Experimental Models of Ischemic Lung Damage for the Study of Therapeutic Reconditioning During Ex Vivo Lung Perfusion

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INTRODUCTION

The development of normothermic ex vivo lung perfusion (EVLP) during the past decade has represented a major breakthrough to increase the pool of donor lungs suitable for clinical lung transplantation.1 EVLP allows the prolonged evaluation of the quality of the graft and may permit to extend the overall lung preservation time without any negative influence on posttransplantation outcomes.2 Additionally, EVLP may serve as a therapeutic platform for the repair of a damaged graft, a concept termed therapeutic reconditioning, which has been the matter of multiple experimental studies in various preclinical animal models as extensively reviewed recently.3

A key aspect of the preclinical evaluation of therapeutic reconditioning is the development of reproducible models of lung damage before transplantation. This study aimed to develop relevant rat models of lung damage to study EVLP therapeutic reconditioning for possible translational applications. Methods. Lungs from 31 rats were exposed to cold ischemia (CI) or warm ischemia (WI), inflated at various oxygen fractions (FiO₂), followed by 3h EVLP. Five groups were studied as follow: (1) C21 (control): 3h CI (FiO₂ 0.21); (2) C50: 3h CI (FiO₂ 0.5); (3) W21: 1h WI, followed by 2h CI (FiO₂ 0.21); (4) W50: 1h WI, followed by 2h CI (FiO₂ 0.5); and (5) W2h: 2h WI, followed by 1h CI (FiO₂ 0.21). Following 3h EVLP, we measured static pulmonary compliance (SPC), pulmonary vascular resistance, lung weight gain (edema), oxygenation capacity (differential partial pressure of oxygen), and protein carbonyls in lung tissue (oxidative stress), as well as lactate dehydrogenase (LDH, lung injury), nitrotyrosine (nitro-oxidative stress), interleukin-6 (IL-6, inflammation), and proteins (permeability edema) in bronchoalveolar lavage (BAL). Perivascular edema was quantified by histology. Results. No significant alterations were noted in C21 and C50 groups. W21 and W50 groups had reduced SPC and disclosed increased weight gain, BAL proteins, nitrotyrosine, and LDH. These changes were more severe in the W50 group, which also displayed greater oxidative stress. In contrast, both W21 and W50 showed comparable perivascular edema and BAL IL-6. In comparison with the other WI groups, W2h showed major weight gain, perivascular edema, SPC reduction, drop of differential partial pressure of oxygen, and massive increases of BAL LDH and proteins but comparable increase of IL-6 and biomarkers of oxidative stress. Conclusions. These models of lung damage of increasing severity might be helpful to evaluate new strategies for EVLP therapeutic reconditioning. A model combining 1h WI and inflation at FiO₂ of 0.5 seems best suited for this purpose by reproducing major alterations of clinical lung ischemia-reperfusion injury.

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donation after circulatory death (DCD), in which an unavoidable period of WI occurs before lung procurement. Although lungs can tolerate WI to a certain extent and good long-term outcomes have been reported after transplantation of DCD lung grafts, extended WI may result in significant damage, increasing the risk of ischemia-reperfusion injury and primary graft dysfunction (PGD). Therefore, the ex vivo therapeutic reconditioning of lungs damaged by such extended WI might represent an additional progress to further increase the pool of suitable donor lungs for clinical lung transplantation.

Therefore, the aim of the present study was to develop appropriate and reproducible rat models of ischemic lung damage that would be relevant for the study of ex vivo therapeutic reconditioning. Various conditions of lung preservation were evaluated, including CI or WI of different durations, with lung inflation at different levels of an inspired fraction of oxygen, followed by 3 h normothermic EVLP. At the end of EVLP, we measured biological markers of lung damage, oxidative and nitro-oxidative stress, and various functional variables, including the amount of lung water accumulation, static compliance, vascular resistance, and oxygenation capacity. Based on these results, we propose several models of ischemic lung damage of various severity that will be well suited for the evaluation of novel reconditioning strategies during EVLP.

