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ORIGINAL ARTICLE

Electroacupuncture Pretreatment Attenuates Inflammatory Lung Injury After Cardiopulmonary Bypass by Suppressing NLRP3 Inflammasome Activation in Rats

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Abstract—Cardiopulmonary bypass (CPB) can induce inflammatory lung injury, which is a common complication during cardiac surgery. Nod-like receptor family, pyrin domain-containing 3 (NLRP3) inflammasome-induced inflammation plays a crucial role in lung injury after CPB. Previous studies have shown that electroacupuncture (EA) has potential anti-inflammatory activity. However, the role of EA in CPB is poorly understood. The aim of this study was to determine whether EA was associated with CPB-induced inflammatory lung injury. In the present study, rats were treated with EA for 5 days before CPB. Two hours after CPB, the lung tissue, serum, and bronchoalveolar lavage fluid (BALF) were prepared for assessment. Our results showed that the expression of NLRP3 in the lung tissue increased significantly after CPB. The EA pretreatment suppressed NLRP3 inflammasome activation, reduced lung edema, and inhibited IL-1β release into the serum and BALF after CPB. Our findings suggest that EA pretreatment attenuates inflammatory lung injury after CPB by suppressing NLRP3 inflammasome activation.

KEY WORDS: Electroacupuncture; Inflammatory lung injury; Cardiopulmonary bypass; NLRP3; IL-1β; Caspase-1.

INTRODUCTION

Inflammatory lung injury is an inevitable complication of cardiopulmonary bypass (CPB) during heart surgery [1]. Acute respiratory distress syndrome (ARDS) is the extreme form of inflammatory lung injury, which is characterized by the rapid onset of widespread inflammation in the lungs and is associated with increased mortality [2]. It is crucial to alleviate the inflammatory lung injury induced by CPB. However, the exact mechanism of CPB-induced inflammatory lung injury remains unclear.

An inflammasome is a large intracellular signaling platform that contains a cytosolic pattern recognition receptor, especially a nucleotide-binding oligomerization domain-like receptor (NLR) [3]. Among the NLR inflammasome complexes, the NLR pyrin domain-containing 3 (NLRP3) inflammasome is the most versatile and the most clinically implicated inflammasome [4]. The

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NLRP3 inflammasome controls the maturation of two proinflammatory interleukin (IL)-1 family cytokines, IL-1β and IL-18, through the activation of caspase-1 [5, 6]. Caspase-1 is known as an inflammatory caspase that plays a crucial role in the maturation of pro-IL-1β and pro-IL-18 into active cytokines [7]. Increasing evidence supports the conclusion that the aberrant activation of the NLRP3 inflammasome is associated with various autoinflammatory and chronic inflammatory diseases [8, 9]. Grailer et al. showed that the NLRP3 inflammasome played a critical role during acute lung injury [10]. Thus, reduced activation of the NLRP3 inflammasome may be beneficial in inflammatory lung injury.

Electroacupuncture (EA), a traditional Chinese medicine, has attracted increasing attention. It is a form of acupuncture where a small electric current is passed between pairs of acupuncture needles. Song et al. suggested that EA could significantly attenuate systemic inflammatory responses in rats with lethal endotoxemia [11]. Many animal experiments have also found that EA could inhibit the inflammatory reaction and inflammatory pain [12–14]. However, it remains unclear whether EA has protective effects on lung injury after CPB.

In this study, we hypothesized that EA may attenuate inflammatory lung injury after CPB by suppressing NLRP3 inflammasome activation and downstream proinflammatory factor expression.

MATERIALS AND METHODS

Animals and Experimental Grouping

Male Sprague–Dawley (SD) rats weighing between 400 and 450 g were provided by the Sino-British SIPPR/BK Lab (Shanghai, China). The animals were provided food and water *ad libitum*, housed at a temperature of 23–25 °C and a humidity of 45–55%, and maintained on a 12/12 h light/dark cycle.

Rats were randomly divided into the following four groups ($n = 6$ per group): a control group, a CPB group, an EA + CPB group, and a sham EA + CPB group. Rats in the control group received anesthesia and tracheal intubation without CPB. Rats in the CPB group received the CPB procedure. Rats in the EA + CPB group received EA pretreatment once a day for 5 days prior to the CPB procedure. Rats in the sham EA + CPB group received acupuncture needles without electrical stimulation once a day for 5 days prior to the CPB procedure. All tissue samples were collected at 2 h after CPB.

