Gap Junction Channel Modulates Pulmonary Vascular Permeability through Calcium in Acute Lung Injury: An Experimental Study

Jinzhou Zhang a, Wen Wang b, Jing Sun c, Qiang Li a, d, Jincheng Liu a, Hailong Zhu a, Tao Chen a, Hongbing Wang a, Shiqiang Yu a, Guocheng Sun a, Wensheng Chen a, Dinghua Yi a

Departments of a Cardiovascular Surgery and b Traditional Chinese Medicine, and c Nursing Department, Xijing Hospital, Fourth Military Medical University, Xi’an, and d Department of Cardiothoracic Surgery, Second Affiliated Hospital, Shanxi Medical University, Taiyuan, People’s Republic of China

Key Words
Acute lung injury · Gap junction channel · Gunshot injury · Pulmonary permeability · Thoracic wound

Abstract
Background: Increased pulmonary vascular permeability is a hallmark of acute lung injury (ALI). Gap junction channels (GJCs) connect adjacent cells and facilitate ion exchange. It remained unclear whether GJCs modulate pulmonary permeability in ALI through intracellular calcium. Objectives: This study aimed to verify if GJCs in pulmonary microvessel endothelial cells (PMVECs) modulate pulmonary vascular permeability in ALI via intracellular calcium. Methods: Firstly, an animal model of ALI was studied using connexin 40 (Cx40) immunohistochemistry in the lung with Evans’ blue (EB) leakage. Then cultured PMVECs were divided into three groups: G control, G serum and G blocker. Serum was obtained from animals with ALI following gunshot injury (injured serum). Initially, G blocker was treated with the blocker of GJCs, and then G serum and G blocker were stimulated with the injured serum, respectively. GJCs, the permeability of cell monolayers and intracellular Ca²⁺ were assessed. Results: Cx40 time-dependently decreased, whereas EB leakage increased. Cx40 and EB leakage exhibited a strong inverse correlation ($R = -0.934, p < 0.05$). Injured serum decreased GJCs and expression of Cx40, whereas the blocker aggravated this effect. Similarly, when PMVEC monolayer was treated with injured serum, both permeability and intracellular Ca²⁺ increased. These effects were also aggravated with the blocker. Conclusions: Depression of GJCs of PMVECs increased pulmonary vascular permeability in ALI; this effect may be mediated by the overload of intracellular calcium.

Introduction
Acute lung injury (ALI) is a common, costly and potentially lethal disease, for which treatment of the underlying cause is the first step to recovery [1]. Many clinical and experimental studies have established the concept that increased-permeability pulmonary edema is the primary physiologic abnormality in the early stages of ALI.

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Thus, increased-permeability edema, defined as accumulation of protein-rich edema fluid in the alveoli, has become a hallmark of ALI [3], and modulating pulmonary permeability has become an important strategy to prevent and treat ALI.

Many studies have been performed to define the mechanisms that account for the acute increase in pulmonary vascular permeability in ALI [4]. The integrity of pulmonary microvessel endothelial cells (PMVECs) is a critical requirement for the preservation of pulmonary function [5]. PMVECs function as a semipermeable cellular barrier between the vascular and interstitial compartments; the general consensus is that fluid, macromolecules and leukocytes are transported through a PMVEC monolayer to the interstitial and subsequently the alveolar air spaces via the paracellular route. The essential role of paracellular transport in endothelial permeability has been supported by a substantial body of research, including electron-microscopic studies [5–7]. Garcia and Schaphorst [8] reported that the formation of the paracellular gap between endothelial cells (ECs) is regulated by a balance of competing contractile forces, which generate centripetal tension, and adhesive cell-cell and cell-matrix tethering forces, which together regulate cell shape changes. During this process, a key event that promotes EC contraction is the phosphorylation of regulatory myosin light chains catalyzed by Ca²⁺/calmodulin-dependent myosin light chain kinase [5].

