Injury of Cell Tight Junctions and Changes of Actin Level in Acute Lung Injury Caused by the Perfluoroisobutylene Exposure and the Role of Myosin Light Chain Kinase

Ge Meng\textsuperscript{1,2*}, Jian Zhao\textsuperscript{1*}, He-Mei Wang\textsuperscript{1}, Ri-Gao Ding\textsuperscript{1}, Xian-Cheng Zhang\textsuperscript{1}, Chun-Qian Huang\textsuperscript{1} and Jin-Xiu Ruan\textsuperscript{1}

\textsuperscript{1}Institute of Pharmacology and Toxicology, Academy of Military Medical Sciences and \textsuperscript{2}School of Public Health, Tianjin Medical University, P. R. China

Abstract: Injury of Cell Tight Junctions and Changes of Actin Level in Acute Lung Injury Caused by the Perfluoroisobutylene Exposure and the Role of Myosin Light Chain Kinase: Ge Meng, et al. Institute of Pharmacology and Toxicology, Academy of Military Medical Sciences, P. R. China—Objectives: To investigate the injury of cell tight junctions and change in actin level in the alveolus epithelial cells of the lung after perfluoroisobutylene (PFIB) exposure and the role of myosin light chain kinase (MLCK) in the injury. Methods: Rats and mice were exposed to a sublethal dose of PFIB. The changes in tight junction zonula occludens-1 (ZO-1), actin and myosin light chain kinase (MLCK) were detected by immunofluorescence at 30 min, 1, 2, 4, 8, 16, 24, 48 and 72 h after PFIB exposure. The role of MLCK was analyzed by lung indices and the actin level. Results: The normal ZO-1 immunofluorescence density and those after PFIB exposure were 71.63, 39.41, 37.59, 35.71, 33.22, 31.34, 31.61, 24.51, 40.03 and 44.71 respectively. The normal actin immunofluorescence density and those after PFIB exposure were 31.82, 36.46, 36.57, 41.60, 40.95, 35.41, 30.69, 19.96, 29.30 and 33.00 respectively. The normal MLCK immunofluorescence density and those after PFIB exposure were 61.21, 50.87, 48.37, 41.60, 40.95, 35.41, 30.69, 19.96, 29.30 and 33.00 respectively. The normal MLCK immunofluorescence density and those after PFIB exposure were 61.21, 50.87, 48.37, 41.60, 40.95, 35.41, 30.69, 19.96, 29.30 and 33.00 respectively. When the MLCK inhibitor ML-7 was given in advance, pulmonary edema and actin degradation were suppressed. Conclusions: At an earlier stage, the increased permeability of the blood-air barrier after PFIB exposure is probably the result of injury of cell tight junctions that acts in concert with later changes in actin, resulting in an increase in permeability. MLCK could be a potential target for novel drug development for relief of acute lung injury.

Key words: Actin, Acute lung injury, Blood-air barrier, Myosin light chain kinase, Perfluoroisobutylene, Zonula occludens-1

Perfluoroisobutylene (PFIB, chemical structure: \((\text{F}_3\text{C})_2=\text{CF}_2\)) is a potential pneumoedematogenic gas, that is usually produced as the main by-product of the fluoropolymer industry in quantities of tens of thousands of tons. It is also a main component of the fumes in fire disasters. A small amount of PFIB inhalation can cause severe acute lung injury (ALI), pulmonary edema and even death\cite{1,2}. Unfortunately, no specific antidote or effective therapeutic measures are currently available.