Surgical Procedure

The surgical procedure was performed as previously described [15]. Briefly, SD rats were anesthetized with 5% isoflurane and then underwent tracheal intubation with a 16-G catheter (BD, New Jersey, USA). Rats were maintained with isoflurane at 1.5 to 2.0% and mechanically ventilated with a TOPO Small Animal Ventilator (Kent Scientific, Torrington, USA) during the surgery.

For the initiation of the extracorporeal circulation, a 22-G catheter (BD, New Jersey, USA) was cannulated in the tail artery for arterial inflow, and a 5F special catheter (Cordis, Fremont, California, USA) was cannulated in the right jugular vein for venous return. Consistent blood pressure and arterial blood gases were achieved by cannulation of the left femoral artery. The blood flow was directed from the jugular vein to the CPB device and then back to the corporal circulation *via* the tail artery. The circuit was primed with 8 mL Voluven (hydroxyethyl starch 130/0.4 and sodium chloride injection) and heparin (200 IU/kg). The venous reservoir was placed 35 cm below the heart level to build a potential difference. The blood flow was adjusted to 100 mL/kg/min and maintained at this rate for 60 min.

Electroacupuncture Pretreatment

EA pretreatment was performed as previously described [16] at the Zusanli (ST36) acupoint, which is located between the tibia and fibula, laterally in line with the distal end of the cranial tuberosity of the tibia and approximately 5 mm lateral to the anterior tubercle of the tibia, and the Feishu (BL13) acupoint, which is located between T3 and T4 of the spine approximately 1.5 cm lateral to the midline. All rats received EA pretreatment, and sham EA pretreatment rats were anesthetized with 3% pentobarbital sodium (0.1 ml/100 g, intraperitoneal). The bilateral Zusanli (ST36) and Feishu (BL13) acupoints were stimulated at a frequency of 2/15 Hz for 30 min by an EA treatment instrument (No. SDZ-IIB; Suzhou Medical Appliances, Suzhou, China).

Lung Histopathology

As described previously [15], the right upper lung of each of the rats in the four groups was excised and fixed with 4% paraformaldehyde for 48 h. All samples were

embedded in paraffin before cutting into 5-μm sections, and then the sections were stained with hematoxylin and eosin for observation with an Olympus microscope (Olympus Corporation, Tokyo, Japan). The degree of lung injury in the rats was assessed by two pathologists in a blinded manner using a 0–3 scoring system (0, normal; 1, mild; 2, moderate; and 3, strong) for interstitial/alveolar edema, hemorrhage, alveolar septal thickening, and infiltration of inflammatory cells [17].

Measurement of the Lung W/D Ratio and BALF Protein Concentration

The right upper lung of each rat was excised and weighed to obtain the wet weight and dried in an oven at 60 °C for 1 week to obtain the dry weight. The W/D ratio was calculated by dividing the wet weight by the dry weight as previously described [18]. The protein concentration in the BALF was measured with a BCA Protein Assay kit (Thermo Fisher Scientific, Inc.) to evaluate pulmonary protein infiltration.

Immunohistochemistry for NLRP3

After dewaxing and rehydration, the lung sections were immersed and boiled in TRIS-EDTA buffer for antigen retrieval and blocked for endogenous peroxidase activity by 3% hydrogen peroxide treatment. Then, the lung sections were incubated with a primary antibody against NLRP3 (1:500; cat. no. ab214185; Abcam, Cambridge, MA, USA) overnight at 4 °C, followed by incubation with a secondary anti-rabbit antibody (1:1000; cat. no. A0208; Beyotime, Shanghai, China) and processed with an avidin-biotin-immunoperoxidase technique. Finally, the sections were counterstained with Mayer's hematoxylin.

ELISA for the IL-1β Concentrations in the Serum and BALF

The samples were prepared 2 h after CPB. The protein content was measured with a BCA Protein Assay kit (Thermo Fisher Scientific, Inc.). The levels of IL-1β in the serum and BALF were measured using an ELISA kit (R&D Systems, Inc.) following the manufacturer's protocol.

