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Leumedin（NPC-15669）Ameliorates Ischemia-Reperfusion Injury in Rat Lung Transplantation

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Abstract：Background：Neutrophil-mediated reperfusion injury has been demonstrated to contribute to early graft dysfunction after lung transplantation. NPC-15669, a member of a new class of anti-inflammatory compounds termed "leumedins", is known to block inflammation in several animal models. This agent specifically prevents the recruitment of neutrophils to inflammatory sites and inhibits subsequent tissue damage by inhibiting the cellular response to cytokine activation. As a result, the upregulation of CD11b/CD18（Mac-1）on the neutrophils is inhibited. In the present experiment, we studied the effect of NPC-15669 on reperfusion injury in an orthotopic left lung transplant model in syngeneic Fischer rats.

Methods：Fifteen rats were divided into three groups. Group Ⅰ（n＝5）rats underwent immediate graft implantation. Donor lungs for group Ⅱ（n＝5）and group Ⅲ（n＝5）underwent 18 hours of hypothermic ischemia（1℃）before implantation. All donor lungs were flushed with 20 ml of low-potassium dextran-1% glucose（LPDG）solution and stored at 1℃ until implantation. In group Ⅲ, 1 mg NPC-15669 was added to the last 1 ml of flush. In addition, the rats in group Ⅲ received NPC-15669（10 mg/kg）intravenously（i.v.）prior to reperfusion. Groups Ⅰ and Ⅱ received an equivalent volume of normal saline i.v. Immediately prior to sacrifice at 24 hours after reperfusion, the function of the isolated graft was determined by an arterial blood gas（ABG）analysis. The neutrophil CD11b expression was determined by flow cytometry while isograft neutrophil sequestration was measured by a myeloperoxidase（MPO）assay.

Results：NPC-15669 significantly improved the PaO_2 levels compared with group Ⅱ（339.9±60.1 versus 52.5±3.0 mmHg, $p<0.001$）. The CD11b expression in all groups increased in comparison to normal rats. The up-regulation of the CD11b expression in the NPC-15669 treated recipients decreased in comparison to the 18-hour storage control group（39.1±1.9 versus 53.0±4.5 mean fluorescence intensity, $p<0.01$）. The graft myeloperoxidase activity significantly decreased in group Ⅲ（group Ⅱ versus group Ⅲ, 0.291±0.033 versus 0.172 ± 0.041 ΔOD/mg/min, $p<0.05$）.

Conclusion：These results suggest that NPC-15669 reduced the degree of neutrophil mediated reperfusion injury and improved the post-transplant lung graft function.

Key words：Ischemic reperfusion injury, NPC-15669, Rat lung transplantation, Mac-1

Introduction

The mechanisms of ischemia－reperfusion injury of vascularized organs have been intensely investigated. The mechanism of reperfusion injury involves a variety of factors such as neutrophils,[1)2)] platelets,[3)] oxygen free radicals,[4)5)] and cytokines.[6)] Neutrophils have been identified as mediators of post-transplant reperfusion injury.[7)] Re-

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cent data have demonstrated that neutrophils may be the primary mediators of the actual tissue injury.[8)9)] Neutrophil binding to the vascular endothelium is an essential stage for both the activation and production of toxic oxygen free radicals.[10)11)] A number of reports have demonstrated that the adherence and subsequent migration of neutrophils through the vascular endothelium are dependent on interactions between leukocytes, glycoproteins and their ligands.[12)–15)] The integrin CD11b plays a major role in neutrophil adherence.[14)16)] The interaction of the neutrophil CD11b with the endothelial intercellular adhesion molecule (ICAM–1) results in firm adhesion, a necessary step for subsequent neutrophil migration.[8)]

NPC–15669, a member of a new class of anti－inflammatory compounds termed leumedins, specifically prevents the recruitment of neutrophils to inflammatory sites and subsequent tissue damage by the inhibition of the cellular response to inflammatoy mediators.[8)17)18)] In consequence, this agent is effective in inhibiting the upregulation of CD11b/CD18 on the neutrophil without affecting the endothelial ligand.