Our laboratory has focused on the pathogenesis, treatment and prevention of ALI caused by PFIB inhalation for quite a few years. We found that PFIB inhalation could quickly initiate alveolar edema and an increase in protein content in bronchoalveolar lavage fluid (BALF), which was accompanied by the infiltration of a large quantity of inflammatory cells, thickening of the alveolar wall and apoptosis and necrosis of alveolar type I epithelial cells (AT-I), alveolar type II epithelial cells (AT-II) and pulmonary microvascular endothelial cells (PMVEC)\cite{3,4}. These results implied that the blood-air barrier was probably damaged and its permeability increased. In other kinds of ALI induced by lipopolysaccharide (LPS), thrombin, ischemia and reperfusion and mechanical ventilation, evidence of blood-air barrier injuries has also been observed\cite{5,6,7,8}. It is generally consented that the dysfunction of the blood-air barrier is a central event in ALI.
The blood-air barrier is composed of the epithelial barrier and endothelial barrier. It depends on the delicate balance between the barrier-protective tethering forces of cell junctions and the barrier-disruptive cellular contractile forces of the cytoskeleton to maintain its structure and function. Three major types of animal cell junctions, tight junctions, anchoring junctions and gap junctions, have been found to mediate the cell-cell and cell-extracellular matrix interactions. Zonula occludens-1 (ZO-1) is one of the first proteins identified and localized to the tight junction. The cytoskeleton is a complex network of actin microfilaments, intermediate filaments and microtubules. Cellular contractile forces are generated by ratcheting of bonds between actin and myosin and result in the formation of the actin stress fibers and the increased tension in these stressed fibers. Activation of cell contraction and disturbance of cell junctions usually result in the induction of gaps in the blood-air barrier followed by enhancement of its permeability.

Others have reported that phosphorylation of myosin light chain (MLC) was probably involved in the injury of tight junctions and cell cytoskeleton. Phosphorylation induces a conformational change in MLC that enables actin-myosin interaction and cell contraction, which lead to injury of tight junctions and actin and finally the increased permeability of the blood-air barrier.

The major signaling pathways related to MLC phosphorylation are Ca²⁺/calmodulin-dependent myosin light chain kinase (MLCK) and Rho kinase (RhoK) mediated inhibition of MLC phosphatase. The increased activation of MLCK and RhoK enhances the actin-myosin interaction, central stress fiber formation and endothelial cell contraction and gap formation, which ultimately cause disruption and increased permeability of the blood-air barrier. Recent studies have shown that the phosphorylation of MLC at Ser-19 and Thr-18 by activated MLCK plays a key role in the development and regulation of contractile forces within cells.

Studies concerning injury of tight junctions and changes in actin level in PFIB-induced ALI have rarely been reported. In the current paper, we described the time course of injury of cell junctions and the cytoskeleton after PFIB exposure and preliminarily investigate the role of MLCK in PFIB-induced ALI.

Materials and Methods

General

1) Animals

Eighty specific pathogen-free male Wistar rats (180–220 g, 8 wk old) and sixty-four pathogen-free male Kunming mice (18–22 g, 4 wk old) obtained from the Center of Medical Experimental Animals in the Academy of Military Medical Sciences (Beijing, P. R. China) were used in this study. The animals were housed in quiet, humidified, clean rooms with a light-cycle of 12 h/12 h for 1 wk before use. They were fed laboratory pellet food and tap water ad libitum except when they were in the exposure chamber. All the animal experiments were performed in accordance with the Guidelines for Animal Experiments of the Chinese Academy of Medical Sciences, Beijing, P. R. China.

2) Doses of PFIB for animal exposure

PFIB was obtained from the Shanghai Institute of Organic Fluorine Materials with a purity of 98%. As was described previously, the mice were exposed to PFIB at a dose of 130 mg/m² x 5 min with a flow-past whole-body exposure apparatus, and the rats were exposed to a dose of 140 mg/m² x 5 min with a head-nose exposure apparatus.

3) Overview of the objectives and approaches

In the first part of the study, immunofluorescence was used to observe the concentration changes in the tight junction protein ZO-1 and actin of the alveolus epithelial cells in rats. Samples were taken at different times after PFIB exposure in order to study the basic pathological change in the blood-air barrier.

Since MLC was probably involved in the changes in the tight junctions and actin in ALI caused by PFIB and MLCK was the main regulatory factor of MLC, the changes of MLCK in rats were studied by immunofluorescence in the second part of the study. Also, an MLCK inhibitor, ML-7, was used in the mice to study the role of MLCK in ALI. Pulmonary edema was evaluated by lung indices, and the change in actin level was analyzed by western blot.

