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The effects of FK409 on pulmonary ischemia-reperfusion injury in dogs

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Abstract FK409 is the first spontaneous nitric oxide (NO) donor known to increase plasma cyclic guanosine 3',5'monophosphate levels. In this study, we evaluated the effect of FK409 on pulmonary ischemia-reperfusion injury in an *in situ* warm ischemia canine model. Fourteen dogs were divided into two groups, and the FK409-treated group was given 5 μg/kg per min FK409. Warm ischemia was induced for 3 h. The arterial partial pressure of oxygen (PaO_2), arterial oxygen saturation (SaO_2), cardiac output (CO), left pulmonary vascular resistance (L-PVR), and endothelin-I (ET-I) were measured. A histologic study was performed, and polymorphonuclear neutrophils (PMNs) were also counted. The PaO_2, SaO_2, and L-PVR levels and PMNs after 30 min of reperfusion, ET-I after 2 h of reperfusion, and the 7-day survival rate were significantly ($P < 0.05$) better in the FK409-treated group than in the control group. The histologic damage was reduced in the FK409-treated group compared to the control group. FK409 appears to have a protective effect in ischemia-reperfusion injury of the lung.

Key words Pulmonary ischemia-reperfusion injury · FK409 · Nitric oxide · Endothelin-I

Introduction

Lung transplantation is an acceptable therapeutic approach for patients with end-stage lung disease, and the short-term survival rates have improved recently. Ischemia-reperfusion injury results in the deterioration of pulmonary function after lung transplantation. The ability to preserve pulmonary grafts in clinical lung transplantation lags behind that of other solid organs, such as the kidney, liver, and heart. The acceptable ischemic time for lung grafts remains between 4 and 7 h [4, 14], but inhibiting ischemia-reperfusion injury may prolong this time. FK409 (±)-(E)-ethyl-2-((E)-hydroxyamino)-5-nitro-3-hexenemide (Fujisawa Pharmaceutical Co., Osaka, Japan) is a spontaneous nitric oxide (NO) releaser, which has potent vasorelaxant and antiplatelet effects [16]. FK409 is the first NO donor known to increase plasma cyclic guanosine 3',5'monophosphate (cyclic GMP) levels [16]. FK409 is reported to prevent myocardial infarction following occlusion and reperfusion of rat coronary arteries [16]. The NO donor may also improve early lung function and increase the supply of available lung grafts by extending the safe preservation time. In this study, the effect of FK409 on pulmonary ischemia-reperfusion injury was investigated in an *in situ* warm ischemia model of the canine lung.

Materials and methods

Animals and operative procedures

A total of 14 adult mongrel dogs, weighing 8–13 kg, were randomly allocated into two groups. The control group consisted of 7 dogs, and the remaining 7 dogs made up the FK409-treated group, which received FK409 (5 μg/kg per min) continuously for 30 min prior to ischemia and for 60 min beginning 15 min prior to the onset of reperfusion.

After administering ketamine hydrochloride (2 mg/kg i.m.), the animals were anesthetized with pentobarbital sodium (15 mg/kg) and pancuronium bromide (0.1 mg/kg), intubated, and connected to a ventilator (MD800TM, Senko Med. Co., Tokyo, Japan) at a tidal volume of 20 ml/kg and a rate of 15 breaths/min. An inspired oxygen fraction of 0.3 was maintained. Positive end-expiratory pressure was maintained at 5.0 cm H_2O. After 2 h of reperfusion, the animals were ventilated with room air. Muscular relaxation was obtained with additional pancuronium bromide (0.1 mg/kg). An arterial line was inserted into the right carotid artery to monitor blood pressure and blood gases. The right jugular vein was used as a venous infusion line. A thoracotomy was performed in the left fourth intercostal space. Hilar stripping of the left lung was performed; the nerves, bronchial arteries, and lymphatic system were transected completely. Warm ischemia was induced for 3 h by clamping the left pulmonary artery and veins. The left mainstem bronchus was bisected and reanastomosed 3 h later.

All of the animals were cared for in compliance with the Principles of Laboratory Animal Care formulated by the National Society for Medical Research and the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health [NIH publication No. 86–23, revised 1985].