The purpose of this study was to determine whether the inhibition of neutrophil adhesion by NPC–15669 results in a reduction of post–ischemic reperfusion injury in preserved syngeneic rat lung grafts.

Materials and Methods

Animals：Pathogen–free inbred male F344 rats (270 to 300 gr) (Harlan Sprague Dawley, Inc. Indianapolis, IN) were used as both donors and recipients. All animals received human care 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" prepared by the National Academy of Sciences and published by the National Institutes of Health (NIH Publication No. 86–23, revised 1985).

Experimental Groups：An orthotopic rat left lung transplantation was performed with a modification of the "cuff" technique originally described by Mizuta et al.[19)] Fifteen pairs of rats were divided into three groups. In group I (n＝5), the rats underwent immediate graft implantation. The donor lungs for group Ⅱ (n＝5) and group Ⅲ (n＝5) were stored for 18 hours at 1℃ before implantation. Donor lungs were flushed with 20 ml of cold (4℃) low－potassium dextran－1% glucose (LPDG) solution and stored in the same solution until implantation. The donor lungs in group Ⅲ received the same flush solution with NPC–15669 (Scios Nova, Mountain View, CA) (1.0 mg) added. In addition, the recipients in group Ⅲ received NPC –15669 (10 mg/kg) intravenously into the penile vein immediately prior to reperfusion. Rats in group I and Ⅱ received an equivalent amount of normal saline i.v. before reperfusion.

Donor Procedure：Donor animals were anesthetized with intraperitoneal pentobarbital (20 mg/kg), and intubated with a polyethylene catheter. Mechanical ventilation was established with room air using a Harvard rodent ventilator (Harvard Apparatus, South Natick, MA) (tidal volume 10 mL/kg, respiratory rate 60/min, positive end–expiratory pressure 1.0 cm H_2O). A median sternotomy was performed and the donors were given intravenous heparin (1,000 units/kg). After ligating the inferior vena cava, the donor lungs were flushed via the pulmonary artery with cold (4℃) LPDG solution (20 ml) at 20 cm H_2O gravity pressure. As noted above, the lungs in group Ⅲ received the same volume of LPDG solution containing NPC–15669 (1 mg). The heart–lung block was removed with the lungs inflated at end tidal volume. A cuff made from a 14–gauge grooved polyethylene catheter was placed on each vascular stump.

Recipient Procedure：The recipient animals were anesthetized with intramuscular ketamine (25 mg/kg) and atropine (0.25 mg/kg), intubated and ventilated with 99.5% oxygen and 0.5% halothane. A left thoracotomy was performed in the fifth intercostal space. The pulmonary artery and vein were isolated, crossclamped and anastomosed with a cuff technique. The left main bronchus was anastomosed with a running suture (8–0 Prolene, Ethicon, Somerville, NJ) for the cartilaginous portion and interrupted suture (9–0 Prolene) for the membranous portion. During implantation, the donor lung was kept inflated and cooled by a continuous application of cold (4℃) normal

saline. Following a restoration of ventilation and perfusion to the graft, the thorax was closed. A single chest tube was placed for negative pressure drainage and removed when the rat recovered from the anesthesia.

Assessment: The animals were re-anesthetized 24 hours after reperfusion using the donor technique noted above. The anesthetized animals were mechanically ventilated with 100% oxygen. Median sternotomy was performed and the right (contralateral) hilum clamped for five minutes to assess function of the left lung isograft (tidal volume 1.5 ml, respiratory rate 100/min). Blood samples were obtained from the abdominal aorta for an arterial blood gas analysis, white blood cell count, and a differential analysis as well as a flow cytometric analysis. Animals were then sacrificed by a pentobarbital overdose while completely anesthetized. Following sacrifice, the lung grafts were flushed with cold normal saline, removed from the thoracic cage, and snap frozen in liquid nitrogen for the myeloperoxidase assay.

Histology: Additional transplants were performed in each group (n= 5) for histologic examinations to prevent artifacts, such as structural damage by mechanical ventilation during the arterial blood gas analysis.

