Systemic Administration of FC-77 Dampens Ischemia–Reperfusion-Induced Acute Lung Injury in Rats

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Abstract—Systemic administration of perfluorocarbons (PFCs) reportedly attenuates acute lung injury induced by acid aspiration and phorbol myristate acetate. However, the effects of PFCs on ischemia–reperfusion (IR)-induced lung injury have not been investigated. Typical acute lung injury was induced in rats by 60 min of ischemia and 60 min of reperfusion in isolated and perfused rat lung model. Rat lungs were randomly assigned to receive PBS (control), 1 % FC-77, IR only, or IR with different doses of FC-77 (0.1 %, 0.5 %, or 1 %). Subsequently, bronchoalveolar lavage fluid (BALF), perfusate, and lung tissues were collected to evaluate the degree of lung injury. IR caused a significant increase in the following parameters: pulmonary arterial pressure, capillary filtration coefficient, lung weight gain, lung weight/body weight ratio, wet/dry lung weight ratio, and protein concentration in BALF. TNF-α and cytokine-induced neutrophil chemoattractant-1 concentrations in perfusate samples and MDA concentration and MPO activities in lung tissues were also significantly increased. Histopathology showed increased septal thickness and neutrophil infiltration in the lung tissues. Furthermore, NF-κB activity was significantly increased in the lungs. However, pretreatment with 1 % FC-77 prior to IR significantly attenuated the increases in these parameters.

In conclusion, our results suggest that systemic FC-77 administration had a protective effect on IR-induced acute lung injury. These protective mechanisms may have been mediated by the inhibition of NF-κB activation and attenuation of subsequent inflammatory response.

KEY WORDS: ischemia–reperfusion; acute lung injury; perfluorocarbon; FC-77.

INTRODUCTION

Perfluorocarbons (PFCs) are inert chemicals having high density and good oxygen-dissolving capacity; moreover, they are highly hydrophobic and lipophobic [1]. Because of these advantages, liquid ventilation with PFCs was initially shown to improve acute lung injury in animal models and a few human studies. Important mechanisms that attenuate lung inflammation include the distention of collapsed alveoli to improve lung compliance, improvement of oxygenation by increasing oxygen diffusion, and removal of inflammatory mediators from the alveolar regions of the lung [2–4]. However, two subsequent large clinical trials of patients with acute respiratory distress syndrome (ARDS) showed that liquid ventilation was not beneficial for improving survival [5, 6].

Ischemia–reperfusion (IR) lung injury is a common complication of lung transplantation procedures, pulmonary embolism, shock, and trauma, and it results in increased microvascular permeability and neutrophil infiltration in the lung tissue, damaged pulmonary endothelium, and pulmonary hypertension [7, 8]. Despite advances in intensive care, ARDS-associated morbidity and mortality remain high [7, 8]. Therefore, it is important to develop new strategies to treat these potentially reversible pulmonary injuries.
The results of a number of recent in vitro and in vivo studies have suggested that PFCs have anti-inflammatory effects that may directly modulate inflammatory cell function and decrease the production of inflammatory mediators [9–12]. Emulsified PFCs can be safely injected into the blood system of humans as a blood substitute [13]. Emulsified PFCs have also been used as part of management of a variety of diseases, including polytrauma, anemia, burns, hemorrhagic shock, toxic infectious shock, and blood vessel occlusion [14].

During the early stage of acute lung injury, the origin of activated neutrophils is intravascular and not intra-alveolar; therefore, intravascularly administered PFCs will make direct contact with inflammatory cells and may be a more potent means to decrease harmful inflammatory reactions. In addition, systemic FC-77 administration has been shown to attenuate acute lung injury induced by acid aspiration and phorbol myristate acetate (PMA) [15, 16]. Although PFCs reportedly limit the infarction size in IR heart injury [17, 18], the beneficial effects of FC-77 on IR-induced acute lung injury remain unclear. Therefore, the present study was designed to determine whether systemic FC-77 administration ameliorates acute lung injury induced by IR. In this experiment, we used a well-established model of isolated rat lungs because it enabled us to measure the pulmonary filtration coefficient ($K_{ef}$), which is the most accurate indicator of pulmonary capillary permeability [19].