Experimental methods

1) Immunofluorescence

Eighty rats were randomly divided into 10 groups, and each group contained 8 rats. One group was used as the normal group, and the others were exposed to PFIB. The rats in the 9 exposed groups were sacrificed separately at 30 min, 1, 2, 4, 8, 16, 24, 48 and 72 h after PFIB exposure. They were anesthetized using 25% ethyl carbamate (0.5 ml/100 g bw, i.p.) and then exsanguinated via abdominal aorta transection. The accessory lobes of the right lungs were collected for immunofluorescence.

Five-micrometer frozen sections were fixed in ice-cold acetone-methanol (1:1, v/v) at –20°C for 5 min. They were then permeabilized with 0.01% (v/v) Triton X-100 for 10 min and blocked with 1% (w/v) BSA dissolved in PBS at room temperature for 1 h. After removing the blocking solution, the sections were incubated with antibodies against ZO-1 (H-300), actin (H-300) or MLCK (H-195) (1:100, Santa Cruz, CA, USA) at room temperature for 1 h. After three washing in PBS, they were incubated with FITC-conjugated goat anti-rabbit IgG (1:100, Santa Cruz) and rinsed three times in PBS. The fluorescent labeling was observed immediately with a Radiance 2100™ confocal laser scanning microscope (Bio-Rad, Hercules, CA, USA). Three visual fields were...
observed in each simple, and three cells were selected in each visual field. The immunofluorescence density of each cell was determined with the Image-Pro Plus 6.0 software, and the SAS 6.12 software was used for statistical analysis.

2) Protection against PFIB-induced lung injury by ML-7

Sixty-four mice were randomly divided into 4 groups with 16 mice in each group. One group was used as the normal group, and the other three were exposed to PFIB. Before exposure to PFIB, two groups were treated with 10% ethanol (0.2 ml) or MLCK inhibitor ML-7 (dissolved in 10% ethanol, Sigma-Aldrich, St. Louis, MO, USA) intraperitoneally administered twice daily for 2 days. The first bolus of ML-7 was administered at 4.3 mg/kg body weight, and three successive administrations were then performed at 1.4 mg/kg body weight. The last administration of ethanol and ML-7 was performed 1 h before PFIB exposure. The mice were sacrificed 24 h later. A lobe of the lung was used for Western blot, and the rest was used to analyze the lung indices.

1) Lung indices

Lung indices were assayed as described previously. The lungs were rinsed in saline, blotted and then weighed to obtain the wet weight. They were then put in an oven at 80°C for 24 h to obtain the dry weight. The lung indices were calculated by the following formulas: lung wet-to-dry weight ratio = mass(wet lung)/mass(dry lung); dry lung-to-body weight ratio = (mass(dry lung)/mass(body)) × 10,000; and wet lung-to-body weight ratio = (mass(wet lung)/mass(body)) × 10,000.

2) Western blot analysis

Tissue specimens were pulverized in liquid nitrogen and homogenized in an ice-cold lysis buffer containing a cocktail of protease inhibitors (Complete, EDTA-free: Roche, Mannheim, Germany). The homogenates were centrifuged at 15,000 g at 4°C for 1 h, and the proteins (supernatant) were extracted and stored in liquid nitrogen until electrophoresis. The protein concentration was determined by the Bradford assay. Ten-microgram samples were diluted with 2 × loading buffer [50 mmol/l Tris-HCl, 50 mmol/l dithiothreitol (DTT), 2% sodium dodecyl sulphate (SDS), 0.1% bromphenol blue and 10% glycerol] and heated in boiling water for 5 min. The proteins were separated by 10% SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to a nitrocellulose membrane. The membrane was washed in Tween-20 Tris-buffered saline (TTBS) [20 mmol/l Tris · HCl (pH 7.5), 0.15 mol/l NaCl, 0.05% Tween-20] for 10 min and blocked with 10% skimmed milk at room temperature for 1 h. Thereafter, the membrane was incubated with a polyclonal anti-actin antibody (1:1,000; Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 4°C for 1 h. After three washes in TTBS, it was incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (1:5,000; Beijing Zhong Shan-Golden Bridge Biological Technology Company, Beijing, China) at room temperature for 1 h and then rinsed three times in TTBS. It was then stained with enhanced chemiluminescence reagent (ECL, Amersham Life Science, Arlington Heights, IL, USA). Densitometric analysis of actin detected on Western blot was performed with the Image-Pro Plus 6.0 software. The SAS 6.12 software was used for statistical analysis. The actin density of the normal group was designated as 1, and the actin densities of the other groups were control led with that of normal group. This experiment was repeated three times.