Western Blot Analysis

Western blot analysis was performed as previously described [15]. The lung tissue of the rats in the four groups was prepared 2 h after CPB. The protein concentrations were determined using a BCA Protein Assay kit (Thermo Fisher Scientific, Inc.). Proteins (30 μg) were separated by 12% SDS-PAGE gel and transferred to a nitrocellulose membrane (BioRad, Hercules, California, USA). The nitrocellulose membrane was blocked with 5% non-fat dry milk for 2 h at room temperature, followed by an incubation with a primary antibody against NLRP3 (1:500; cat. no. ab214185; Abcam, Cambridge, MA, USA), caspase-1 (1:500; cat. no. ab1872; Abcam, Cambridge, MA, USA), IL-1β (1:500; cat. no. ab150777; Abcam, Cambridge, MA, USA) at 4 °C overnight and then an incubation with a secondary antibody (goat anti-rabbit IgG; 1:10,000; Cat. No. ab6721; Abcam) conjugated with horseradish peroxidase for 2 h at room temperature. The proteins were detected using Pierce ECL Plus Western Blotting Substrate (cat. no. 32134; Thermo Fisher Scientific, Inc.). β-actin levels (Sigma-Aldrich-Merck KGaA, Darmstadt, Germany; 1:500) were used as the control. A densitometry analysis was performed using ImageJ software (version 1.8.0; National Institutes of Health, Bethesda, MD, USA).

Data Analysis and Statistics

The data are expressed as the mean ± SD. The statistical analysis was performed using SPSS v25 software (IBM, Armonk, NY, USA). Differences among groups were tested using one-way analysis of variance followed by Tukey's tests. $P < 0.05$ was considered statistically significant.

RESULTS

EA Pretreatment Attenuated NLRP3 Inflammasome Activation in the Lung After CPB

NLRP3 protein expression was detected 2 h after CPB (Fig. 1a). As presented in Fig. 1b, the expression of NLRP3 in lung tissue increased significantly in the CPB group ($P < 0.05$, $n = 6$) when compared with the control group. Compared with the CPB group, the rats that received EA pretreatment prior to CPB showed a significant decrease in the expression of NLRP3 ($P < 0.05$, $n = 6$). However, there was no significant difference in NLRP3 expression in lung tissue between the CPB and sham EA + CPB groups, suggesting that EA pretreatment may prevent the upregulation of NLRP3 expression.

Immunohistochemical staining was also used to evaluate the levels of NLRP3 in the four groups. As illustrated in Fig. 2 (× 200), NLRP3 staining in the lung was abundant