**MATERIALS AND METHODS**

**Preparation of Isolated and Perfused Rat Lungs**

We prepared the isolated and perfused rat lungs in situ as previously described [20, 21]. The animals used for this study were cared for in accordance with the guidelines of the National Institutes of Health (National Academy Press, 1996), and approval for our study protocol was obtained from the National Science Council and Animal Review Committee of the National Defense Medical Center. Male Sprague–Dawley rats (weight 250–350 g) were anesthetized with intraperitoneal injections of pentobarbital sodium (20–25 mg/rat). Tracheostomy was performed to enable ventilation with a rodent ventilator (7025; Ugo Basile, Comerio, VA, Italy). The rat lungs were ventilated with 5 % CO$_2$ in air at 65–70 breaths/minute with a tidal volume of 2 mL. After a median sternotomy was performed, heparin (1 U/g of body weight) was injected into the right ventricle, from which 10 mL of blood was collected. This blood sample was mixed with 10 mL of normal saline containing 1.5 % human serum albumin. This was subsequently used as a perfusing fluid for the isolated lungs. For constant-flow perfusion of the isolated lungs, a cannula was inserted into the pulmonary artery via a right-ventricular puncture. A tight ligature was placed around the main trunk of the pulmonary artery. A wide-bore cannula was inserted into the left atrium via the left ventricle to divert pulmonary venous outflow into a reservoir. The wide-bore cannula was then fixed with a ligature at the apex of the heart. Another ligature was placed above the atrioventricular junction to prevent the flow of the perfusate into the ventricles. Both the pulmonary arterial pressure (PAP) and the pulmonary venous pressure (PVP) were recorded from side arms of the inflow and outflow cannula. A roller pump was used to provide constant perfusion flow at a rate of approximately 8–10 mL/min to stabilize PAP at 15–20 cmH$_2$O. PVP was set at 4–6 cmH$_2$O by adjusting the height of the venous reservoir. With the isolated perfused lungs remaining in situ, the whole rat was placed on an electronic balance. The digital signals of the electronic balance were converted to analog signals with a digital-to-analog converter and were recorded on an oscillograph recorder. Isolated lung preparations were used in this study only if they satisfied three criteria: (1) no leakage at the sites of cannula insertion, (2) no evidence of bleeding or edema, and (3) an isogravimetric state.

**Induction of Lung Ischemia and Reperfusion**

The lungs were then subjected to 60 min of ischemia by stopping ventilation and perfusion. After this period of ischemia, the lungs were reperfused for 60 min and ventilated with 5 % CO$_2$–95 % air.

**Microvascular Permeability**

$K_{ef}$, which is an index of microvascular permeability to water, was determined from the lung weight change caused by increased venous pressure. During ventilation and lung perfusion, PVP was rapidly elevated by 10 cmH$_2$O for at least 7 min. The slow, steady phase of weight gain as a function of time ($\Delta W/\Delta t$) was plotted on a semi-logarithmic paper. The slow component was then extrapolated to zero time to obtain the initial rate of transcapillary filtration. From this plot, $K_{ef}$ was defined as the $y$-intercept (g min$^{-1}$) divided by PVP (10 cmH$_2$O) and lung weight. $K_{ef}$ was expressed in units of grams per minute of cmH$_2$O$^{-1} \times 100$ g [20, 21].
Determinations of Lung Weight/Body Weight (LW/BW) and Wet/Dry Lung Weight (W/D) Ratios

After experiments, the right lung was removed from the hilar region and the wet weight was determined to calculate the LW/BW ratio. A part of the right upper lung lobe was placed in an oven at 60 °C for 48 h to determine the W/D lung weight ratio.

Protein Concentration in Bronchoalveolar Lavage Fluid (BALF)

BALF was obtained at the end of the experimental by irrigating the left lung with saline (2 × 2.5 mL). This fluid was centrifuged at 250×g for 10 min, and the protein concentration in the supernatant was determined using a BCA protein assay (Pierce, Rockford, IL, USA).

Measurement of TNF-α and Cytokine-Induced Neutrophil Chemoattractant (CINC)-1 Concentrations in the Perfusate

TNF-α and CINC-1 concentrations in the perfusate after the experiment were determined using an ELISA kit (R&D Systems Inc., Minneapolis, MN, USA) according to the manufacturer’s instructions.

Lung Malondialdehyde Concentration

Lung tissue was homogenized in a 1.15 % KCL solution. An aliquot (100 μL) of the homogenate was added to a reaction mixture containing 200 μL of 8.1 % thiobarbituric acid and 700 μL of distilled water. Samples were then boiled for 30 min at 100 °C and centrifuged at 3,000×g for 10 min. The absorbance of the supernatant was measured spectrophotometrically at 532 nm.