Immunofluorescence Analysis by Flow-Cytometry: Blood was withdrawn from an indwelling arterial catheter (22-gauge, Terumo Medical Corp., Elkton, MD) into an EDTA-containing tube and immediately placed on ice. Monoclonal antibodies were purchased from Harlan Bioproducts Science, Inc. (Indianapolis, IN) as follows: FITC conjugate of mouse anti-rat CD11 antibody, isotype control mouse IgG2a-FITC, and Erythrolyse red blood cell lysing buffer. Phosphate-buffered saline (PBS) was purchased from Sigma Chemical Company (St. Louis, MO). Binding-buffer (90 μL, pH 7.4)consisting of PBS, 0.2% bovine serum albumin, and 0.1% NaN, were admixed with pretitered 10 μL of anti-CD11b-FITC antibody and anti-IgG2a-FITC, respectively. These pre-diluted antibodies were incubated with 200 μL of whole blood for 30 minutes on ice in the dark. After two washes in ice-cold PBS, the erythrocytes were lysed using the lysing buffer for 10 minutes, followed by two additional washes in PBS. The leukocytes were collected by centrifugation at 4 ℃, and fixed in ice-cold PBS containing 4 % paraformaldehyde.

The flow-cytometric analysis was performed with a FACScan flow cytometer (Becton Dickinson Facscan, San Jose, CA) using the FACScan software for data acquisition (Consort 30 and Cellquest, Becton Dickinson, San Jose, CA) gated by a forward angle scatter to exclude any dead cells. After suitable instrument setting and spectral correction carried out during initial pilot experiments, the settings were not changed throughout the experiment. Fluorescence from 10^4 cells was analyzed on a logarithmic scale and expressed as the change in the mean fluorescence intensity (MFI). As a preliminary investigation, the changes in the peripheral neutrophil CD11b expression after reperfusion in control animals receiving 18 hours preserved lung grafts were measured in two-hour intervals during a 24-hour reperfusion period in order to investigate optimal timing of the measurement of CD11b expression. The baseline neutrophil CD11b expression was assessed in the blood specimens obtained from normal F344 rats (n=5).

Lung Tissue Myeloperoxidase: The lung samples were immediately frozen in liquid nitrogen and subsequently stored at −80 ℃. Quantitative MPO assays were performed as described previously.[20]

Statistical Analysis: Values are given as the mean±standard error of the mean (SEM). Comparisons among groups were made by the one-way analysis of variance (ANOVA) followed by Bonferroni's test for multiple comparisons. Differences were considered to be significant if the p-value was less than 0.05.

Results

The total ischemic time was 84.2±0.6, 1081.4±2.0 and 1083±1.4 minutes in groups I, Ⅱ and Ⅲ, respectively. Warm ischemic time, the period from the start of implantation to reperfusion, was 22.8±0.4, 23.0±0.4 and 23.8±0.4 minutes, respectively. There was no significant difference among the groups regarding the number of peripheral white blood cells and the absolute neutrophil count (Table 1).

Table 1. Characteristics of the Experimental Groups

Group	Total Ischemic Time (min)	Warm Ischemic Time (min)	PMN ($1,000/mm^3$)	ANC ($/mm^3$)
I (n=5)	84.2±0.6	22.8±0.4	1.98±0.34	1,268±280
II (n=5)	1,081.4±2.0	23.0±0.4	1.80±0.19	818±126
III (n=5)	1,083.3±1.4	23.8±0.4	2.02±0.27	1,134±192

PMN, Whole blood polymorphonuclear count ; ANC, Whole blood absolute neutrophil count.
Tabulated data indicate the mean and standard error of the mean

