Hydrogen Preconditioning During Ex Vivo Lung Perfusion Improves the Quality of Lung Grafts in Rats

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Background. Although the benefits of ex vivo lung perfusion (EVLP) have been globally advocated, the potentially deleterious effects of applying EVLP, in particular activation of proinflammatory cascades and alteration of metabolic profiles, are rarely discussed. This study examined proinflammatory events and metabolic profiles in lung grafts on EVLP and tested whether preconditioning lung grafts with inhaled hydrogen, a potent, cytoprotective gaseous signaling molecule, would alter the lungs' response to EVLP.

Methods. Rat heart-lung blocks were mounted on an acellular normothermic EVLP system for 4 hr and ventilated with air or air supplemented with 2% hydrogen. Arterial and airway pressures were monitored continuously; perfusate was sampled hourly to examine oxygenation. After EVLP, the lung grafts were transplanted orthotopically into syngeneic rats, and lung function was examined.

Results. Placing lung grafts on EVLP resulted in significant upregulation of the messenger RNAs for several proinflammatory cytokines, higher glucose consumption, and increased lactate production. Hydrogen administration attenuated proinflammatory changes during EVLP through upregulation of the heme oxygenase-1. Hydrogen administration also promoted mitochondrial biogenesis and significantly decreased lactate production. Additionally, in the hydrogen-treated lungs, the expression of hypoxia-inducible factor-1 was significantly attenuated during EVLP. These effects were maintained throughout EVLP and led to better posttransplant lung graft function in the recipients of hydrogen-treated lungs.

Conclusions. Lung grafts on EVLP exhibited prominent proinflammatory changes and compromised metabolic profiles. Preconditioning lung grafts using inhaled hydrogen attenuated these proinflammatory changes, promoted mitochondrial biogenesis in the lungs throughout the procedure, and resulted in better posttransplant graft function.

Keywords: Ex vivo lung perfusion, Hydrogen, Lung transplantation, Pulmonary metabolism.

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Ex vivo lung perfusion (EVLP) has proved its high value as an essential tool for the reassessment of donor lungs that might otherwise have been rejected for transplantation and has revolutionarily changed the face of lung transplantation (*1*). Ex vivo lung perfusion has also accelerated translational research in lung transplantation because it can be used as a platform for assessing many kinds of treatment interventions to repair injured donor lungs (*2*). Whereas these benefits of EVLP have been globally addressed, EVLP-related adverse effects have rarely been discussed. However, it is well established that extracorporeal circulation is associated with coagulation disorders and inflammatory responses, both of which can lead to organ damage (*3*). In addition, some studies have suggested increased lactate production in lung grafts on EVLP, indicating the anaerobic metabolism of glucose within the lung tissue. This increase in lactate production seems to be associated with upregulation of hypoxia-inducible factor 1 (HIF-1), a key mediator of adaptation to hypoxia (*4, 5*). In the lung grafts on EVLP, the circulation to the bronchial artery is disrupted. Notably, the current evidence strongly suggests that loss of the bronchial artery circulation may be a contributing factor to developing airway hypoxia resulting in bronchial obliterans syndrome (*6–8*). Although there are many benefits of organ reconditioning using EVLP including dehydration of the lung tissue, removal of remaining donor blood, and the recruitment of atelectatic lungs (*9*), lung grafts on EVLP also may incur some procedure-related adverse effects.

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Since the discovery of the antioxidant effects of hydrogen, a number of experimental and clinical studies have indicated that hydrogen gas can be a useful new therapeutic modality for various diseases including acute lung injuries (*10–13*). Hydrogen also exhibits potent anti-inflammatory and antiapoptotic properties and may act as a gaseous signaling molecule, similar to nitric oxide (*14*). We have recently explored some of these therapeutic properties of hydrogen in lung and heart transplantation (*12, 15, 16*).

In this study, we tested the hypothesis that lung grafts on EVLP would incur damage from activation of proinflammatory cascades and altered metabolism. We also tested whether exposure to hydrogen gas during EVLP would ameliorate these adverse effects. We found that preconditioning lung grafts with hydrogen during EVLP reduced the inflammatory response, improved metabolism, and yielded superior function after transplantation as compared with lung grafts on conventional EVLP.

RESULTS

Proinflammatory and Metabolic Profiles of Lung Grafts on EVLP

Five lung grafts were placed on normothermic EVLP and perfused for 4 hr. Pulmonary function (P:F ratio; PaO_2-F_IO_2), pulmonary vascular resistance, and dynamic lung compliance remained stable in the perfused lungs throughout 4 hr of EVLP, although they gradually declined during the last hour (Fig. 1).

