transient cerebral ischemia/reperfusion-induced acute lung injury in rats associated with protein kinase C alpha expression

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Abstract: Objective: The pathogenesis and development timing of acute lung injury (ALI) following cerebral ischemia/reperfusion (I/R) are not fully understood. In this study, the development timing of ALI induced by transient global cerebral I/R as well as the underlying mechanisms of action were investigated. Methods: A cerebral I/R-induced ALI model in Wistar rats was established by electrocoagulation of bilateral vertebral arteries combined with ligation of the transient bilateral common carotid arteries. Rats were randomly divided into control and cerebral I/R groups. The latter was subdivided into 3 h, 24 h, 48 h and 72 h post reperfusion. Lung injury was assessed by histological examination. The mRNA and protein expression of protein kinase C alpha (PKCα) were determined using qRT-PCR and immunofluorescence analysis, respectively. Results: Lung histological injury could be detected as early as 3 h after global cerebral I/R, and was significant between groups at 48 h and 72 h. Compared with the control group, mRNA expression of PKCα in the lung was enhanced in rats in the cerebral I/R groups ($P<0.001$), and the highest expression was observed at 48 h ($P<0.001$). The intensity of PKCα reactivity gradually increased starting at 3 h, and peaked at 72 h after cerebral I/R ($P<0.05$). Conclusions: The lung is very susceptible to transient global cerebral I/R injury *in vivo*. Lung histological injury occurred within hours of cerebral I/R induction and aggregated in a very short period after cerebral I/R. Moreover, PKCα expression was implicated in the pathogenesis of cerebral I/R-induced ALI.

Keywords: Stroke, cerebral ischemia and reperfusion, acute lung injury, inflammation, PKCα

Introduction

Stroke is one of the most common cerebrovascular disorders and the global burden of stroke is still rising [1]. The prognosis of ischemic stroke largely depends on the incidence of complications [2]. Lung injury is the most frequent severe complication and cause of death in stroke patients [3-5].

Brain-lung crosstalk as a complex interaction is gradually being recognized [6-8]. The mechanisms underlying acute lung injury (ALI) may involve activation of inflammatory pathways, increased endovascular permeability, enhanced oxidative stress, and a change in stress hormones [9]. However, the development of ALI after stroke is not well understood and "crosstalk time" has not yet been elucidated. The purpose of this study was to investigate the development timing of ALI after transient global cerebral ischemia/reperfusion (I/R) and to elucidate the possible underlying mechanisms involved.

Materials and methods

Experimental animals and rat model of cerebral I/R injury

Mature male Wistar rats (14 weeks old, weighing 200-250 g) were provided by Shanghai SLAC Laboratory Animal Co, Ltd. (Shanghai, China). All animal experiments were reviewed and approved by the Laboratory Animal Ethics Committee of Jinhua Hospital of Zhejiang

University (Zhejiang, China), and were conducted according to the guidelines of the National Institutes of Health for the care and use of laboratory animals. Rats were randomly divided into a control group and cerebral I/R group. Furthermore, the cerebral I/R group was subdivided into four subgroups based on the time after cereb-ral I/R, which was 3 h, 24 h, 48 h or 72 h. Bilateral vertebral artery electrocoagulati-on combined with bilateral common carotid artery ligation was used to est-ablish a cerebral I/R model (four blood vessels obstruction, 4-VO) as previously described [10, 11]. Cerebral blood flow was interrupted for 10 min before reperfusion. In the control group, bilateral foramen alare of the first cervical vertebrae and common carotid arteries were anatomically exposed, however, the blood vessels were not blocked. Animals were housed at a circadian rhythm of a 12 h interval in a room at a temperature of 21-25°C and a humidity of 45-50%, and had access to normal chow and water ad libitum. Rats were sacrificed at different times after cerebral I/R.

Histological procedures

Lung tissue was harvested, embedded in paraffin, cut into 5-μm thick sections, and stained with hematoxylin and eosin (HE) using a well-established protocol [11]. Subsequently, the changes in lung histology were determined by using an optical microscope (DM4000; Leica, Heidelberg, Germany). Lung tissue destruction was evaluated based on the following five aspects: alveolar congestion, hemorrhage, neutrophilic infiltration, thickness of the alveolar wall, and hyaline membrane formation. Pathological scores were referenced to a previously published study [9].