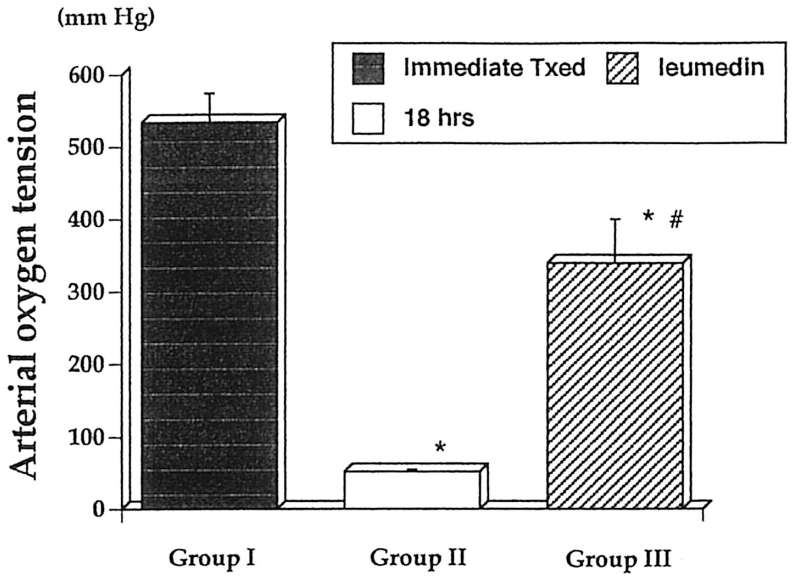

Figure 1. Arterial oxygen tension (PaO_2) for group I (n=5, immediate lung transplantation), group II (n=5, 18-hour stored control), and group III (n=5, NPC-15669-treated animals). A significant difference in PaO_2 was observed between group I and either group II or group III ($p<0.001$). The NPC-15669-treated group indicates a significant amelioration of arterial oxygenation in comparison to group II ($p<0.01$). *$p<0.01$, group I versus groups II and III ; #$p<0.01$, group II versus group III.

Arterial Blood Gas Analysis

The mean arterial oxygenation 24 hours after reperfusion in NPC-15669 treated recipients (group III)was superior to that in group II (339.9±60.1 versus 52.5±3.0 mmHg, $p<0.001$), but did not reach the level of the immediately transplanted lungs (group I) (534.7±39.9 mmHg, $p<0.01$) (Figure 1).

Flow-Cytometric Analysis

All samples labeled with the anti-CD11b-FITC antibody or anti-IgG2a-FITC antibody demonstrated one-peak histograms on the logarithmic scale. An assessment of neutrophil CD11b expression in two-hour intervals during a 24-hour post reperfusion period demonstrated a marked increase 14 hours after reperfusion (53.8 MFI) and remained at this level until the last assessment 24 hours after reperfusion (51.0 MFI). There was no significant difference in the MFI of the negative control among the groups (unpublished data). The baseline expression of peripheral neutrophil CD11b in the normal rat was 22.1±2.6 MFI. The neutrophil CD11b expression in all groups increased in comparison to the baseline levels in normal rats. There was no significant difference between the normal rats and group I. However, the neutrophil CD11b expression in the NPC-15669 treated recipients (group III) was significantly decreased in comparison to the 18-hour control group (group II) (39.1±1.5 versus 53.0±4.5 MFI, $p<0.01$)