Hemodynamic and blood gas measurements

Blood samples were taken prior to warm ischemia and before, immediately after, and 30 min after reperfusion. At these times, except before reperfusion, both lungs were ventilated and perfused. The arterial partial pressure of oxygen (PaO_2) and arterial oxygen saturation (SaO_2) were analyzed (ABL 520 Blood Gas System, Radiometer Co., Copenhagen, Denmark). A 5-min clamping test of the right pulmonary artery was performed prior to ischemia and 30 min after reperfusion. The left pulmonary artery pressure (L-PAP) was measured simultaneously by inserting a 24-G needle into the main pulmonary artery and connecting it to a transducer (Spectramed TA 1017, Sanei Co., Tokyo, Japan). Cardiac output (CO) was measured by placing an electromagnetic blood flow meter (MF V-3100, Nihonkohden Co., Tokyo, Japan) on the ascending aorta. The left pulmonary vascular resistance (L-PVR) was calculated according to the following formula:

$$\text{L-PVR (dyne} \times \text{s} \times \text{cm}^{-5}) = \text{mean L-PAP (mmHg)} \times 79.92/\text{CO (l/min)}.$$

Endothelin-I measurements

The endothelin-I (ET-I) levels in arterial blood were measured 2 h after reperfusion. Blood samples for ET-I measurement were drawn into ice-chilled tubes containing K_2 EDTA and trasylol, and the plasma was immediately separated by centrifugation at 4 °C and stored at –80 °C until the assay. Plasma samples were extracted in octylsilane-silica cartridges with 2 ml 60% acetonitrile and 0.09% trifluoroacetic acid, and evaporated in a centrifugal concentrator. The dried residue was reconstituted in the assay buffer and subjected to radioimmunoassay. Incubation of 0.1 ml assay buffer and 0.1 ml anti-endothelin-I serum at a final dilution of 1:300000 was undertaken at 4 °C for 20 h. Then 0.05 ml of 83 pmol/ml ^{125}I-endothelin-I with a specific activity of 74TBq/mmol (Amersham International, Buckinghamshire, UK) was added and incubated at 4 °C for 48 h. The bound and the free ligands were separated by the double-antibody/polyethylene glycol method [2].

Histological studies

Lung specimens were harvested for histological examination 30 min after reperfusion, at the time of death, or on the 7th postoperative day, and the specimens were fixed in 10% formalin. The tissues were dehydrated, embedded in paraffin, sectioned into 3- to 5-μm specimens, and mounted. After the tissues were removed from the paraffin, they were stained with hematoxylin and eosin and naphthol AS-D chloroacetate esterase to count polymorphonuclear neutrophils (PMNs). PMNs were counted using a light microscope at × 400 magnification in ten fields per specimen, and the number was totaled. The data were expressed as PMNs/alveolus.

Statistical analyses

The results are expressed as the mean ± the standard error of the mean. Statistical significance between the FK409-treated and control groups was determined using the Mann-Whitney U-test and the Log-rank (Cox-Mantel) test. A Friedman two-way analysis of variance was performed on PaO_2 and SaO_2 within each group. If the Friedman two-way analysis of variance revealed a significant interaction, the statistical significance between measurement at each time was determined using Sheffe's test. A P value of less than 0.05 was considered to be statistically significant.

Results

Prior to ischemia, there were no significant differences in mean arterial pressure, L-PVR, CO, PaO_2 or SaO_2 between the FK409-treated and control groups. Just before and after, and 30 min after reperfusion, PaO_2 was significantly ($P < 0.05$) higher in the FK409-treated group (167.8 ± 12.7, 161.6 ± 11.3, and 121.7 ± 12.3 mmHg, respectively) than in the control group (127.9 ± 15.6, 98.7 ± 16.2, and 68.5 ± 8.8 mmHg, respectively) (Fig. 1). However, there was no significant difference between before ischemia and any time after reperfusion within the FK409-treated and control groups. Immediately and 30 min after reperfusion, SaO_2 was significantly ($P < 0.05$) higher in the FK409-treated group (99.9 ± 0.1 and 98.5 ± 1.0%, respectively) than in the control group (94.5 ± 3.5 and 75.1 ± 6.5%, respectively). There was no significant difference between before ischemia and any time after reperfusion in the FK409-treated group. In the control group, at 30 min after reperfusion, SaO_2 (75.1 ± 6.5%) was significantly lower than before ischemia (99.9 ± 0.1%). After 30 min of reperfusion in both groups, CO decreased in comparison with the preischemic condition. CO in the FK409-treated and control groups was 0.62 ± 0.05 and 0.50 ± 0.06 l/min, respectively. CO was almost identical in both groups after 30 min of reperfusion. After 30 min of reperfusion, L-PVR values were significantly ($P < 0.05$) higher in the control group (9027 ± 1810 dyne × s × cm^{-5}) than in the FK409-treated group (4514 ± 866 dyne × s × cm^{-5}) (Fig. 2). After 2 h of reperfusion, ET-I