3) Statistical analysis

The SAS 6.12 software was used for statistical analysis. Data are shown as the mean ± SD. Unless particularly indicated, one-way analysis of variance followed by Dunnett’s test was used to detect the difference between groups.

Results

Expression profile of ZO-1 in the blood-air barrier of the rats after PFIB exposure

ZO-1 was located in the paracellular space between the endothelial cells and the epithelial cells. Its expression in the blood-air barrier was abundant and showed strong staining in the normal group. At 30 min after PFIB exposure, the immunofluorescence density of ZO-1 became weaker and weaker, and the numerical value started to decrease persistently, and reached the lowest level at 24 h. Afterwards, the staining began to increase gradually (Table 1 and Fig. 1). The lung wet-to-dry weight ratio, dry lung-to-body weight ratio and wet lung-to-body weight ratio respectively
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represents the changes of volatile substances (such as water), involatile substances (such as protein) and both volatile and involatile substances during the course of pulmonary edema induced by PFIB. The lung wet-to-dry weight ratio, dry lung-to-body weight ratio and wet lung-to-body weight ratio of the normal group were 4.90 ± 0.05, 11.72 ± 0.86 and 57.44 ± 4.15 (n=16). They were significantly increased at 24 h after PFIB exposure (the numerical values were 6.53 ± 0.88, 20.02 ± 2.42 and 131.83 ± 31.20, n=16). Preinhibition of MLCK by ML-7 could significantly suppress the increase (the numerical values were 5.62 ± 0.41, 16.41 ± 2.16 and 92.55 ± 16.60, n=16). The solvent (10% ethanol) for ML-7 could not influence the pulmonary edema induced by PFIB (Fig. 4). The results indicate that severe pulmonary edema was induced by PFIB. Increased permeability of the blood-air barrier resulted in an increase of water and protein in the lung. Preinhibition of MLCK by ML-7 could significantly suppress the increase of water and protein in the lung.

2) ML-7 protected actin in the blood-air barrier in the mice exposed to PFIB

The western blot results showed that actin decreased at 24 h after PFIB exposure in the group with and without ethanol injection compared with the normal group. In the group with ML-7 pretreatment, the actin level did not change too much, which suggested that MLCK played an important role in reducing the degradation of actin and stopping the leakage of the blood-air barrier in the ALI caused by the PFIB exposure (Fig. 5).