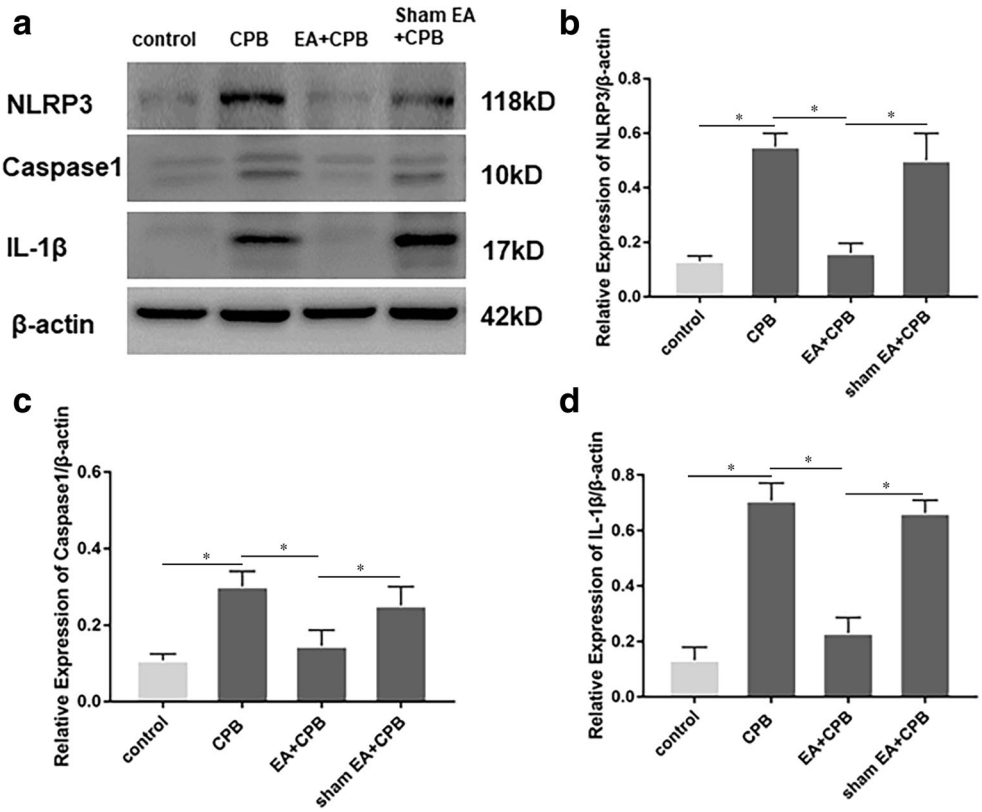

Fig. 1. Expression of NLRP3, caspase-1, and IL-1β in the lung tissue of the four groups. **a** Representative results of Western blotting for NLRP3, caspase-1, and IL-1β expression in the lung tissue are shown. **b**–**d** Immunoblot analyses of NLRP3, caspase-1, and IL-1β expression in the lung tissue of each group ($n = 6$, $^*P < 0.05$ CPB group *vs.* control group. $^*P < 0.05$ EA + CPB group *vs.* CPB group, sham EA + CPB group) are shown.

in the CPB group. In contrast, the expression of NLRP3 decreased significantly in the EA + CPB group, indicating that EA pretreatment markedly attenuated NLRP3 inflammasome activation.

EA Pretreatment Suppressed the Expression of Proinflammatory Factor Downstream of NLRP3

NLRP3 inflammasome activation could lead to caspase-1 activation, which causes the maturation of IL-1β and IL-18 [5, 6]. To detect the activation of the NLRP3 downstream pathway, Western blot analysis was performed to evaluate the expression levels of caspase-1 and IL-1β in the lung tissue 2 h after CPB. As shown in Fig. 1c, d, the expression levels of both caspase-1 and IL-1β were upregulated in the CPB group ($P < 0.05$, $n = 6$) when compared with the control group. In contrast, the expression of caspase-1 and IL-1β decreased significantly in the lung tissue after EA pretreatment in the EA + CPB group compared with the CPB group and sham EA + CPB group ($P < 0.05$, $n = 6$). These findings showed that EA pretreatment markedly suppressed the activation of the NLRP3 downstream pathway.

EA Pretreatment Reduced the Inflammatory Response in the Lung Tissue, Protein Infiltration, and Lung Water Content

As presented in Fig. 3a (× 200), H&E staining showed the intact and thin alveolar walls in the control group; however, the alveolar walls in the CPB group were not intact and were instead full of inflammatory cells. In the EA + CPB group, relatively intact and thin alveolar walls were observed. The alveolar walls in the sham EA + CPB group were similar to those in the control group. The lung injury scores also showed significantly decreased injury with EA pretreatment prior to CPB ($P < 0.05$) (Fig. 3b). These