Transmission electron microscopy was performed to assess ultrastructural changes in lung tissue. The procedures were performed as follows: the right lung of experimental animals was removed and immediately fixed in 2.5% glutaraldehyde at 4°C. Lungs were rinsed with phosphate-buffered saline (PBS) and fixed in 1.0% osmic acid for 2 h. Then lung tissue underwent acetone gradient dehydration and was stained overnight with uranium acetate. Epoxy resin-embedded specimens were cut into ultrathin sections (0.1 μm) and examined by electron microscopy (H-7500; Hitachi, Tokyo, Japan).

PKCα expression by qRT-PCR

Total RNA was extracted from lung tissues using TRIZOL reagent (no. 15596026; Ambion, USA) according to the manufacturer's guidelines. In brief, total RNA was reverse transcribed in a final volume of 20 μl using random primers under standard conditions for the PrimeScript RT reagent Kit (RR037A; TaKaRa, Dalian, China). Quantitative reverse-transcription polymerase chain reaction was performed using SYBR Premix Ex Taq™ II (Tli RNaseH Plus) (RR420A; TaKaRa, Dalian, China) according to manufacturer's guidelines. Specific primers used were as follows: protein kinase C alpha (PKCα)-F: 5'-AATACGTCAACGGTGGGGAC-3'; PKCα-R: 5'-GTGAAGAAAGAACAGTCCGATGG-3'; β-actin-F: 5'-GGAGAAGATTTGGCACCACAC-3'; β-actin-R: 5'-ACACAGCCTGGATGGCTACG-3'. PCR was conducted as follows: 95°C for 30 sec for initial denaturation, followed by 40 cycles of 95°C for 5 sec, and at 60°C for 34 sec using a LightCycler® 96 real-time PCR system (Roche Diagnostics). Results are presented as the mean ± S.D. for duplicate runs. Relative quantification of PKCα expression was calculated using the $2^{-\Delta\Delta CT}$ method relative to β-actin, which was used as an internal control.

Immunofluorescent staining

Frozen sections were thawed at room temperature (RT) for 5 min, and rehydrated in double-distilled water for 5 min, then incubated twice with 0.01 M PBS for 5 min at RT. Then, sections were incubated with 0.1% Triton X-100 for 10 min at RT, and 5% goat serum was added for 30 min. An anti-PKC alpha antibody (rabbit monoclonal antibody, 1:200, Abcam) was added and sections were incubated in a wet box for 40 h at 4°C. After washing three times 5 min with PBS, a goat anti-rabbit IgG (H+L) secondary antibody (green) (Beyotime Biotechnology, Nanjing, China) was added and sections were incubated in a wet box in the dark for 2 h at 37°C. Then, 4',6-diamidino-2-phenylindole (DAPI; 1:1000; Sigma) was added for 2 min at 37°C. Next, a fluorescence quenching agent (Beyotime Biotechnology) was added and the sections were sealed. Sections were observed and images were taken using a fluorescent microscope (Leica, Solms, Germany).