Discussion

We studied injury of the cell tight junctions and change

<p>| Table 1. Immunofluorescence density of ZO-1, actin and MLCK |</p>
<table>
<thead>
<tr>
<th>Time</th>
<th>n</th>
<th>ZO-1</th>
<th>Actin</th>
<th>MLCK</th>
</tr>
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<tbody>
<tr>
<td>Normal</td>
<td>9</td>
<td>71.63 ± 11.12**</td>
<td>31.82 ± 2.37**</td>
<td>61.21 ± 5.11**</td>
</tr>
<tr>
<td>0.5 h after PFIB exposure</td>
<td>9</td>
<td>39.41 ± 7.28**</td>
<td>36.46 ± 4.84**</td>
<td>50.87 ± 8.69**</td>
</tr>
<tr>
<td>1 h after PFIB exposure</td>
<td>9</td>
<td>37.59 ± 6.17**</td>
<td>36.57 ± 2.64**</td>
<td>48.37 ± 11.78**</td>
</tr>
<tr>
<td>2 h after PFIB exposure</td>
<td>9</td>
<td>35.71 ± 6.78**</td>
<td>41.60 ± 9.64**</td>
<td>43.65 ± 5.82**</td>
</tr>
<tr>
<td>4 h after PFIB exposure</td>
<td>9</td>
<td>33.22 ± 1.98**</td>
<td>40.95 ± 9.96**</td>
<td>41.96 ± 5.16**</td>
</tr>
<tr>
<td>8 h after PFIB exposure</td>
<td>9</td>
<td>31.34 ± 3.27**</td>
<td>35.41 ± 4.59**</td>
<td>35.44 ± 8.38**</td>
</tr>
<tr>
<td>16 h after PFIB exposure</td>
<td>9</td>
<td>31.61 ± 3.11**</td>
<td>30.69 ± 6.18**</td>
<td>31.77 ± 7.56**</td>
</tr>
<tr>
<td>24 h after PFIB exposure</td>
<td>9</td>
<td>24.51 ± 2.92**</td>
<td>19.96 ± 6.67**</td>
<td>30.85 ± 4.27**</td>
</tr>
<tr>
<td>48 h after PFIB exposure</td>
<td>9</td>
<td>40.03 ± 4.60**</td>
<td>29.30 ± 8.26**</td>
<td>33.10 ± 5.02**</td>
</tr>
<tr>
<td>72 h after PFIB exposure</td>
<td>9</td>
<td>44.71 ± 4.89**</td>
<td>33.00 ± 8.19**</td>
<td>38.20 ± 7.34**</td>
</tr>
</tbody>
</table>

Mean ± SD, n=9, ANOVA. **: p<0.01 versus the normal group. * and **: p<0.05 and p<0.01 versus the 24 h after PFIB exposure group.
in actin level of the blood-air barrier in the ALI caused by PFIB exposure and also explored the role of MLCK in maintaining the integrity of the blood-air barrier. Cell junctions, in concert with the dynamic cytoskeleton, play an important role in cell–cell adhesion and the blood-air barrier regulation. The tight junctions are located at the paracellular space between the endothelial cells and the epithelial cells. Their role is to control and regulate the paracellular permeability of these cell layers. The tight junction proteins are composed of transmembrane proteins and cytoplasmic plaque proteins. The transmembrane proteins mainly include occludin, claudins, junctional adhesion molecules and so on. The cytoplasmic proteins are located at the cytoplasmic plaque domain of the tight junctions and include ZOs, membrane-associated guanylate kinase inverted (MAGI), cingulin, ZO-1-associated nucleic acid-binding protein, and so on. ZO-1, a 220-kDa phosphoprotein, is one of the major cytoplasmic proteins of the tight junctions. It is a scaffolding protein that links transmembrane proteins, occludin and claudin, to F-actin in the cytoplasm. Actin, a 43-kDa protein, is very important in maintaining
Fig. 4. Pretreatment with ML-7 significantly reduced the PFIB-induced (130 mg/m³×5 min) pulmonary edema in mice. A: Lung wet-to-dry weight ratio. B: Dry lung-to-body weight ratio. C: Wet lung-to-body weight ratio. Compared with the normal group, the three lung indices (lung wet-to-dry weight ratio, dry lung-to-body weight ratio and wet lung-to-body weight ratio) were significantly increased at 24 h after PFIB exposure in the groups with and without 10% ethanol injection. Pre-inhibition of MLCK by ML-7 could significantly suppress the increase of the four lung indices in the ALI caused by PFIB. The solvent (10% ethanol) of ML-7 cannot influence the pulmonary edema induced by PFIB. Mean ± SD, n=16, ANOVA. * and **: p<0.01 and p<0.001 versus the normal group. # and ##: p<0.01 and p<0.001 versus the PFIB exposure group. + and ++: p<0.01 and p<0.001 versus the pretreatment with 10%Et + PFIB group. Et: Ethanol.