Gap junction channels (GJCs) connect the cytoplasm between adjacent cells to allow rapid exchange of ions and metabolites up to approximately 1 kDa in size, including second messengers such as cyclic AMP, inositol triphosphate, and Ca²⁺. The crucial contribution of GJCs to disease etiopathology has been the subject of intensive research in recent years [9, 10], and connexin 40 (Cx40) has been identified as the critical component of GJCs in ECs [11]. An abundance of data has proven the necessity of GJC communication to coordinate vascular behaviors during blood flow regulation, while Cx40-dependent coupling is necessary to regulate vascular behaviors and peripheral resistance [12]. Recent research revealed that thrombin inhibited intercellular calcium wave propagation in corneal ECs by modulation of hemi-channels and GJCs [13].

Based on the previous findings, we postulated that GJCs in PMVECs modulate pulmonary vascular permeability in ALI via intracellular calcium. Here, we first correlated the Cx40 level with pulmonary vascular permeability after ALI induced by thoracic gunshot wounds. Then the effects of a GJC blocker on the stimulation of PMVECs by serum acquired from rabbits subjected to ALI were studied to determine the possible use of GJCs as a novel therapeutic target for the treatment of ALI.

Materials and Methods

Animal Experiments
All animal experiments were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Ethics Committee of the Fourth Military Medical University. All surgeries were performed in designated veterinary surgical suites using sterile instruments and procedures. Healthy, White New Zealand rabbits (weighing 2–3.1 kg, Experimental Animal Center, Fourth Military Medical University) were randomly assigned to one of the following five groups (n = 8 in each group) irrespective of gender: before (or sham-operated group; the interventions were the same as in the other groups except for gunshot injury) and 6, 12, 24 and 48 h after gunshot injury.

ALI was induced in the animals as described previously [14]. After shooting, first-aid treatment was immediately started, including closing of wounds, management of hemopneumothorax with thoracentesis and control of bleeding. Fluid was infused as needed to compensate for blood loss and to maintain systolic blood pressure ≥ 90 mm Hg. Normal saline was used depending on the bleeding rate. In ALI-surviving animals, a Swan-Ganz catheter was inserted through the right jugular vein to measure pulmonary artery wedge pressure excluding cardiogenic edema [15]. When pulmonary artery wedge pressure was > 2.6 mm Hg, the animal was excluded from the following investigations [16].

Arterial blood was sampled before and 6, 12, 24 and 48 h after gunshot injury. Analyses done immediately included arterial blood gases, hemoglobin and electrolytes. Blood samples for later analysis were obtained at the same time points. Serum values of the early cytokines TNF-α and IL-8 were determined with standard kits by radioimmunoassay (Byk & DiaSorin Diagnostics). For the stimulation in the ex vivo study, serum was obtained 12 h after gunshot injury (serum12h). Furthermore, control serum was taken from rabbits before gunshot injury.

At each time point, 4 animals were killed with an overdose of anesthetic, the lungs were isolated, the trachea and bilateral bronchi were excised, and part of the left lung was removed, immediately weighed, left overnight in an oven at 110°C and again weighed to compute the wet/dry weight ratio. At the same time, part of the right lung tissue was sampled 2 cm away from the wound site for immunohistochemistry. The other 4 animals were selected to assess microvascular permeability of the lung tissue using Evans blue (EB) dye leakage.

Immunohistochemistry

Cryosections (5 μm thick) were fixed and incubated with 0.2% BSA-C (Aurion) followed by incubation with primary antibodies for 1 h. Biotin-SP-conjugated AffiniPure mouse anti-rabbit IgG (Dianova) and secondary antibodies were incubated at a dilution of 1:200 for 1 h. The sections were covered and then viewed with a microscope (H600L, Nikon). Further documentation and image analysis were performed in a blinded fashion using a digital camera (DXM1200F, Nikon) and image processing software (ACT-1.
Isolation of PMVECs and Primary Cell Culture
PMVECs were isolated and cultured using a modified method described by Stevens et al. [17]. Cultured primary EC colonies were isolated, trypsinized, resuspended, seeded and then grown at 37°C with 5% CO₂ for ~2–3 days. Eventually, the primary PMVECs grew together into a slab-like continuous monolayer. Cells were characterized using anti-factor VIII by immunocytochemistry (Sigma).