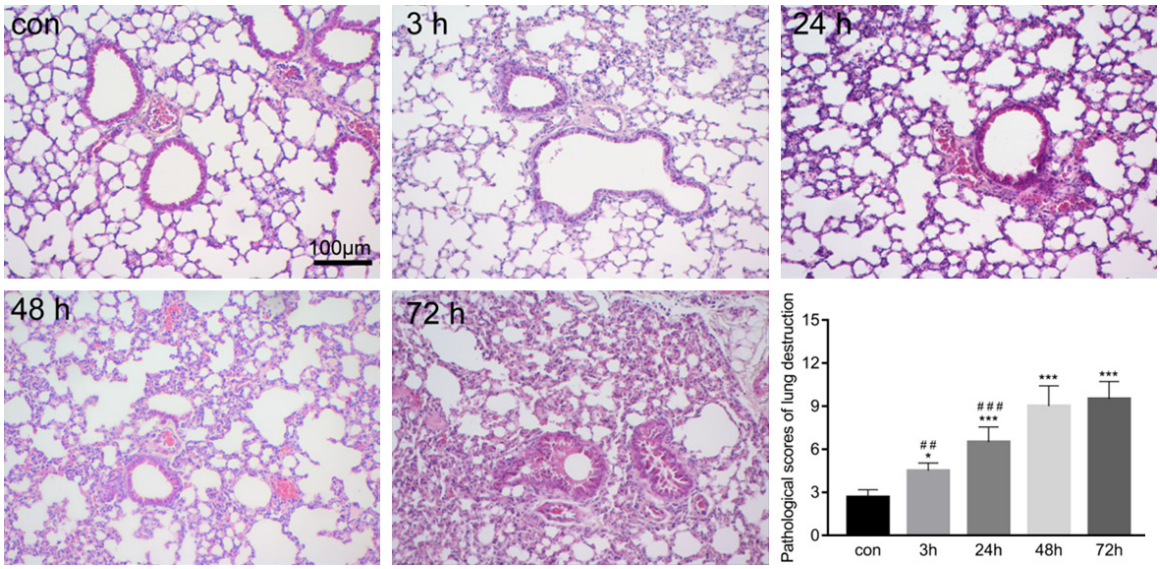

Figure 1. Hematoxylin and eosin staining demonstrates pulmonary histological changes after cerebral I/R (×200). Alveolar septal proliferation, inflammatory cells infiltration, and congested vessels in cerebral I/R groups. The pathological scores of lung tissue destruction: compared with the control group, **P*<0.05, ****P*<0.001; in cerebral I/R groups, compared with 48 h group, ##*P*<0.01, ###*P*<0.001.

Composite figures were analyzed using Image-Pro Plus 6.0 software systems (Media Cybernetics, Inc, Maryland, USA).

Statistical analysis

All data are expressed as the mean ± S.D. (standard deviation) and analyzed using a GraphPad software package (Prism 7.0) for Windows. Significant differences were assessed using one-way analysis of variance (ANOVA) analysis, and Tukey's multiple comparison tests were performed for statistical comparison of multiple groups. $P<0.05$ was considered statistically significant.

Results

Histological presentations

HE staining showed alveolar septal proliferation, alveolar congestion, and infiltration of inflammatory cells in rats in the cerebral I/R groups (**Figure 1**). No significant destruction was observed in alveoli and alveolar septa in rats in the control group. In addition, the pathological scores of lung tissue destruction were higher in the 3 h group when compared to the control group ($P<0.05$). The most severe injury of lung tissue was observed at 48 h and 72 h after induction of cerebral I/R. The ultrastructural, pulmonary changes observed using electron microscopy included the occurrence of alveolar infiltration of inflammatory cells, alveolar septal cell proliferation, interstitial edema, as well as swelling of type I epithelial cells, and capillary endothelial cells (**Figure 2**).

Expression of PKCα mRNA

In lung tissue of rats in the cerebral I/R groups, the expression of PKCα mRNA was significantly increased compared to that in the control group (**Figure 3**, $P<0.001$). Moreover, expression of PKCα mRNA in the lung peaked at 48 h after cerebral I/R.

PKCα immunofluorescent staining

Positive PKCα expression, as characterized by green fluorescence in immunofluorescent staining, was found in the cytoplasm and plasma membrane (**Figure 4**). Statistical significant diferences in PKCα expression in rat lung were observed between each cerebral I/R group and the control group ($P<0.001$). The intensity of PKCα staining after cerebral I/R gradually increased starting at 3 h after induction of cerebral I/R and peaked at 72 h after cerebral I/R. However, no statistically significant differences

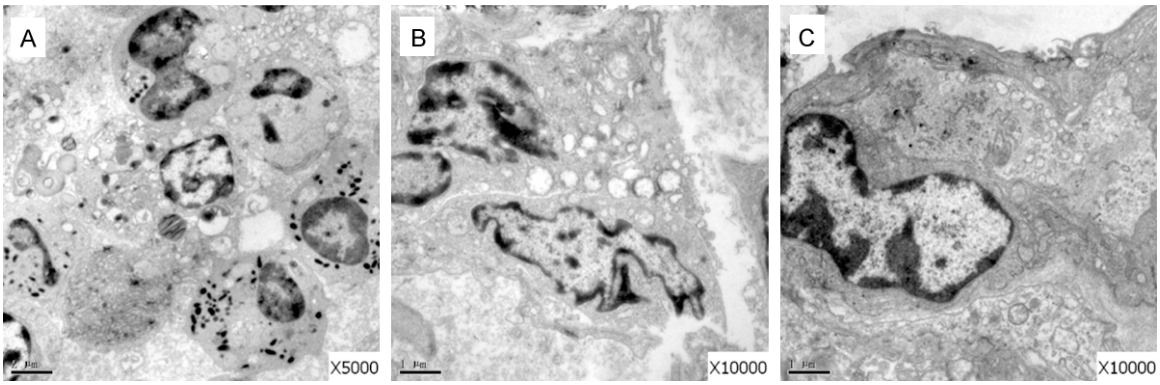

Figure 2. Electron microscopy shows ultrastructure of lung tissue after cerebral I/R. inflammatory cells infiltration (A, ×5000), Type I epithelial cell hyperplasia with interstitial edema (B, ×10000), capillary endothelial cells swelling (C, ×10000).