**MATERIALS AND METHODS**

**Animals**

Thirty-one male adult Sprague-Dawley rats (mean weight 377 g) were used in this study in compliance with the “Guide for the Care and Use of Laboratory Animals” (National Institutes of Health Publication no. 86–23). The experimental protocol was approved by our local ethical animal committee (authorization no. 2637).

**Animal Model Preparation and Study Design**

The rats were anesthetized with intraperitoneal pentobarbital (50 mg/kg), and they were tracheotomized and mechanically ventilated (model 683 rodent ventilator, Harvard Apparatus, Holliston, MA) with a fraction of inspired oxygen (FiO₂) of 0.5, a tidal volume (V̇ₜ) of 7 mL/kg, and a respiratory rate of 7 strokes/min. Following a midline sternotomy, the animals were killed by exsanguination. Cannulas were inserted into the pulmonary artery (PA) and the left atrium (LA), as extensively described previously, and the animals were assigned to 1 of 5 experimental groups (Figure 1).

1. **C21 group** (n = 6): lungs were flushed (at an infusion pressure of 20 cm H₂O) through PA and LA cannulas with 15 mL of 4 °C Perfadex (Xvivo Perfusion, Göteborg, Sweden) and inflated at a FiO₂ of 0.21, and the heart-lung block was removed from the animal and preserved at 4 °C into Perfadex for 3 h, followed by 3 h of EVLP (see ahead).
2. **C50 group** (n = 6): the same as mentioned previously was performed but with lungs inflated at a FiO₂ of 0.5 to stimulate the production of oxygen-derived oxidants and free radicals.12
3. **W21 group** (n = 6): lungs were kept deflated in situ at room temperature for 1 h, then flushed as mentioned previously, inflated at a FiO₂ of 0.21, and preserved at 4 °C Perfadex for 2 h, followed by 3 h of EVLP.
4. **W50 group** (n = 7): the same as the W21 group was performed, except that lungs were inflated at a FiO₂ of 0.5 since the onset of 4 °C preservation. This group had 7 animals; bronchoalveolar lavage (BAL) fluid analysis and histology could not be done in 1 animal because of a technical issue.
5. **W2h group** (n = 6): the same as the W21 group mentioned previously was performed, except that the duration of WI was 2 h instead of 1 h.

**Ex Vivo Lung Perfusion**

Following cold storage, the heart-lung block was weighed (PB-602C, Mettler-Toledo, Greifensee, Switzerland) and mounted in a rat EVLP circuit (Harvard IL-2 System, Hugo Sachs Electronic, Hugstetten, Germany). Perfusion was started in flow-controlled mode, with the initial flow set at 2 mL/min and temperature set at 10 °C, using Steen solution (XVIVO Perfusion AB, Göteborg, Sweden), deoxygenated by a gas mixture of 6% O₂, 10% CO₂, and 84% N₂ through a gas exchange membrane (Hemofilter D150; MEDICA S.P.A., Italy). The perfusion flow and temperature were progressively increased during 30 min to 7 mL/min and 37.5 °C, respectively. The LA pressure was maintained throughout the experiments at 4 cm H₂O. Once normothermia was reached, perfusion was switched to a pressure-controlled mode (constant PA pressure of 15 cm H₂O), and mechanical ventilation was started using a Flexivent FX3 ventilator (SCIREQ Inc, Montréal, Canada). Settings included a V̇ₜ of 3 mL/kg for 10 min and then 6 mL/kg, a respiratory rate of 7 strokes/min, a FiO₂ of 0.21, and a positive end-expiratory pressure of 3 cm H₂O, as described previously.11 EVLP was maintained for a total of 3 h.