PMVECs were divided into the following groups: control (Gcontrol), injured serum (Gserum) and block agent (Gblocker), that were all incubated in DMEM initially. In Gblocker, the blocking agent (1-heptanol; 0.5 mM) was added to the medium for 1 h. In Gserum and Gblocker, cells were stimulated with serum12 h, i.e. 20% FBS was replaced by the same amount of serum12 h. Gcontrol cells underwent the same procedures except for blocker and serum12 h treatments.

Scrape-Loading and Dye Transfer Technique
A dye transfer assay was used to evaluate gap junction communication. PMVECs were rinsed with 2 ml PBS. Two milliliters of 0.075% Lucifer yellow CH (molecular weight 443 Da, L-0259, Sigma) dissolved in PBS were added to the cells, and two scrape lines (parallel, equidistant scrapes per well) were made by gently passing a diamond-tipped pen (tip diameter 0.25 mm) across the cultures. Subsequently, cells were examined with an inverted confocal microscope (FV1000 IX81, Olympus) at emission/excitation wavelengths of 528/425 nm. Images were quantified by counting the number of donor and recipient cells and calculating a cell coupling index with the ratio of recipient to donor cells. The value of dye transfer, defined as the number of secondary recipient cells visualized by Lucifer yellow CH, was recorded for only one side of the scrape.

Measurement of Monolayer Endothelial Permeability
PMVEC permeability was assessed by EB-labeled albumin transfer, as previously described [18]. Clearance of EB albumin was measured up to 90 min. Measurements of transendothelial albumin flux are expressed as the percentage of clearance of bovine serum albumin compared with untreated controls.

Western Blot
Expression levels of Cx40 and their phosphorylated isoforms were measured by Western blotting in PMVECs. Cells were washed with PBS and lysed in buffer. After centrifugation, the supernatant was collected and protein content was quantified using the bichinchoninic acid protein assay (Pierce) in comparison to albumin standards. To measure signal intensity, blots were covered with enhanced chemiluminescence reagents (Amersham Biosciences) for 5 min and then exposed to film. β-Actin expression was used as internal standard.

Measurement of Intracellular Ca²⁺
The concentration of intracellular Ca²⁺ was measured with Flu-3 acetoxymethyl ester (Fluo-3 AM, 50013, Biotium). InterVision software was used to calculate the value of [Ca²⁺], in selected regions from the changes in fluorescence of the Fluo-3 indicator according to the following equation: [Ca²⁺] = Kd × (Fmax − F), where Fmax is maximum fluorescence and Fmin minimum fluorescence. The Kd value for calcium binding to Fluo-3 is 450 nM at 25°C.

Intracellular free Ca²⁺ concentration changes were estimated as ∆F/F0, where ∆F is the fluorescence intensity of the response minus the baseline intensity and F0 was derived from the averaged intensity of the first 10–20 frames minus the background in the cell-free region. Cells that changed fluorescence intensity >10% over the course of an experiment were eliminated from further analysis.

Statistical Analysis
Statistical analysis was performed with SPSS 14.0 for Windows. Numeric variables are expressed as means ± SD. Comparisons among experimental conditions were performed using one- or two-way ANOVA if the variables depended on time. The Bonferroni post-test was performed to determine significant differences between experimental conditions. Probability values <0.05 were considered statistically significant. The Pearson’s correlation test was used to analyze EB permeability and optical density (OD) of Cx40.

Results
General Conditions and Laboratory Examination
Fifteen rabbits were killed instantly or within 2 h and excluded from the experiment. At autopsy, there was abundant hemorrhage in the pleural cavity, 4 showed rupture of the right branch of the pulmonary artery, 3 ruptures of the right ventricular anterior wall, 3 hematomas in the superior right lung lobe, 2 thoracic spinal column fractures with inferior caval vein rupture, 2 intercostal vessel hematomas and 1 cervical vertebral fracture.