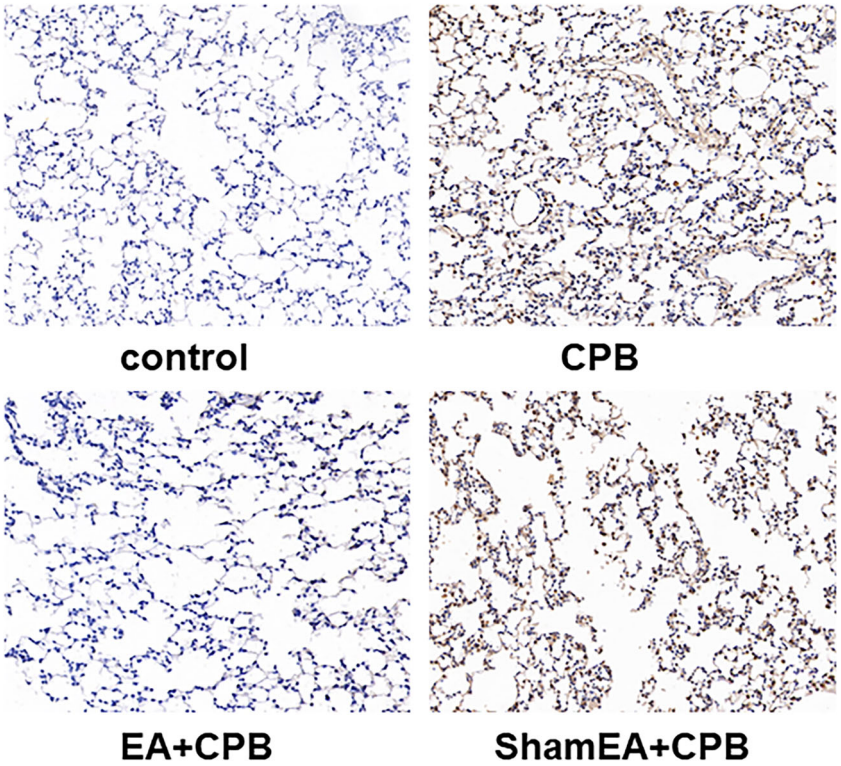

Fig. 2. Immunohistochemical staining for NLRP3 in the lung tissue (magnification, × 200). NLRP3 expression was abundant in the CPB group. The EA pretreatment prevented the upregulation of NLRP3 expression after CPB in the lung tissue.

results demonstrated that EA pretreatment could attenuate lung injury after CPB.

The W/D ratio of the lung and the protein concentration in the BALF were examined to estimate lung edema and vascular permeability. As shown in Fig. 3c, the lung W/D ratio in the CPB group was significantly increased when compared with that in the control group ($P < 0.05$). However, this increase was significantly suppressed by EA pretreatment in the EA + CPB group ($P < 0.05$).

In contrast to the control group, the protein concentration in the BALF was increased in the CPB group ($P < 0.05$) (Fig. 3d). This increase was also significantly suppressed by EA pretreatment in the EA + CPB group ($P < 0.05$). These results suggested that EA pretreatment could suppress CPB-induced lung edema and vascular permeability.

EA Pretreatment Decreased the IL-1β Release

Considering that IL-1β is an important mediator of the inflammatory response, we examined the levels of IL-1β in the serum and BALF using an ELISA kit. As indicated in Fig. 4, the concentrations of IL-1β in the serum and BALF were significantly increased in the CPB group compared with the control group ($P < 0.05$, $n = 6$). In contrast to the CPB group, pretreatment with EA remarkably decreased the IL-1β release into the serum and BALF in the EA + CPB group ($P < 0.05$, $n = 6$). Moreover, there were no significant differences in the IL-1β concentrations in the serum and BALF between the CPB group and sham EA + CPB group. The results suggested that EA pretreatment could decrease IL-1β release into the serum and BALF.

DISCUSSION

Inflammatory lung injury is a common complication after CPB during cardiac surgery.

Ample evidence exists regarding the massive inflammatory response that is initiated by CPB [1, 19, 20]. The inflammatory response can result in organ dysfunction, including respiratory failure, renal failure, and altered hepatic function; acute respiratory distress syndrome; and systemic inflammatory response syndrome [20, 21]. ARDS, which carries a 50% mortality rate, exists in 2% of patients. Additionally, up to 20% of patients need to be