**Measurements**

**Physiological Measurements**

Static pulmonary compliance (SPC, in mL/cm H₂O) was determined at 180 min of EVLP as the slope of the lung pressure-volume curve generated by stepwise low-flow lung inflation up to 4 mL/kg. Pulmonary vascular resistance (PVR, in cm H₂O/mL/min) was calculated at 180 min of EVLP as follows: PVR = (mean PA pressure − LA pressure)/flow. The partial pressure of O₂ (PO₂) of the Steen solution was determined at 180 min of EVLP in the inflow and outflow cannulas, and the differential partial PO₂ (DppO₂) was calculated to assess the lung oxygenation capacity. At the end of EVLP, the weight gain of the heart-lung block was determined as an index of lung water accumulation. These measurements were obtained in 6 animals per group, except in the W50 group (n = 7).

**BAL, Lung Tissue Preparation, and Biochemical Measurements**

At the end of EVLP, a BAL was performed using 5 mL of PBS instilled into the trachea. The BAL fluid was cleared by centrifugation, and the supernatants were stored at −80 °C until further assayed. The lung tissue was flash frozen and pulverized in liquid nitrogen. The powder (50 mg) was lysed in a buffer (TrisHCL 10 mMol/L, NP40 0.5%, NaCl 0.15 M, Na₂VO₄ 1 mM, NaF 10 mM, phenylmethylsulfonyl fluoride 1 mM, EDTA 1 mM, aprotinin 10 µg/mL, leupeptin 10 µg/mL, and pepstatin 1 µg/mL), sonicated (10 s, 3 times), and centrifuged (15 000 revolutions per minute, 10 min). Proteins were quantified by the bicinchoninic acid assay (Thermo Scientific Pierce, Rockford, IL).

The concentration of proteins in BAL fluid (bicinchoninic acid assay) was determined as an index of high permeability lung edema (expressed in milligram per milliliter BAL fluid). Lactate dehydrogenase (LDH) activity was measured in BAL fluid.
fluid as an index of cell damage (Cytotoxicity Detection Kit PLUS; Roche, Basel, Switzerland) and expressed in arbitrary units. The concentration of 3-nitrotyrosine (3-NT) was measured in the BAL fluid (Rat 3-nitrotyrosine ELISA kit; Amsbio, Abingdon, United Kingdom) as a marker of nitro-oxidative stress and expressed in nanomole per milliliter BAL fluid. The concentration of interleukin-6 (IL-6) was measured in BAL fluid (Rat IL-6 ELISA kit, R&D system, Minneapolis, MI) and expressed in pg/mL BAL fluid. The accumulation of protein carbonyl adducts was quantified in lung tissue (OxiSelect Protein Carbonyl ELISA Kit; Cell Biolabs Inc, San Diego, CA) to assess lung oxidative stress, expressed in nanomole per milligram lung protein. All dosages in BAL fluid were performed in 6 animals per group.

FIGURE 1. Experimental design. CI, cold ischemia; C21 (control), 3 h CI (FiO2 0.21); C50, 3 h CI (FiO2 0.5); EVLP, ex vivo lung perfusion; FiO2, inspired fraction of oxygen; WI, warm ischemia; W21, 1 h WI, followed by 2 h CI (FiO2 0.21); W50, 1 h WI, followed by 2 h CI (FiO2 0.5); W2h, 2 h WI, followed by 1 h CI (FiO2 0.21).

FIGURE 2. Lung mechanics and vascular resistance at the end of EVLP. A, SPC at 180 min EVLP. B, PVR at 180 min EVLP. *P<0.05 vs C21; #P<0.05 W21 vs W2h. CI, cold ischemia; C21 (control), 3 h CI (FiO2 0.21); C50, 3 h CI (FiO2 0.5); EVLP, ex vivo lung perfusion; PVR, pulmonary vascular resistance; SPC, static pulmonary compliance; WI, warm ischemia; W21, 1 h WI, followed by 2 h CI (FiO2 0.21); W50, 1 h WI, followed by 2 h CI (FiO2 0.5); W2h, 2 h WI, followed by 1 h CI (FiO2 0.21).
Histological Assessment of Perivascular Edema