The oxygenation index (PaO₂/FiO₂; mm Hg) decreased after injury, being <300 mm Hg 12 h after injury (PaO₂/FiO₂, 385 ± 22, 342 ± 19, 283 ± 25, 227 ± 16 and 184 ± 21, fig. 1a). The chest radiograph taken 12 h after gunshot injury revealed rib fractures and diffuse bilateral infiltrates (fig. 1b).

Serum Concentrations of TNF-α and IL-8
Surviving (n = 32) and sham-operated rabbits (n = 8) received the following treatments. TNF-α and IL-8 concentrations were significantly higher 6, 12, 24 and 48 h after than before gunshot injury (TNF-α, 0.505 ± 0.071, 0.927 ± 0.134, 1.006 ± 0.097, 0.646 ± 0.083 vs. 0.236 ± 0.049, fig. 2a; IL-8, 5.32 ± 0.44, 5.9 ± 1.12, 5.64 ± 0.71, 5.29 ± 0.96 vs. 3.81 ± 0.28, fig. 2b; p < 0.05). In all groups, the lung water content (wt/dry ratio) was significantly higher after compared with before gunshot injury (5.50 ± 1.03, 9.27 ± 1.77, 7.73 ± 1.59, 8.13 ± 2.01 vs. 4.13 ± 0.92, fig. 2c; p < 0.05).
Gap Junction Channels in Acute Lung Injury

**Cx40 Level and EB Leakage in Injured Lung Tissue**

Immunostaining with Cx40 antibodies was observed throughout the pulmonary EC membrane. The mean OD values of Cx40 staining (fig. 3f) decreased after gunshot injury \[176.20 \pm 25.34 \text{ (fig. } 3a) \text{ vs. } 160.34 \pm 16.34 \text{ (fig. } 3b), 134.42 \pm 8.12 \text{ (fig. } 3c), 118.83 \pm 12.95 \text{ (fig. } 3d) \text{ and } 92.19 \pm 16.34 \text{ (fig. } 3e; p < 0.05) \text{ before vs. } 6, 12, 24 \text{ and } 48 \text{ h after gunshot injury, respectively].}

EB leakage rates from lung tissue time-dependently increased after gunshot injury \[12.02 \pm 3.19 \text{ vs. } 26.76 \pm 5.77, 67.62 \pm 11.36, 53.56 \pm 17.94 \text{ and } 81.69 \pm 16.58, \text{ before vs. } 6, 12, 24 \text{ and } 48 \text{ h after gunshot injury, respectively].

\[\text{Fig. 1. a Oxygenation index before and after gunshot injury. b Rib fractures and diffuse bilateral infiltrates were seen on the chest radiograph taken 12 h after gunshot injury.}\]

\[\text{Fig. 2. a TNF-}\alpha \text{ concentration at different times after gunshot injury. IL-8 concentration (b) and water content (c) significantly increased after gunshot injury (} * p < 0.05).\]
Dye Transfer

Similar cell numbers were initially loaded with dye in all treatment groups (fig. 5). However, fewer neighboring cells were labeled with Gserum (fig. 5b) and Gblocker (fig. 5c), with the lowest count occurring after Gblocker treatment. The recipient/donor cell ratios of Gcontrol (fig. 5a), Gserum and Gblocker were 23 ± 5, 11 ± 3 and 7 ± 2, respectively (p < 0.05; fig. 5d).

PMVEC Permeability Assay

Treatment with serum and a gap junction blocker time-dependently increased PMVEC permeability (two-way ANOVA). There are significant increases in the clearance ratio compared with baseline (60, 90 or 30 min) both in Gserum (170 ± 28%, 210 ± 30% vs. 105 ± 21%, p < 0.05) and Gblocker (185 ± 29%, 240 ± 35% vs. 105 ± 19%, p < 0.05; fig. 6).