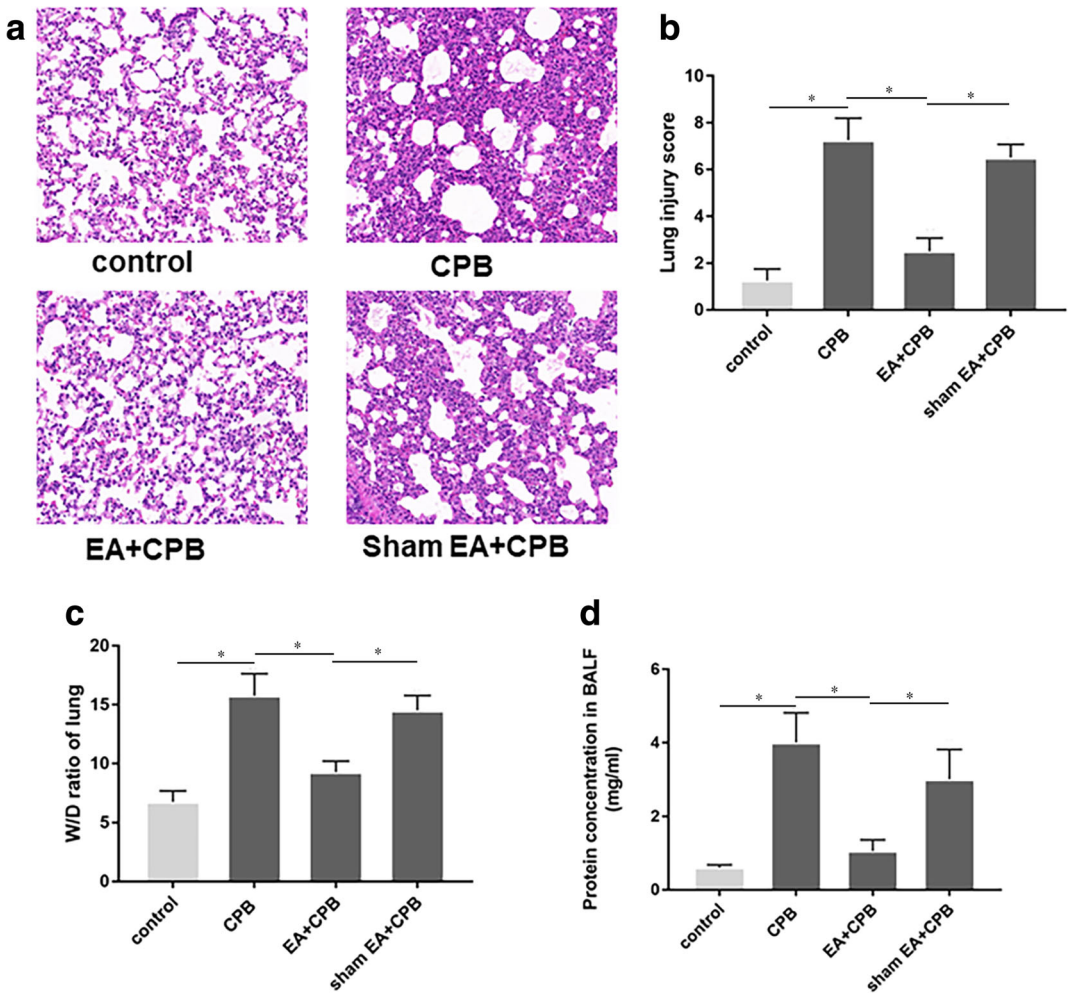

Fig. 3. EA pretreatment reduced pulmonary histopathological injury, protein infiltration, and lung water content in rats after CPB. a Representative photographs of lung samples from the four groups stained with H&E are shown (magnification, 200x). **b** The lung injury scores are shown. **c** The W/D ratios of the lung tissue samples are shown. The W/D ratio of the CPB group was significantly higher than that of the control group ($n = 6$, $^*P < 0.05$); however, the W/D ratio decreased significantly after EA pretreatment in the EA + CPB group ($n = 6$, $^*P < 0.05$ *vs.* CPB group, sham EA + CPB group). **d** The protein concentrations in the BALF were quantified with a BCA protein assay. The protein concentration was significantly higher in the CPB group ($n = 6$, $^*P < 0.05$ *vs.* control group). The EA pretreatment significantly decreased the protein concentration in the EA + CPB group ($n = 6$, $^*P < 0.05$ *vs.* CPB group, sham EA + CPB group).

ventilated for more than 48 h following cardiac surgery involving CPB [20]. Considering the severe clinical effects of CPB-induced inflammatory lung injury during cardiac surgery, studies on inflammatory lung injury are of crucial importance. The aim of this study was to evaluate the role of EA pretreatment in CPB-induced inflammatory lung injury.

In the present study, we used a classic rat CPB model [22], and EA pretreatment was performed as previously described [16]. The lung tissue after CPB displayed features of lung injury, including alveolar septal thickening, interstitial edema, and neutrophil infiltration. EA pretreatment, a combination of traditional Chinese medicine and modern techniques, significantly reduced the morphological inflammatory response of the lung tissue, the protein concentration in the BALF, which was related to the pulmonary capillary permeability, and the W/D ratio of the lung tissue, which was measured to analyze the water content of the lung. We further found that EA pretreatment suppressed NLRP3 inflammasome activation and downstream IL-1β expression. Our findings indicate that EA pretreatment may target the NLRP3 inflammasome in the lung to attenuate CPB-induced inflammatory lung injury, suggesting a potential role for EA