At the end of EVLP, the degree of perivascular edema in the right lung (n = 6/group) was determined in 5-µm sections stained with hematoxylin and eosin, digitalized (Hamamatsu NanoZoomer HT Digital slide scanner, Hamatsu Photonics, Japan) and analyzed using Slidepath (Leica Biosystems). Perivascular edema was quantified by determining the ratio of perivascular edema thickness to the inner vessel diameter, averaged from 20 cross-sectioned vessels per lung (arteries and veins), as described previously.11

Statistical Analysis

Results were presented as means ± SEM. The normality of data distribution was determined using the Kolmogorov-Smirnov test. All data were compared using one-way analysis of variance followed by Dunnett’s test with a control. Further pairwise comparisons were made between the different WI groups (W21, W50, and W2h) using Bonferroni’s adjustments. All analyses were performed using GraphPad Prism 6 (GraphPad Software Inc, La Jolla, CA), and a P value <0.05 was considered statistically significant.

RESULTS

Physiological Variables

After 3 h of EVLP, SPC (Figure 2A) was significantly reduced in the 3 WI groups in comparison with C21 (control group). The decrease in SPC was significantly more pronounced in the W2h group in comparison with the W21 and W50 groups. No significant differences were noted across the different groups with respect to PVR (Figure 2B). The lung oxygenation capacity (DppO2; Figure 3) was comparable in all groups except for the W2h, in which DppO2 was significantly reduced at 180 min EVLP compared with the control group. Also, DppO2 was significantly less in W2h than in the W50 group.

Lung Weight Gain and Biochemical Measurements

Compared with the control (C21) group, lung weight gain was significantly greater in the W50 and W2h groups (Figure 4A). The increase was particularly marked in the W2h group, in which it was significantly greater than in the W21 and W50 groups. BAL proteins (Figure 4B) were also significantly increased in the 3 WI groups, the change being particularly more pronounced in the W2h group. LDH release in BAL (Figure 5A), a sensitive marker of cellular injury, was not observed in C21 and C50 groups, whereas it was significantly increased in the 3 WI groups, and the change was statistically more pronounced in the W50 and W2h groups. The levels of 3-NT in BAL fluid (Figure 5B) were significantly greater in the W21, W50, and W2h groups, with no significant differences between them. The formation of carbonyl adducts on tissue proteins (Figure 5C) was increased only in the W50 and W2h
groups, the change being slightly more important in the W50 group. IL-6 release within the BAL was significantly greater in W21, W50, and W2h groups than in the C21 group, with no significant differences between these 3 groups.

**Histological Analysis**

Figure 6A–E shows representative lung sections at the end of EVLP in the 5 experimental groups. The most noticeable alteration was the presence of perivascular edema, which was quantified by the ratio of edema thickness to the diameter of the examined vessels (Figure 6F). In comparison with the C21 group, perivascular edema was increased in all 3 WI groups, most significantly in the W2h group.

**DISCUSSION**

EVLP has been developed to increase the number of donor lungs available for lung transplantation by serving 4 distinct purposes. First, it allows the assessment of marginal donor lungs to evaluate their suitability for transplantation. Second, it may permit to significantly extend the duration of graft preservation. Third, it can lead to the identification of novel molecular and cellular pathways, using metabolomic and transcriptomic analyses. Finally, EVLP may serve as a therapeutic platform for the repair of damaged organs before transplantation. An essential aspect of the translational application of experimental EVLP reconditioning relies on selecting appropriate animal models, which are able to reproduce, on the bench, key features of the clinical problem. Most notably, lung ischemia-reperfusion injury is a crucial pathophysiological mechanism of lung damage and dysfunction during lung transplantation, which may be treated by targeted interventions during EVLP. In this respect, we assessed several ex vivo models of rat lung ischemia-reperfusion and evaluated their impact on major end points of EVLP (lung mechanics, edema and oxygenation capacity, and injury and oxidative/nitro-oxidative stress). Three distinct variables were incorporated in our models, namely, CI or WI preservation, various
levels of oxygen (FiO$_2$ 0.21 or 0.5) for lung inflation before EVLP, and 2 durations (1 or 2 h) of WI.