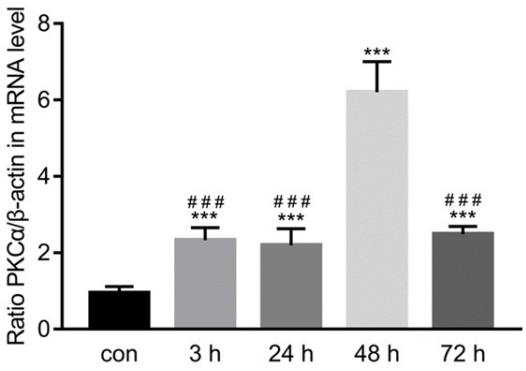

Figure 3. PKCα gene expression is highly up-regulated in cerebral I/R groups compared with that in the control group. Expression of PKCα mRNA: compared with the control group, ***P<0.001; in cerebral I/R groups, compared with 48 h group, ###P<0.001.

were observed between the 48 h group and the 72 h group (P>0.05).

Discussion

In the present study, we demonstrate that transient global cerebral I/R resulted in ALI as early as several hours after induction of cerebral I/R *in vivo*. Furthermore, histological analysis showed that significant differences in lung injury were observed within a very short period after transient global cerebral I/R induction. In addition, cerebral I/R-induced ALI was associated with increased PKCα expression.

After a stroke, there are several complications that are still major challenges to healthcare professionals. Among them, pulmonary dysfunction, such as ALI, acute respiratory distress syndrome [12], pulmonary edema [6, 13], and lung infection [14] are severe and often lead to increased mortality or poor prognosis [12, 15, 16]. Therefore, it is of utmost importance to explore effective strategies and intervention opportunities for the treatment of lung injury. However, clinicians rarely treat lung injury until significant pulmonary complications occur. One of the main reasons is the limited knowledge of intervention strategies of pulmonary dysfunction after stroke.

To the best of our knowledge, limited data is available that could serve as a reference for the most appropriate timing of treatment of ALI. Wu et al. [17] demonstrated that middle cerebral artery occlusion (MCAO) for 2 h, followed by 24 h of reperfusion resulted in ALI in rats. Moreover, Hu et al. [18] established a focal cerebral ischemia model in rats by applying the MCAO method without reperfusion, and show-ed that lung tissue destruction was observed in this brain injury model. However, in their study, time-related lung injury was not investigated. The findings of the current study demonstrated that 10 min of global cerebral ischemia followed a reperfusion period of 3 h resulted in lung injury, which was gradually aggravated after 24 h, 48 h and 72 h of reperfusion. Therefore, it may be beneficial to perform lung protective strategies as soon as possible after cerebral I/R injury. The 4-OV model that was used in the present study, resulted in global cerebral ischemia, and is considered to more closely resemble the clinical stroke process when compared to the MCAO model.