Lung Myeloperoxidase Activity

A part of the right lower lung lobe was freeze–thawed and sonicated three times. Homogenates were centrifuged at 15,000×g for 10 min at 4 °C. An aliquot (100 μL) of the supernatant was mixed with 900 μL of 50 mM phosphate buffer (pH 6.0) containing 0.167 mg/mL of o-dianisidine dihydrochloride and 0.0005 % hydrogen peroxide. One unit of peroxidase activity was defined as the amount of enzyme that decomposed 1 μmol of hydrogen peroxide per minute at 25 °C. Hydrogen peroxide decomposition was determined from the oxidation of o-dianisidine using an absorption coefficient of 11.3 mM cm at 460 nm.

NF-κB Activity

NF-κB activity was assessed by the nuclear translocation and DNA binding of the p65 subunit in lung tissues, using a commercially available ELISA kit (TransAM NF-κB p65; Active Motif, Carlsbad, CA, USA) according to the manufacturer’s instructions.

Histological Assessment

A part of the right lower lung lobe was stained with hematoxylin and eosin. The histopathologic assessment was performed by two pathologists blinded to the experimental condition. For each section, 10 random areas were examined at a magnification of ×400. Within each field, lung injury was scored according to (1) infiltration or aggregation of neutrophils in the airspace or vessel wall, and (2) thickness of the alveolar wall. Each assessment was graded on the following four-point scale: 0, 1, 2, or 3, for no, mild, moderate, or severe injury, respectively. The resulting two scores were added and presented as the lung injury score for that section [22].

Experimental Protocol

An isolated lung preparation was allowed to equilibrate for 20 min. We recorded baseline PAP, PVP, and weight change and determined the initial $K_f$ for 7 min. Then, all parameters were allowed to return to their baseline values for 10 min. Rat lungs were randomly assigned to receive PBS (control, $n=6$), 1 % FC-77 (drug control, $n=6$), IR only, or IR with different doses of FC-77 (0.1 %, 0.5 %, or 1 %; $n=6$ per group). FC-77 (chemical formula, C8F18; purity, 100 %; 3M Company, St. Paul, MN, USA) was added to the reservoir (containing 20 mL of perfusate). Then the lungs were perfused and ventilated for 60 min following IR, and the $K_f$ measurement was repeated. Due to the hydrophobic property of FC-77, FC-77 was premixed with 3 mL of perfusate (drawn from the reservoir) and vortexed for several seconds before adding to the reservoir.

Statistical Analysis

Results are expressed as mean±SEM. Group comparisons were made by repeated measures one-way or two-way ANOVA, followed by post hoc comparisons using a Newman–Keuls test. Comparisons within each group for a given variable were made by paired Student’s $t$ tests. A $P$ value of $<0.05$ was considered statistically significant.
RESULTS

PAP

PAP was significantly increased in the IR group than in the control group (Fig. 1a). Pretreatment with FC-77 at different concentrations tended to attenuate the increase in PAP induced by IR; however, the attenuation was significant only in the 1 % FC-77 group when compared with that in the IR group (P<0.05).

Lung Filtration Coefficient

Figure 1b shows the microvascular permeability changes (expressed as $K_f$) in the isolated rat lungs due to IR at different doses of FC-77. $K_f$ (P<0.05) significantly increased after 120 min of IR, whereas $K_f$ did not change after 120 min of perfusion in the control group. Pretreatment with FC-77 at different concentrations tended to attenuate the increase in $K_f$; however, significant attenuation was seen only in the 1 % FC-77 group when compared with that in the IR group (P<0.05).

Lung Weight Gain

The lung weights in the control group remained essentially constant during the 120-min experimental period (Fig. 2). In contrast, IR caused a progressive increase in lung weight. The lung weight change expressed as the lung weight gain was significantly higher in the IR group than in the control group (P<0.05). The decrease in lung weight gain was statistically significant in the 1 % FC-77 (P<0.05) group, but not in the 0.1 % or 0.5 % FC-77 groups (P>0.05), when compared with that in the IR group.

LW/BW and Wet/Dry Ratios

The LW/BW and W/D ratios were significantly increased in the IR group compared with those in the control group (Fig. 3, P<0.05). These ratios tended to decrease in the 0.1 % and 0.5 % FC-77 groups, albeit with no statistical significance when compared with those in the IR group. However, the LW/BW and W/D ratios significantly decreased in the 1 % FC-77 group compared with those in the IR group (P<0.05).

Protein Concentration in BALF

Protein concentration in BALF was significantly higher in the IR group than in the control group (Fig. 4, P<0.05). A significant protective effect was observed in the 0.5 % and 1 % FC-77 groups (P<0.05), but not in the 0.1 % FC-77 group (P>0.05), when compared with the IR group.