Rat lungs exposed to only CI (3 h) did not develop any overt signs of damage and dysfunction after 3 h EVLP, whereas they inflated at a FiO$_2$ of 0.21 or 0.5 to foster the generation of oxygen-derived oxidants and free radicals. Furthermore, the 2 CI groups did not display elevated levels of IL-6 within the BAL, a major proinflammatory cytokine associated with significant prognostic implications in the field of EVLP and LTx. This implies that these 2 models would not be well suited to address therapeutic reconditioning for reversing donor organ injury in the experimental setting. These observations are consistent with previous data from Ohsumi et al, who performed a detailed evaluation of rat lung damage and dysfunction during 4 h EVLP, after preservation with CI times (CITs) ranging from 20 min to 24 h. Lungs preserved up to a CIT of 12 h did not display significant alterations during EVLP, whereas those with a CIT of 18 h disclosed reduction of compliance and increased release of lactate and apoptotic changes, and lungs with a CIT of 24 h immediately deteriorated. The authors concluded that a CIT of 18 h may be the limit for ischemic injury in this rat model of EVLP and would therefore be best suited to evaluate therapeutic interventions to treat damaged donor lungs; however, this conclusion seems different when rat lungs are obtained from brain dead, instead of euthanized, animals, as recently shown by van Zanden et al.

These authors indeed reported significant lung injury and dysfunction during EVLP of lungs procured after 3 h brain death and preserved for CIT of only 1 h.

The 2 next models evaluated included a period of 1 h WI with deflated lungs, followed by 2 h CI with lungs inflated a FiO$_2$ of 0.21 (W21) or 0.5 (W50) before EVLP. The lungs were purposely kept deflated during the WI time to amplify the damaging effect of WI, as previously detailed. Our choice to use a WI time of 1 h may be criticized at first glance because the duration of WI in clinical DCD donation is generally kept to a minimum, with 90% of DCD donors displaying a WI time of <30 min in the most recent International Society for Heart and Lung Transplantation registry; however, these reports did not find any relationship between WI times up to 60 min and early survival, suggesting that the true limits of WI time in DCD lungs may not yet be reached. Therefore, evaluating EVLP reconditioning in lungs exposed to WI times up to ≥60 min is clinically relevant because it might help find innovative solutions to further increase the pool of available donor lungs.

Both conditions promoted a comparable reduction of lung compliance, with an increased weight gain and BAL proteins together with significant perivascular edema on histological analysis and strikingly elevated BAL IL-6. A primary mechanism underlying such alterations is the generation of free radicals and oxidants on reoxygenation of the previously ischemic tissue, promoting diffuse cellular toxicity and dysfunction. Accordingly, both W21 and W50 models displayed an increased formation of 3-NT, a footprint of nitro-oxidative stress and more specifically peroxynitrite, a cytotoxic oxidant playing key pathogenic roles in reperfusion/reoxygenation injury. In contrast, protein carbonyl adducts (marker of protein oxidative damage) increased only in W50
lungs, which is consistent with an enhanced flux of reactive oxidants from enzymes (e.g., xanthine oxidase, NADPH oxidase) and from the mitochondrial electron transport chain at higher local PO2. Furthermore, W50 displayed considerably greater release of LDH, indicating that the higher FiO2 promoted lung injury after WL. Interestingly, this was not paralleled by a larger release of BAL IL-6, implying no direct correlation between the degree of lung injury and inflammation during EVLP, in total agreement with our previous studies on innate immune mechanisms during EVLP and lung transplantation.