Fig. 5. Western blot analysis of actin from pretreatment with ML-7 mice sacrificed at different times before and after PFIB (130 mg/m³×5 min) exposure. The image above the bar graph is a representative western blot photograph of actin. The western blot results showed that actin decreased at 24 h after PFIB exposure in the group with and without ethanol injection compared with the normal group. Pre-inhibition of MLCK by ML-7 could significantly suppress the decrease induced by PFIB. The solvent (10% ethanol) of ML-7 could not influence the change induced by PFIB. Western blot was repeated three times (n=16/group). Mean ± SD, n=16, ANOVA. *: p<0.05 versus the normal group.

the integrity of the blood-air barrier. Disruption of the actin cytoskeleton can increase the permeability of the blood-air barrier in cultured endothelial cells. Both cell tight junctions and actin work in concert to maintain the normal structure and function of the ATs and PMVEC.
In our study, we found that the level of ZO-1 in the cells of the blood-air barrier started to decrease at 30 min after PFIB exposure and reached the lowest level at 24 h. Thereafter, it gradually increased. However, the level of actin in the blood-air barrier increased slowly within the first 4 h after PFIB exposure. It began to decrease at 8 h and reached the lowest level at 24 h. Then it recovered gradually. These data can be used to interpret our previous experiment results. In our last paper, we observed that the protein concentration of BALF, an index of the integrity of the blood-air barrier, remained unchanged within the first 4 h (30 min, 1, 2 and 4 h) after PFIB exposure, increased significantly at 8 h, peaked at 24 h and then declined. According to our results, we assume that injury of cell tight junctions results in the increased permeability of the blood-air barrier in the early stage of the ALI induced by PFIB exposure. As time passes, a change in actin begins to occur, and the permeability of the blood-air barrier increases seriously.

Recently, quite a few publications have confirmed that MLCK participates in the regulation of the cell junctions and cytoskeleton. MLCK includes an endothelial form and a smooth muscle form. They encode a 210-kDa protein and a 108-kDa protein respectively. The endothelial MLCK is probably more important in regulating the function of the blood-air barrier. In the mice with endothelial MLCK knockout, the susceptibility to inflammatory lung disease and mechanical injury induced by positive pressure ventilation was reduced obviously. Phosphorylation of MLC at Ser19/Thr18 by activated MLCK leads to myosin, which associates with actin stress fibers to produce cell contraction, and plays an important role in the development and regulation of contractile forces within cells. Thus, the delicate balance between the barrier-protective tethering forces and the barrier-disruptive cellular contractile forces is destroyed, and injury of the cell junctions and cytoskeleton occurred.

In our study, we observed the change in MLCK in the blood-air barrier at different time points after PFIB exposure. The level of MLCK decreased persistently until at 24 h. Thereafter, it began to increase gradually. Why did both ZO-1 and MLCK decreased persistently after PFIB exposure, but actin increased slowly within the first 4 h and then began to decrease at 8 h? The decreases in ZO-1 and MLCK were probably the results of PFIB direct attack and indirect attack (inflammatory reaction). However, during the earlier period in which barrier integrity was destroyed, actin was probably subjected to rearrangement and upregulation in order to oppose the destruction of the barrier. During the course of ALI, damage factors, such as interleukin (IL), tumor necrosis factor (TNF) and so on, were induced. Some damage factors, such as thrombin and TNF-α, can produce a significant decrease in circumferential actin staining in association with a dramatic increase in stress fiber formation that spans the length of the cell. This may be the reason for the increase in actin within the first 4 h after PFIB exposure. Of course, when acute lung injury exceeds the repair ability of the organism, a decrease in actin is inevitable. Thus, actin decreased at 8 h after PFIB exposure.

ML-7, a selective MLCK inhibitor that acts on the adenosine triphosphate-binding site of the active MLCK, was used to elucidate the role of MLCK in the ALI. Preinhibition of MLCK by ML-7 reduced the pulmonary edema and the decrease in actin level induced by PFIB. The same results were observed in other ALI models, such as ALI induced by LPS, PAF, thrombin and I/R. All these data verified the important role of MLCK in ALI.

In conclusion, the level of ZO-1, actin and MLCK decreased obviously after PFIB exposure. ML-7 could inhibit MLCK and reduced the pulmonary edema and actin level.

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References