TNF-α and CINC-1 Concentrations in Perfusates

TNF-α and CINC-1 concentrations in perfusates significantly increased in the IR group compared with those in the control group (P<0.05). Although pretreatment with 0.5 % FC-77 tended to attenuate this increase, the attenuation was not statistically significant when compared with that in the IR group. On the other hand,
the attenuation was significant in the 1 % FC-77 group when compared with that in the IR group (P<0.05; Fig. 5).

**MDA Concentration and MPO Activity in Lung Tissue**

As shown in Fig. 6, MDA concentration and MPO activity in lung tissues significantly increased in the IR group compared with those in the control group (P<0.05). Pretreatment with 0.1 % and 0.5 % FC-77 tended to attenuate these increases, although the attenuation was not statistically significant when compared with that in the IR group. However, MDA concentration and MPO activity were significantly attenuated in the 1 % FC-77 group compared with those in the IR group (P<0.05).

**NF-κB Activity**

NF-κB activity in the lungs was significantly increased after IR (Fig. 7). NF-κB activity in the lungs tended to decrease in the 0.1 % and 0.5 % FC-77 groups compared with that in the IR group, although the difference was not statistically significant. However, the attenuation was significant in the 1 % FC-77 group when compared with that in the IR group (P<0.05).

**Histopathological Findings**

The control group showed normal lung tissue architecture and no inflammation (Fig. 8a), whereas the IR group exhibited septal thickening and marked inflammatory cell infiltration in the interstitium and alveoli (Fig. 8b). Lung inflammation was significantly lower in the 1 % FC-77 group than in the IR group (Fig. 8c). Lung injury scores also provided evidence that pretreatment with 1 % FC-77 significantly attenuated IR-induced acute lung injury (Fig. 8d; P<0.05).

**DISCUSSION**

In this study, we demonstrated that pretreatment with FC-77 had beneficial effects on IR-induced increases in PAP, $K_c$, lung weight gain, LW/BW ratio, W/D lung ratio, protein concentration in BALF, and TNF-α and CINC-1 concentrations in perfusate; and MDA...
concentration, MPO activity, and neutrophil infiltration in lung tissues. In addition, FC-77 pretreatment also inhibited NF-κB activation in the lungs. The protective effects of FC-77 may be mediated by the inhibition of NF-κB activation and attenuation of inflammatory responses in the lungs.

The major characteristic of IR-induced acute lung endothelial injury is an increase in pulmonary microvascular permeability. In this study, we demonstrated that FC-77 pretreatment could attenuate the increase in vascular permeability on the basis of evaluations of different variables, including \( K_e \), W/D lung ratio, LW/BW ratio, and protein concentration in BALF. These findings were consistent with those of previous reports where PFCs attenuated endothelial injury in acid and PMA-induced acute lung injury [15, 16].

Studies of IR-induced lung injury in animal models have confirmed that sequestration of neutrophils plays an important role in IR-induced lung injury [23]. Activation of neutrophils and their adhesion to endothelial cells are observed during the initial inflammatory response. Activated neutrophils that infiltrate the lung release reactive oxygen species, proteases, cytokines, and vasoconstricting lipids; upregulate the expression of adhesion molecules; and subsequently cause lung injury [23]. An in vitro study showed that PFCs could diffuse from the alveolar space into an adjacent pulmonary vascular endothelial layer that modulated neutrophil adhesion and decreased the number of activated neutrophils entering the injured lung [24].

However, whether PFCs had the same effects in vivo needed to be confirmed.

A previous in vitro study reported that PFCs decreased the production of hydrogen peroxide and superoxide anion by rabbit and piglet alveolar macrophages after chemical stimulation [12]. Steinhorst et al. also demonstrated that PFCs attenuated oleic acid-induced lung injury by reducing the production of reactive oxygen species [25]. PFCs have also been shown to inhibit neutrophil activation and chemotaxis via decreased Syk phosphorylation [26, 27]. Furthermore, systemic administration of PFCs can present a barrier function and prevent direct

**Fig. 4.** Effect of FC-77 on the protein concentration in bronchoalveolar lavage fluid (BALF). Ischemia–reperfusion (IR) caused a significant increase in the protein concentration in BALF. The increase in the BALF protein concentration was significantly attenuated in the 1 % FC-77 group compared with that in the IR group. \(*P<0.05\) versus control group; \(#P<0.05\) versus IR group.