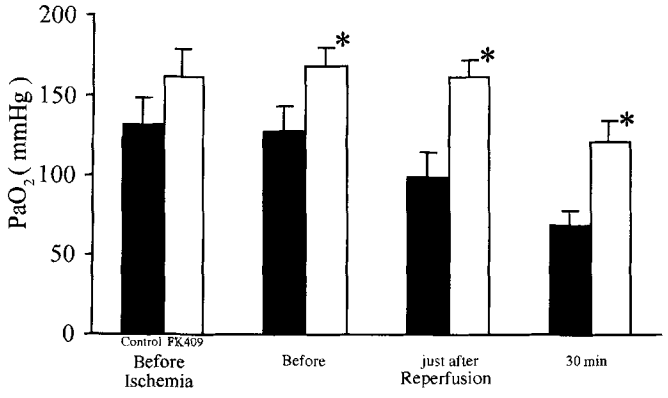

Fig. 1 Arterial partial pressure of oxygen (PaO_2). PaO_2 levels just before, just after, and 30 min after reperfusion were higher in the FK409-treated group than in the control group. However, there was no significant difference between before ischemia and any time after reperfusion within the FK409-treated and control groups. (At these times, except before reperfusion, both lungs were ventilated and perfused). *: $P < 0.05$ vs control group

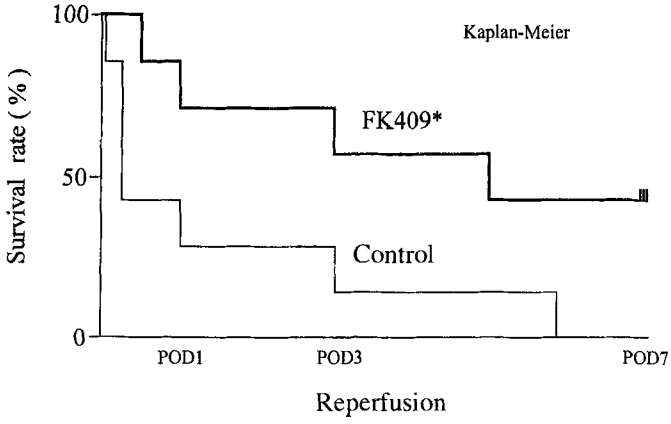

Fig. 3 Survival curve. The 3-day and 7-day survival rates were 71 and 43%, respectively, in the FK409-treated group, and 28 and 0%, respectively, in the control group. There was a significant difference between the survival rates of the two groups. *: $P < 0.05$ vs control group (*POD* postoperative day)

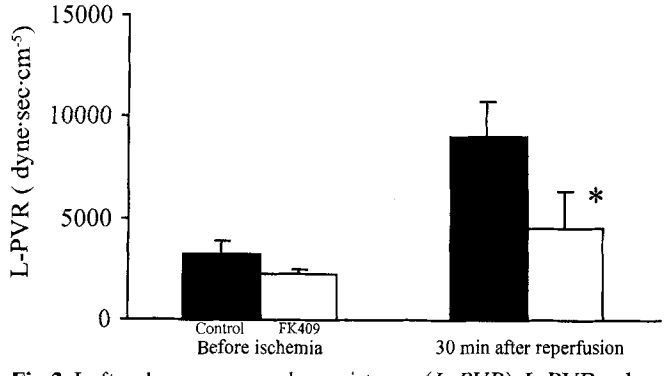

Fig. 2 Left pulmonary vascular resistance (*L-PVR*). L-PVR values after 30 min of reperfusion were higher in the control group than in the FK409-treated group. *: $P < 0.05$ vs control group

levels were significantly ($P < 0.05$) higher in the control group (22.0 ± 1.5 pg/ml) than in the FK409-treated group (16.9 ± 1.0 pg/ml).

Two of the seven dogs in the FK409-treated group died within 24 h, five (71%) survived for 3 days, and three (43%) survived for 7 days. In the control group, five (71%) of the seven dogs died within 24 h and two (28%) survived for 3 days. None of the control dogs survived for 7 days. There were statistically significant differences ($P < 0.05$) between the survival rates of the two groups (Fig. 3). The dogs in the control group died as a result of pulmonary edema after discharging copious amounts of foamy bloody sputum from the tracheal tube.