Western Blot

PMVECs grown in injured medium for 2 h exhibited reduced Cx40 level (fig. 7a). In cells grown in the three media, Cx40 protein level was reduced by injured serum and blocker conditions (54.78 ± 15% and 43.68 ± 13% of control, p < 0.05; fig. 7b).

Intracellular Ca²⁺ Concentration

The three groups of PMVECs loaded with Fluo-3 were imaged on an inverted confocal microscope, and fluorescence intensity was higher in Gserum and Gblocker than in Gcontrol. Thus, the concentrations of intracellular Ca²⁺ in PMVECs in the Gserum and Gblocker groups were higher than in the Gcontrol group (494.8 ± 41.94, 569.8 ± 6.70 vs. 158.8 ± 13.09 nM, respectively, p < 0.05; fig. 8, 9).

Fig. 3. Immunohistochemistry for Cx40 showed staining in pulmonary EC membranes before (a), and 6 (b), 12 (c), 24 (d) and 48 h (e) after gunshot injury. Original magnification, ×400. f OD values significantly decreased with time.
Fig. 4. **a** EB leakage from lung tissues after gunshot injury (* p < 0.05). **b** There was a significant, negative correlation between OD values and EB permeability rates ($\rho = -0.934, p < 0.05$), with a correlation coefficient ($\rho^2$) of 0.8723.

Fig. 5. **a** Dye transfer in ECs on slides; a region of ECs scrape-loaded with dye is shown. **b** $G_{\text{control}}$, **c** $G_{\text{serum}}$, **c** $G_{\text{blocker}}$. (Green indicates Lucifer yellow, including cells initially loaded with dye and recipient cells linked to the donors by gap junctions.) **d** Quantification of dye transfer in $G_{\text{control}}$, $G_{\text{serum}}$, and $G_{\text{blocker}}$ (* p < 0.05).
Discussion

Thoracic gunshot injury decreased Cx40 level and increased pulmonary vascular permeability, with a significant correlation between Cx40 staining intensity and vascular permeability. Stimulation of PMVECs with serum12 h decreased Cx40 levels and GJC function but increased PMVEC monolayer permeability, especially following treatment with a gap junction blocker. Furthermore, intracellular free calcium concentration increased. These effects were aggravated when a GJC blocker was added to the PMVECs prior to serum12 h treatment. Thus, these findings indicate that GJCs of PMVECs modulate pulmonary vascular permeability following thoracic gunshot injury, contributing to the development of ALI possibly via changes in intracellular calcium. This is the first study to elucidate the relationship between GJC function and pulmonary vascular permeability, and the results suggest that GJCs might provide a novel therapeutic target for the treatment of lung inflammation, including ALI.

Though various factors lead to the occurrence of ALI [19–21], severe trauma is still a major cause of ALI. In a study by Treggiari et al. [22], the incidence of ALI was 4–12% in 1,296 patients who survived 24 h or more after trauma. We previously established an animal model of
Thoracic gunshot wounds by decreasing the initial velocity of a bullet, thereby reducing kinetic energy [14]. In this study, the oxygenation index (PaO2/FiO2, fig. 1) was <300 mm Hg ≥12 h after shooting, indicating refractory respiratory failure. In addition, diffuse infiltrates were seen bilaterally on the chest radiograph taken 12 h after gunshot injury. Lung water content and EB leakage rates increased after injury. In order to exclude cardiogenic edema, animals with a pulmonary artery wedge pressure >2.6 mm Hg were excluded from this study. These findings were consistent with the diagnosis of ALI [23].

As the bullet passes through the lung and transfers its kinetic energy, it produces a pressure wave that creates a temporary cavity which is greater than the bullet’s diameter (cavitary effects) [24]. Bellamy and Zajtchuk [25] postulated that the ballistic pressure waves are the main cause for the remote lung injury. Moreover, in our opinion, in addition to lung injury around the ballistic trajectory, gunshot wounds trigger an inflammation cascade involving inflammatory mediators contributing to systemic inflammatory responses and ALI [26, 27] following gunshot wounds. Here, serum levels of TNF-α and IL-8 were significantly higher after gunshot injury, i.e., lung inflammation occurred.