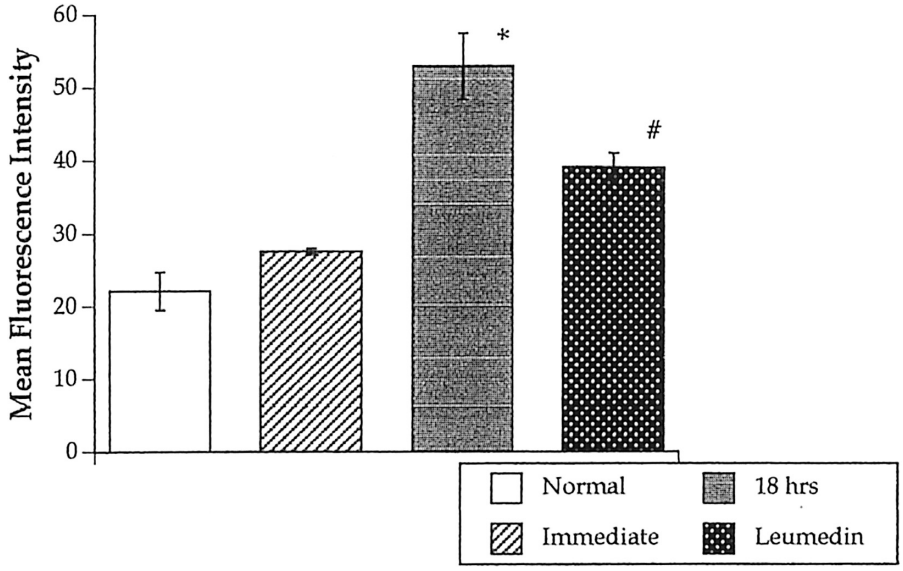

Figure 2. Neutrophil CD11b expression according to a flow cytometric analysis. Normal means the blood samples obtained from normal F344 rats (n=5) as a baseline expression of neutrophil CD11b. All the samples in groups I, II, and III indicate a higher fluorescence expression than that seen in normal rats. A significant difference was observed between group I and either groups II or III ($p<0.05$). The mean fluorescence intensity in the NPC 15669-treated group significantly decreased in comparison to the 18-hour stored control group ($p<0.01$). *$p<0.05$, group I versus group II and III; #$p<0.01$, group II versus group III.

(Figure 2).

Myeloperoxidase Assay

The MPO activity in the flushed rat lung tissue was not detectable, thus indicating no neutrophil sequestration in these lungs. The graft MPO activity in group I was 0.069 ± 0.023 ΔOD/mg/min. In groups II and III, the MPO activity significantly increased in comparison to group I. The Group III lung grafts demonstrated significantly less MPO activity than group II (0.172 ± 0.041 versus 0.291 ± 0.033 ΔOD/mg/min, $p<0.05$) (Figure 3).

Histology

Grafts treated with NPC-15669 demonstrated neutrophil and mononuclear cell infiltrates which were confined to the perivascular portion. There was no evidence of either any severe edema or hemorrhaging. However, the immediately transplanted group demonstrated less injury in comparison to the NPC-15669 treated group. In the 18-hour preserved grafts of group II (control), hemorrhaging and marked edema were seen along with neutrophil migration into the alveolar spaces.

Discussion

Lung transplantation has become an accepted treatment for end-stage pulmonary disease.[21] In spite of the significant progress in the methods of preservation and operative technique, reperfusion injury remains a major and unpredictable clinical problem. In an effort to reduce ischemia-reperfusion injury, many experiments have been performed in association with free radical scavengers,[22] leukocyte depletion,[1)2)] and cytokine antagonists.[6] The pathogenesis of post-transplant lung reperfusion injury is not fully understood despite extensive clinical and laboratory investigations.

Neutrophil-mediated reperfusion injury has been demonstrated to play an important role in the response to lung allograft ischemia reperfusion injury.[1)2)] NPC-15669, a member of a new class of anti-inflammatory compounds termed "leumedins", inhibits neutrophil recruitment to inflammatory