Histologically, after 30 min of reperfusion, alveolar damage with interstitial edema and focal hyaline membrane formation was observed in the control group (Fig. 4a), whereas only slight interstitial edema was observed in the FK409-treated group (Fig. 4b). PMN infiltration was also significantly higher in the control group (1.30 ± 0.38/alveolus) than in the FK409-treated group (0.67 ± 0.13/alveolus). Lung specimens taken from the control group at the time of death showed alveolar damage with interstitial edema, neutrophil and leukocyte infiltration, and hyaline membranes localized along the predominant alveolar ducts and collapsed alveoli. In contrast, after 7 days of reperfusion, only slight interstitial edema was observed in specimens from the FK409-treated dogs.

Discussion

We studied the effects of FK409 on ischemia-reperfusion injury of the lung using an *in situ* warm lung ischemia canine model. The biochemical, functional, and morphological ischemia-reperfusion injury after pulmonary warm ischemia is reported to be very similar to the ischemia-reperfusion injury after lung transplantation following cold ischemia [28]. Halldorsson et al. reported that controlled reperfusion using both a modified reperfusate and a white blood cell filter limits the reperfusion injury after 2 h of warm pulmonary ischemia [8], and that the same method limits the pulmonary reperfusion injury after 24 h of pulmonary cold ischemia [9]. According to these reports, we concluded that an *in situ* warm lung ischemia canine model using a simpler experimental procedure is more stable than a transplantation model and suitable for investigating the efficacy of FK409.

After reperfusion, the SaO_2, PaO_2, ET-I and L-PVR levels were significantly better in the FK409-treated

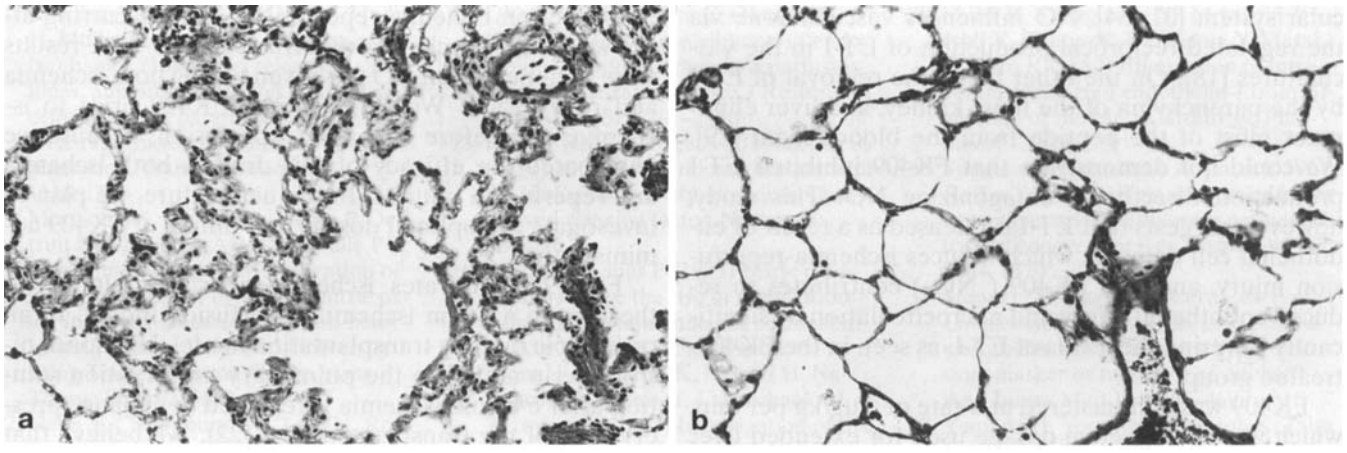

Fig. 4 a, b Histologic findings after 30 min of reperfusion. Focal hyaline membrane formation and interstitial edema were observed in the control group (**a**), while only mild interstitial edema was seen in the FK409-treated group (**b**)

group than in the control group. In the histologic study, there was a greater reduction of pulmonary injury after 30 min of reperfusion in the FK409-treated group than in the control group. Furthermore, PMN infiltration was significantly lower in the FK409-treated group than in the control group. The 7-day survival rate was 43% in the FK409-treated group and 0% in the control group. The difference between the two groups was statistically significant ($P < 0.05$). Therefore, we concluded that this drug provides organic protection against pulmonary ischemia-reperfusion injury.