Although several therapeutic strategies have been developed clinically, the mortality and morbidity associated with ALI and the acute respiratory distress syndrome (ARDS) remain very high [28]. Increased pulmonary permeability has been recognized as a hallmark of ALI/ARDS [3]. Thus, modulating pulmonary permeability has become an important strategy to prevent and treat ALI/ARDS. To date, the mechanism inducing increased pulmonary vascular permeability in lung inflammation has not been elucidated yet. Paracellular gap formation has been reported as a factor contributing to the increased pulmonary permeability in lung inflammation [5] and is regulated by competing contractile forces as well as adhesive cell-cell and cell-matrix tethering forces [8]. A key EC contractile event is the phosphorylation of regulatory

**Fig. 8.** Images from the three groups of PMVECs 30 s after excitation. a Gcontrol. b Gserum. c Gblocker. The fluorescence intensity was higher in Gserum and Gblocker than in Gcontrol. Thus, the [Ca^{2+}]i concentration in PMVECs was higher in Gserum (494.8 ± 41.94 nM) and Gblocker (569.8 ± 6.70 nM) than in Gcontrol (158.8 ± 13.09 nM, p < 0.05).

**Fig. 9.** Effects of serum (obtained from animals subjected to gunshot injury) and GJC blocker on [Ca^{2+}]i increase.
myosin light chains catalyzed by Ca\(^{2+}\)/calmodulin-dependent myosin light chain kinase [5].

In this study, evidence indicated hyperpermeability after gunshot injury, as lung water content and EB leakage increased. Furthermore, immunohistochemistry with Cx40 antibodies showed staining throughout the pulmonary EC membrane. Statistical analysis revealed that a significant negative correlation existed between EB leakage rates and OD values \((p = -0.934, p < 0.05, \rho^2 = 0.8723)\). In an in vitro study in rabbits, serum stimulation increased the clearance of EB-labeled albumin in a time-dependent manner but concomitantly decreased the function of GJCs evaluated by scrape-loading and dye transfer studies and expression of Cx40 in PMVEC. However, pre-incubation with a GJC blocker markedly aggravated these effects. Consequently, Cx40 expression decreased in PMVECs, while scrape-loading and dye transfer studies provided evidence that this serum inhibited GJC function, and this inhibition was aggravated by a GJC blocker. These findings strongly suggest that the increased pulmonary permeability in ALI/ARDS following chest trauma is mediated by cell-cell communication via GJCs, being primarily composed of Cx40. This decrease in barrier function occurs despite transient cell adhesion due to stimulation of focal adhesion kinase activity and resultant remodeling of the actin cytoskeleton [29].

GJCs provide a pathway for the diffusion of ions and small molecules such as cyclic AMP, cyclic GMP, Ca\(^{2+}\) and inositol triphosphate, and contributes to disease etiology [9, 10, 30]. The function of GJCs is finely regulated by cyclic AMP, inflammatory mediators (e.g. TNF-\(\alpha\) and IL-1\(\beta\)), intracellular Ca\(^{2+}\), pH, protein phosphorylation and trans-junctional applied voltage [31–35]. In the present study, the decreased function of GJCs led to an overload of intracellular calcium, and this effect was amplified by a GJC blocker, being consistent with the results described by D’hondt et al. [13]. It is believed that a rise in cytosolic calcium concentration causes centripedal contraction of PMVECs [36] resulting in the formation of paracellular gaps.

Conclusions

In summary, our findings indicate that inflammatory factors, e.g. TNF-\(\alpha\) and IL-8, inhibit Cx40 expression, decreasing gap junction function and causing PMVECs to lose intracellular communication, thus leading to intercellular calcium overload and increases in pulmonary vascular permeability. The limitation of this study is that part of the findings were obtained from in vitro studies, which might be different from in vivo studies. Therefore, further studies in vivo on GJC changes are needed to determine GJC as a novel approach for treating ALI after trauma.