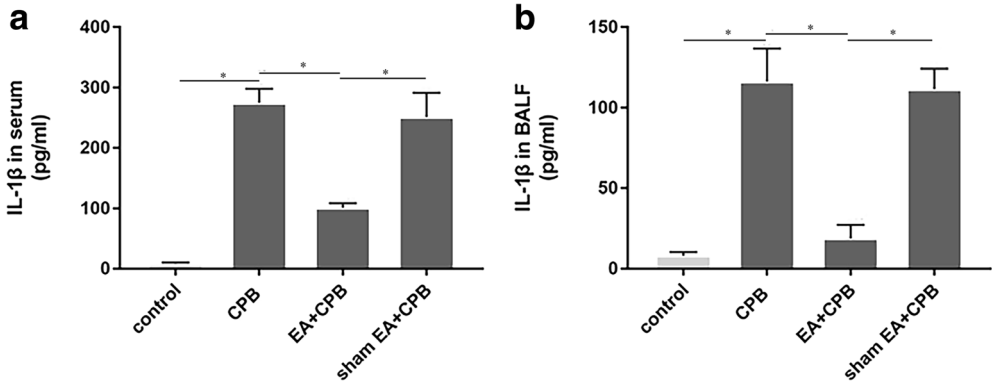

Fig. 4. IL-1β concentrations in the serum and BALF. In both the serum (**a**) and BALF (**b**), IL-1β concentrations increased significantly in the CPB group compared with the control group ($n = 6$, $^*P < 0.05$). The EA pretreatment remarkably decreased IL-1β release into the serum and BALF in the EA + CPB group ($n = 6$, $^*P < 0.05$ *vs.* CPB group, sham EA + CPB group).

pretreatment in prophylactic therapy for CPB-induced inflammatory lung injury.

Previous studies have demonstrated that the cardioprotective mechanism of acupuncture during cardiac surgery involved the inhibition of the β1-adrenoceptor signaling pathway and upregulation of vascular endothelial growth factor expression [23–25]. Although there have been a few studies on the pulmonary protection after CPB mediated by acupuncture [15, 26], the exact protective mechanism in lung injury was unclear. Increasing evidence has already shown that the NLRP3 inflammasome is involved in various inflammatory diseases [6, 8, 9, 27–29]. Grailer et al. demonstrated that the development of acute lung injury requires the engagement of the NLRP3 inflammasome, and extracellular histones were identified as activators of the NLRP3 inflammasome [10]. The NLRP3 inflammasome triggers proteolytic cleavage of dormant procaspase-1 into active caspase-1, which converts pro-IL-1β into mature and biologically active IL-1β [6, 7, 30, 31]. IL-1β is a potent proinflammatory mediator in many immune reactions, including the recruitment of innate immune cells to infected sites and modulation of adaptive immune cells [32]. He et al. demonstrated that upregulated IL-1β expression mediated LPS-induced alveolar macrophage pyroptosis and subsequently enhanced lung inflammation [33]. However, the role of EA pretreatment in NLRP3 expression in the lung after CPB remains unclear. In this study, we found that CPB increased the expression of NLRP3, caspase-1, and IL-1β in the lung. EA has been known to play a crucial role in inducing anti-inflammatory responses [12–14, 34]. In our previous study, we observed that EA could attenuate acute lung injury in rats after CPB [26]. We detected the protein expression of NLRP3 and downstream proinflammatory factors to confirm the suppressive effect of EA pretreatment on NLRP3 inflammasome activation. However, the suppressive impact of EA pretreatment on CPB-induced NLRP3 inflammasome activation might be just one aspect of the protective effect of EA. Other molecules and pathways could also be regulated by EA pretreatment directly or indirectly. The efficacy of EA pretreatment in specific immune cell types should be investigated. Additionally, the exact mechanism of the inhibition of NLRP3 inflammasome activation mediated by EA pretreatment needs further research.

In conclusion, EA pretreatment reduces CPB-mediated inflammatory lung injury by suppressing the activation of the NLRP3 inflammasome, decreasing caspase-1 and IL-1β levels, and reducing the inflammatory response. Hence, EA pretreatment may become an underlying clinical strategy for preventing inflammatory lung injury after CPB through attenuating NLRP3 inflammasome activation.

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AUTHORS' CONTRIBUTIONS

WY conceived and designed the study. DH, MC, ZW, and LH performed the experiments and analyzed the data. DH and MC wrote the manuscript. WY reviewed and edited the manuscript. All the authors read and approved the manuscript.

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COMPLIANCE WITH ETHICAL STANDARDS

All animal experiments were approved by the Animal Care and Use Committee of Shanghai Jiao Tong University, School of Medicine. All the animal procedures conformed to the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 8023, revised 1978).

Competing Interests. The authors declare that they have no competing interests.

Informed Consent. Informed consent was obtained from all individual participants included in the study.

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