Cytokine-activated complement, arachidonic acid metabolite, superoxide, and NO are all related to ischemia-reperfusion injury [13, 20]. The effect of NO on ischemia-reperfusion injury has been widely interpreted. NO may attenuate tissue injury by maintaining circulation as an endothelium-derived relaxing factor [25], directly scavenging superoxide [11], attenuating leukocyte adhesion [19], and inhibiting platelet aggregation [27]. NO inactivates phagocyte nicotinamide adenine dinucleotide phosphate oxidase and xanthine oxidase [3, 7], both of which are key sources of superoxide in ischemia-reperfusion injury [15]. NO protects organs by improving the organ blood flow in ischemia-reperfusion injury [17, 26]. There are some reports that exogenous inhaled NO can mediate vasodilation and improve pulmonary function after ischemia-reperfusion and lung transplantation [5, 6, 21]. This protective effect is due to the prevention of ischemia-reperfusion-injury-induced pulmonary vasoconstriction – resulting from the inhibition of neutrophil sequestration in the reperfused lung [5, 21] – and the suppression of oxygen free radicals [6], and is altered by differences in the timing and dosage of NO inhaled during ischemia-reperfusion [5, 21].

FK409 is a semisynthetic fermentation product of *Streptomyces griseosporeus* with vasodilating activity and nitric oxide-donating ability [10]. FK409 produces vasorelaxation via an increase in intracellular cyclic GMP [32] and inhibits platelet aggregation [10].

Some researchers reported seeing increased vascular permeability, alveolar damage, alveolar collapse, and interstitial edema in ischemia-reperfusion injury of the lung. This produces circulatory collapse, reduced oxygen-exchange capability, and increased pulmonary vascular resistance [12, 30]. The histologic findings in animals after reperfusion reveal a marked collapse of capillaries, intra-alveolar hemorrhage, and interstitial edema [23]. In this study, the histologic findings after 30 min of reperfusion in the control group revealed alveolar damage, interstitial edema, and hyaline membranes localized along the alveolar ducts. L-PVR levels were double the preischemic levels. After 30 min of reperfusion, L-PVR levels in the FK409-treated group were significantly lower than those in the control group. In addition, the histologic damage was not as severe in the FK409-treated group. These results suggest that FK409 may play an important role in stabilizing the lung microcirculation.

PMNs play a pivotal role in ischemia-reperfusion injury by becoming activated, adhering to the endothelium, and releasing reactive oxygen species into the surrounding tissue [21]. Since the PMN-endothelium interaction is the central mechanism in ischemia-reperfusion-induced lung endothelial injury, we assessed the sequestration of PMNs in the lung. PMN infiltration was significantly lower in the FK409-treated group than in the control group. FK409 prevents the pulmonary sequestration of PMNs in ischemia-reperfusion injury of the lung.

ET-I, a 21-amino acid peptide derived from vascular endothelial cells, produces prolonged constriction of mammalian blood vessels [33]. ET-I is a potent vasoconstrictive mediator, which is released during hypoxia [18] and contributes to ischemia-reperfusion injury [24]. ET-I is also generated in physiological disorders of the vas-

cular system [31, 34]. NO influences vascular tone via the regulated reciprocal production of ET-I in the vasculatures [18]. On the other hand, the removal of ET-I by the parenchyma of the lung, kidney, and liver eliminates most of the peptide from the bloodstream [29]. We could not demonstrate that FK409 inhibited ET-I production directly by antagonizing NO. This study, however, suggests that ET-I is released as a result of endothelial cell damage, which induces ischemia-reperfusion injury, and that FK409 (NO) contributes to reduced endothelial injury and microcirculation by significantly lowering the levels of ET-I, as seen in the FK409-treated group.

FK409 was administered at a rate of 5 μg/kg per min, which was the effective dosage used for extended liver resection in a canine ischemia model [1]. FK409 was administered for 30 min prior to ischemia and for 60 min beginning 15 min prior to the onset of reperfusion. Murakami et al. [21] reported that inhaled NO, used either during ischemia, reperfusion, or both, has a beneficial effect on ischemia-reperfusion injury occurring after warm lung ischemia-reperfusion. The best results were obtained when NO was given during both ischemia and reperfusion. We administered FK409 prior to ischemia, and before and after reperfusion, because we anticipated the efficacy of this drug in both ischemia and reperfusion injuries. In the near future, we plan to investigate the optimal dosage and timing of FK409 administration.

FK409 ameliorates ischemia-reperfusion injury of the lung in a warm ischemia-reperfusion model. In an orthotopic rat lung transplantation model, NO donor nitroglycerin added to the pulmonary preservation solution after 6 h cold ischemia is reported to enhance preservation of the transplanted lung [22]. We believe that FK409 may have clinical applications in lung transplantation.

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