**Fig. 5.** Effect of FC-77 on TNF-α and cytokine induced neutrophil chemotactrant (CINC)-1 concentrations in perfusates. TNF-α and CINC-1 concentrations in perfusates were significantly increased in the ischemia–reperfusion (IR) group compared with those in the control group. These increases were significantly attenuated in the 1 % FC-77 group compared with those in the IR group. \(*P<0.05\) versus control group; \(#P<0.05\) versus IR group.
contact between neutrophils and endothelial cells in vessels [17]. In this study, these characteristics may have contributed to the capability of systemic FC-77 to attenuate MPO activity and lipid peroxidation in the lung and decrease the intensity of the inflammatory response. These results are in agreement with recent findings of decreased neutrophil accumulation, MPO activity, and oxidative damage in lung tissues by systemic PFC administration in a rat lung injury model [15].

TNF-α and IL-8 are early response mediators in the pathophysiology of acute lung injury and ARDS [28]. Previous investigations reported that anti-TNF-α or IL-8 antibodies significantly attenuated IR-induced acute lung injury [29, 30]. PFCs can reduce the production of various inflammatory cytokines in vitro and in vivo, such as TNF-α and IL-8 [9, 11, 31–34]. In this study, the decreased pulmonary inflammation may have been caused, in part, by decreased early production of TNF-α and IL-8.

NF-κB is a key transcription factor that is activated by a number of stimuli, including hypoxia, ischemia, inflammatory cytokines, chemokines, and oxygen radicals [35]. In addition, the promoter regions of many cytokines such as TNF-α and IL-8 are controlled by NF-κB. NF-κB activation amplifies an early inflammatory response and exacerbates tissue injury [35]. Previous studies demonstrated that inhibition of NF-κB activity decreased lung reperfusion injury in porcine and rabbit models [36, 37]. The inhibition was associated with the suppression of cytokine production and neutrophil infiltration [37]. PFCs have also been shown to inhibit NF-κB activation in LPS-stimulated macrophages, Chlamydophila pneumoniae-mediated pneumocytes, and models of respiratory syncytial virus-induced lung inflammation [15, 34, 38]. Therefore, our results are compatible with those studies.

Although the anti-inflammatory mechanisms of PFCs are not clear, most investigators initially considered that PFCs acted as a physical barrier on cell surfaces to prevent ligand–receptor-induced signal transduction and direct injury caused by inflammatory mediators [2–4]. Further investigations revealed that PFC particles were ingested early into phagocytic cells in a time-dependent manner, thus resulting in the formation of PFC-filled vacuoles [39].
These cellular inclusions may interfere with intracellular signaling pathways and prevent the activation of inflammatory cascades. Similarly, Fernandez et al. demonstrated that PFCs interfered with transmembrane signal transduction by decreasing Syk phosphorylation in neutrophils. PFCs were also reportedly incorporated into the cellular
membranes of alveolar epithelial cells and erythrocytes; this stabilized the cellular membrane and prevented surface receptor activation [34, 40–42]. However, other important inflammatory pathways may be involved in the protective mechanisms of PFCs, and further investigations are required to clarify these pathways.

Under ischemic conditions, oxidative metabolism is impaired and ATP stores in the lung are rapidly depleted. The high oxygen solubility of PFCs provides a higher partial pressure of oxygen in the microcirculation, thereby enhancing the oxygen flux into tissues. It has been reported that PFCs are effective for preventing ischemic heart and brain injuries by enhancing ischemic myocardial and cerebral oxygen concentration [43, 44]. However, it is not clear if PFCs could oxygenate the ischemic rat lungs in this study. Additional studies are warranted to clarify this.

This study had several limitations. First, FC-77 was chosen because it is widely available. However, different PFCs have various pharmacological properties, and new-generation PFCs may be more effective in attenuating lung inflammation. Second, our observation period was 1 h; therefore, the long-term effects of PFCs on IR remained unknown. Studies with a longer experimental time will be necessary before extrapolating our results to the clinic. Finally, although isolated lung models are widely used to investigate various physiological phenomena, the influence of extrapulmonary organs was not tested. Additional studies in intact whole animals to explore these effects are necessary.

In summary, we demonstrated that rats treated with FC-77 prior to the induction of ischemia had attenuated IR-induced lung injury. The observed decrease in lung damage may have been mediated by the inhibition of NF-κB activity, inflammatory reactions, and neutrophil infiltration into the lung tissues. Systemic PFC administration is a new therapeutic approach. Our results provide evidence for the potential of prophylactic therapy with systemic PFCs to prevent reperfusion injury in lung transplantation.

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