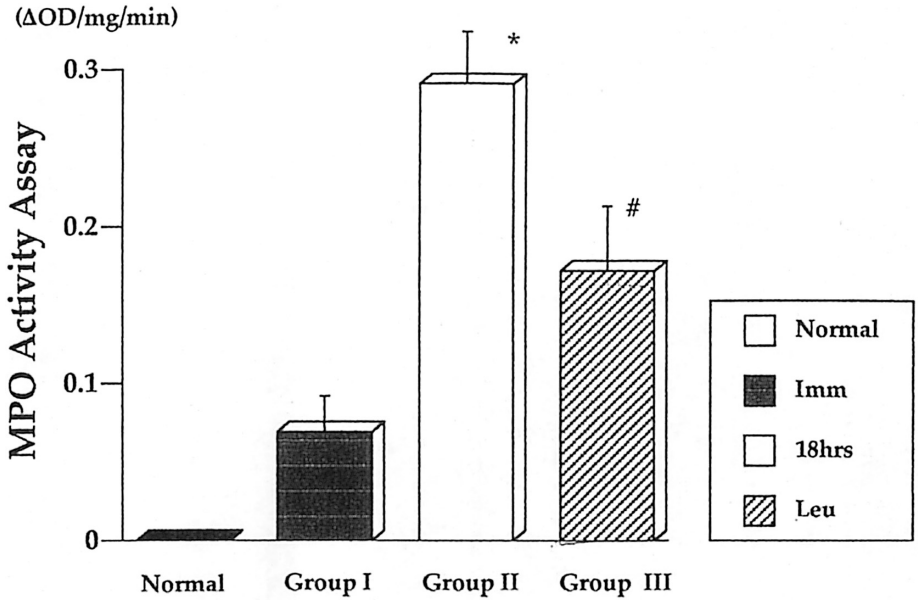

Figure 3. Myeloperoxidase (MPO) activity assay of lung tissue. No MPO activity in the normally flushed lung was detected. A significant difference between group I and group II ($p<0.001$). NPC-15669-treated grafts (group III) contained significantly less activity than the control 18-hour stored lung ($p<0.05$). However, the NPC-15669-treated group and immediately transplanted group showed no differences between the groups. $*p<0.001$, group I versus group II ; $\#p<0.05$, group II versus group III.

sites by inhibiting the functional upregulation of CD11b/CD18 adhesion receptors on stimulated neutrophils,[18] perhaps by acting on some intercellular signaling pathway.[24] The compound is active in vivo, inhibiting neutrophil mediated tissue injury in several animal models. This compound prevents plasma extravasation in the reversed Arthus reaction in a dose-dependent fashion.[25] Noronha-Blob and associates[26] also demonstrated that NPC-15669 inhibits vascular permeability in acetic acid-induced colitis by indicating the vascular integrity using the Evans blue-dye extravasation analysis in rats. It has also been used to decrease the myocardial infarct size after temporary coronary artery occlusion in a pig model.[27] In a recent study, Jornes et al. reported that intratracheal administration of NPC-15669 prevents neutrophil and eosinophil recruitment into the airway due to IL-8 induced neutrophil chemotactic activity in dogs.[28] Uthoff et al. reported that NPC-15669 ameliorated the pulmonary function after 12 and 24 hours of hypothermic preservation at 4 ℃ using isolated rabbit lungs. They used this compound at a dose of 10 mg/kg which we administered systemically to the transplanted rats in the current study. They showed that the administration of NPC-15669 strongly influenced the pulmonary artery pressure and peak airway pressure in a model of extra corporeal rabbit lung perfusion study. This compound also protects cardiac xenografts in discordant species and results in less tissue injury and a prolonged survival.[29]

In the present study, the administration of NPC-15669 significantly improved arterial oxygen tension compared with 18 hours preserved control grafts. In addition, the CD11b expression on peripheral neutrophils was reduced as determined by flow cytometry. Furthermore, lung grafts treated with NPC-15669 demonstrated significantly reduced MPO acitvity, a specific index of neutrophil sequestration, in comparison to controls. A histologic examination of the grafts indicated that, in the NPC-15669 treated group, only mild neutrophil infiltrates were present in comparison to the control grafts stored for 18 hours prior to reperfusion.

In preliminary studies we thus found a superior post-transplant graft function when NPC-15669 was used both systemically and in the flush solu-

tion than when administered systemically to the recipient or in the flush solution only. We tested NPC-15669 in two preliminary studies. In the first group (n=5), NPC15669 was administered a dose of 1.0 mg to the flush solution only. The second group was administered NPC-15669 (10 mg/kg) intravenously right before reperfusion (n=5). Nei-ther group showed a superior PaO_2 level in comparison to the current method. According to Burch, the action of leumedin caused an inhibition of several inflammatory mediators, prevented the interaction of inflammatory mediators with their receptors and inhibited the leukocyte adhesion to the endothelium.[17] They thus concluded that endothelial cells are a target for leumedins. The reason for the efficacy of NPC-15669 in the flush solution might be due to an inhibition of increased capillary permeability by NPC-15669 as demonstrated by Burch et al.,[25] and by Noronha-Blob et al.[26]

In conclusion, the administration of NPC-15669 results in a dramatic improvement of preserved lung graft gas exchange 24 hours after reperfusion. This improved function is associated wirh a reduced neutrophil CD11b expression and decreased lung graft neutrophil sequestration. This study provides further evidence that neutrophils play an important role in ischemia reperfusion injury in lung transplantation. In addition, it appears that anti-neutrophil adhesion strategies are an attractive therapeutic tool for improving the early post-transplant allograft function.

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