By reproducing many characteristics of PGD (oxidative stress, tissue injury, high permeability edema, reduced lung compliance, lung inflammation), these 2 models of W1h are well suited to study ex vivo therapeutic reconditioning, which aims at the repair of damaged lung grafts to reduce the risk of PGD after transplantation. The W50 model may be particularly relevant by amplifying the damage of WI through the simple increase of FiO2. Both models may still be criticized by the absence of impaired oxygenation capacity, which represents the major feature of PGD in the clinical setting; however, it is noteworthy that ex vivo PO2 may remain unaffected despite significant lung injury in conditions of EVLP using an acellular perfusate, as previously demonstrated by Yeung et al in a porcine model of EVLP. This seems related to the linear relationship between oxygen content and PO2 in an acellular perfusate, decreasing the influence of intrapulmonary shunt on PO2 in the effluent of the EVLP circuit. Notwithstanding this limitation, the 1 h WI models are extremely interesting for the evaluation of reconditioning therapies in the EVLP setting, as reviewed recently. Using the W50 model, we recently reported significant benefits of ex vivo treatment with pharmacological inhibitors of poly(ADP-ribose) polymerase and nuclear factor K B, and we also used this model to identify novel mechanisms of lung graft inflammation after EVLP and transplantation.

Extending the duration of WI to 2 h (W2h) promoted major damage and lung dysfunction, as shown by a massive release of LDH, a considerable increase in lung edema, and a marked reduction of compliance. Furthermore, in contrast to the W1h models, there was here a significant impairment of graft oxygenation capacity, reflecting a major reduction of the alveolar gas exchange area with severe diffusion impairment and intrapulmonary shunt. These features were observed while nitrotyrosine, carbonyl proteins, and IL-6 were not increased further in comparison with the W1h models. This suggests that the significant damage after W2h was mainly because of severe, potentially irreversible, ischemic injury but not because of amplification of nitro-oxidative stress and inflammatory signaling during EVLP. Because such, a W2h model may seem extremely stringent to evaluate pharmacological reconditioning for the repair of established ischemic injuries; however, this model has been used by some authors, who reported reconditioning effects of K-ATP channels modulators, plasmamin, and subnormothermic EVLP in rat lungs damaged by W2h.

We acknowledge several limitations to our study. First, we did not compare acellular versus cellular perfusate. Although previous investigators did not find any significant differences between both types of perfusates in terms of functional, immunological, and morphological parameters, others reported less pulmonary edema and lower vascular resistance with cellular perfusate. These latter results indicate that our findings may only apply to acellular EVLP, and future studies will be necessary to assess similar conditions in a cellular EVLP model. Second, we cannot rule out that the true injury pattern promoted by CI or WI might have been modified by the 3 h EVLP. Indeed, we previously reported that EVLP mitigated lung damage produced by WI alone, whereas it slightly enhanced the damage noted at the end of CI preservation. In contrast, EVLP markedly amplified the release of inflammatory cytokines both in WI and CI lungs, consistent with the time required for de novo gene expression on reperfusion. Time-course experiments would therefore be helpful to more precisely assess the evolution of lung injury after ischemia and after various durations of EVLP. Third, we did not evaluate injury patterns after extended durations of CI (>12 h) or prolonged WI times, as can be observed in uncontrolled DCD. Such experiments will be important in the future to help determine the safe upper limits of CIT or WI times in EVLP reconditioning protocols, which presently remain debated. Finally, we did not evaluate the influence of lung transplantation after EVLP; however, because our primary aim was to establish reproducible models of ischemic lung damage for the study of ex vivo therapeutic reconditioning, we chose to limit our investigations to the outcome of EVLP.

In summary, the present study proposes several models of lung ischemic damage of various severity, which will be helpful for the evaluation of novel therapeutic strategies for EVLP reconditioning. A model of WI preservation for 1 h seems the best suited for this purpose, especially when combined with lung inflation at a FiO2 of 0.5 before EVLP, by reproducing typical alterations encountered during clinical lung ischemia-reperfusion injury and PGD.

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