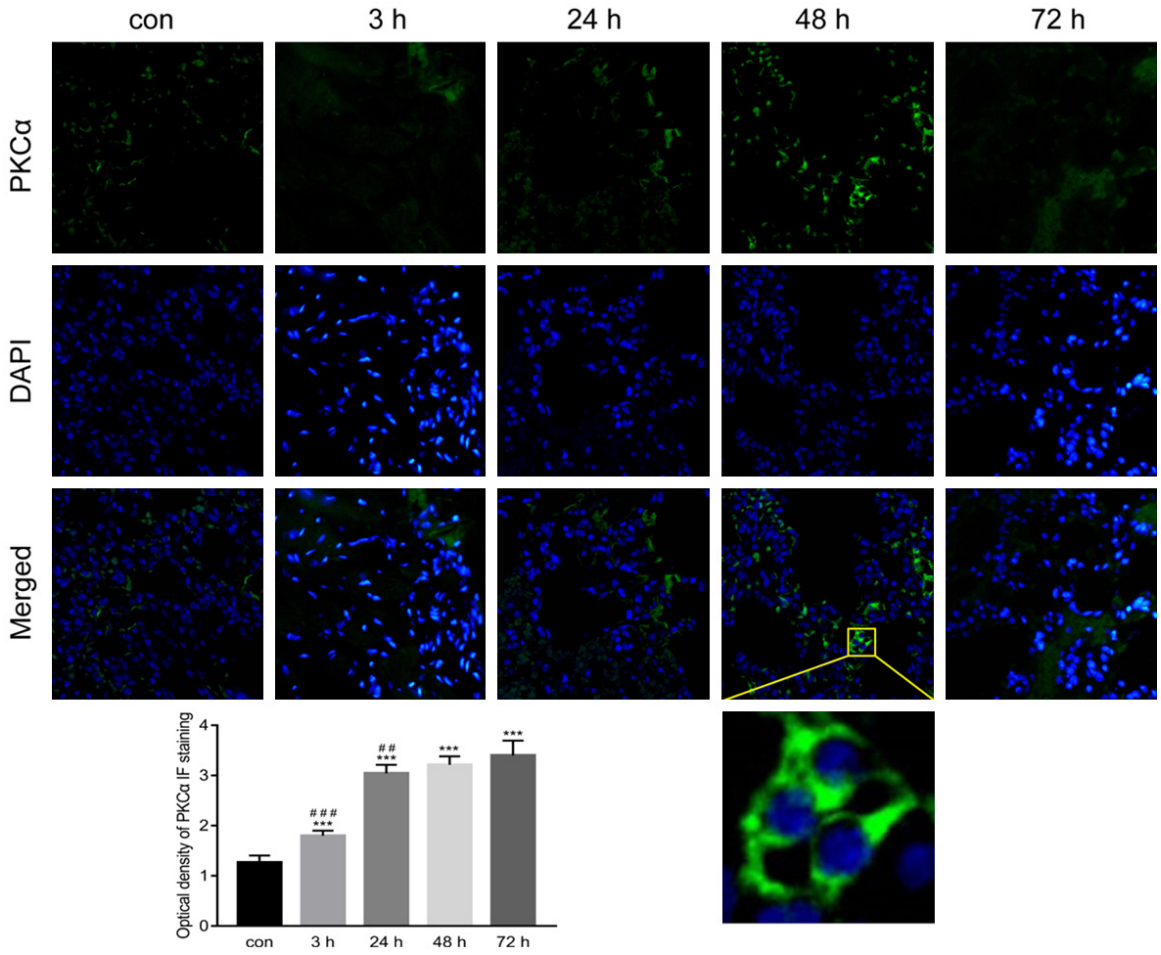

Figure 4. Positive immunofluorescent staining of PKCα characterizes as green in the cytoplasm and plasma membrane. Immunofluorescence optical density values: compared with the control group, ***P<0.001; in cerebral I/R groups, compared with 72 h group, $^{\#\#}P$ < 0.01, $^{\#\#\#}P$ < 0.001.

Currently, the generally recognized mechanism underlying ALI induced by remote organ injury involves inflammatory cascades [19]. Histologically, ALI is ciated with increased neutrophil infiltration, tissue edema, and vascular permeability, all of which are classic characteristics of inflammatory processes. Inflammatory cytokines may be key mediators for pulmonary alveoli injury. In addition, enhanced oxidative stress and stress hormones are considered to be involved in ALI [9].

Previous studies have shown that PKC participated in signal transduction, and its overactivation plays a role in various types of cell injury [20]. PKC could also be activated in oxidative stress-related diseases, such as cancer, cerebral I/R injury, and hepatic damage [21-23]. *In vitro* studies have revealed that PKCα activation includes translocation from the cytosol to the membrane, contributes to increased endothelial permeability [24]. In addition, it has also been reported that PKCα activation mediates tumor necrosis factor-α-induced increases in permeabity of pulmonary microvessel endothelial monolayers [16, 25], and facilitates nuclear translocation of NF-κB/Jun N-terminal protein kinase to augment pro-inflammatory responses [26], leading to acute pulmonary edema in rats following cerebral I/R. PKCα activation also promotes ventilator-induced lung injury by phosphorylating c-Src kinase and further reduced p120-catenin expression [27]. Similarly, in our study, we demonstrated a significant increase of PKCα mRNA and protein expression in lung tissue within three days after transient global cerebral I/R. Therefore, the underlying molecular mechanism of cere-

bral I/R-induced ALI, is at least in part, related to PKCα activation.

In conclusion, in the present study, we demonstrated that the lung is an organ that is very susceptible to stroke. During 10 min of transient global cerebral ischemia, the lung responded within hours and significant lung destruction occurred in one to three days later. Moreover, pulmonary expression of PKCα may be associated with changes in cereb0ral I/R-induced ALI, thereby providing insight into a novel potential therapeutic target for stroke-induced lung injury.

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Disclosure of conflict of interest

None.

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