We subsequently measured cytokine and chemokine expressions in lung tissue exposed to 4 hr of EVLP. Messenger RNA (mRNA) levels for interleukin (IL)-6, IL-1β, and tumor necrosis factor (TNF)-α were significantly increased in EVLP-treated lungs as compared with sham lungs that were exposed to only a short period of ventilation in vivo (Fig. 2). In addition, although perfusate electrolytes were stable during EVLP perfusion, the glucose level in the perfusate dropped significantly, and conversely, lactate level increased during the 4 hr of EVLP (Fig. 3A).

Inhaled Hydrogen Attenuated Proinflammatory Responses and Down-Regulated HIF-1 Expression in Lung Grafts on EVLP

Because hydrogen ameliorates lung injury in several model systems, including ventilator-induced lung injury (*17*) and ischemia-reperfusion injury during lung transplantation (*13*), we tested whether administering 2% hydrogen through the EVLP circuit would mitigate the potentially damaging effects of EVLP. After ventilation with 2% hydrogen in air during EVLP, the lung grafts exhibited significantly attenuated expression of the mRNAs for IL-6, IL-1β, and TNF-α. In fact, the levels of mRNA for these proinflammatory mediators were almost one fourth that seen in lung grafts ventilated with air during EVLP (Fig. 2). Interestingly, HIF-1α mRNA and protein levels were also significantly decreased in the hydrogen-treated lung tissue (Fig. 3B and C).

Hydrogen Markedly Changed the Metabolic Profiles in Lung Grafts on EVLP

Although glucose consumption in lung grafts was not significantly different between grafts ventilated with air during EVLP and grafts ventilated with 2% hydrogen, lactate levels in the perfusate were significantly lower in the hydrogen-treated grafts and remained low throughout EVLP (Fig. 3A).

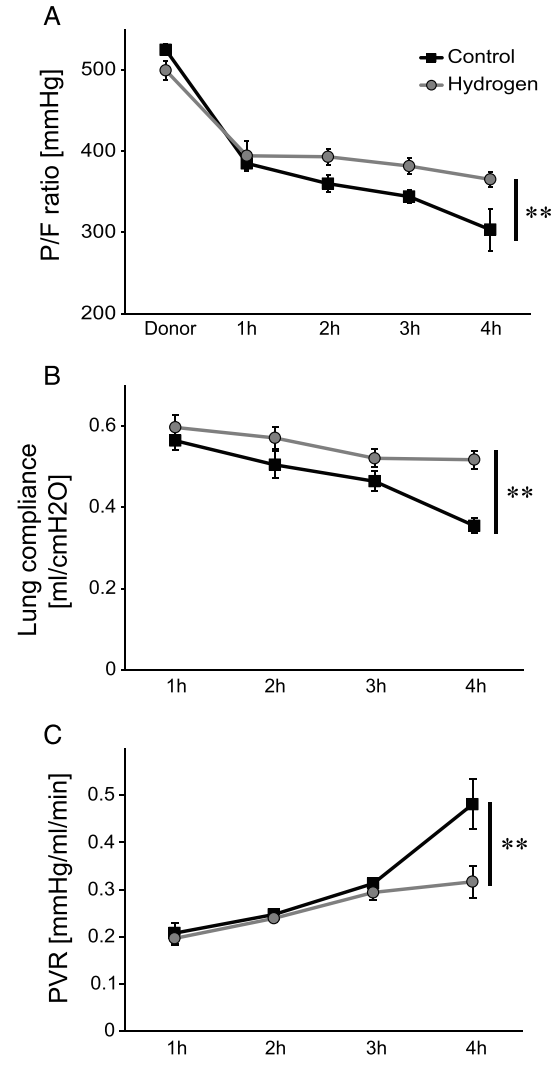

FIGURE 1. Lung function and physiologic parameters on rat ex vivo lung perfusion. A, P/F ratio. B, Lung compliance. C, Pulmonary vascular resistance. n=5 for each group. $^{**}P<0.01$.

In addition, whereas mitochondrial complex I and II enzyme activities in the lung tissue were significantly decreased after EVLP for 4 hr, the activity of both enzymes and the activity of mitochondrial complex IV were significantly increased in the hydrogen-treated lung grafts (Fig. 4A–C). Furthermore, adenosine triphosphate (ATP) production was attenuated in lung grafts 4 hr after EVLP, but ATP levels were significantly increased in lung grafts that received hydrogen treatment during EVLP to levels four times as high as in the lungs without hydrogen on EVLP (Fig. 4D).

Next, we evaluated heme oxygenase (HO)-1, peroxisome proliferator-activated receptor gamma coactivator (PGC)-1α, and nuclear respiratory factor (NRF)-1 mRNA levels to further investigate the mechanism behind the mitochondrial biogenesis notably seen in hydrogen-treated lung grafts. All of these mitochondrial biogenesis-related genes were significantly upregulated in grafts ventilated

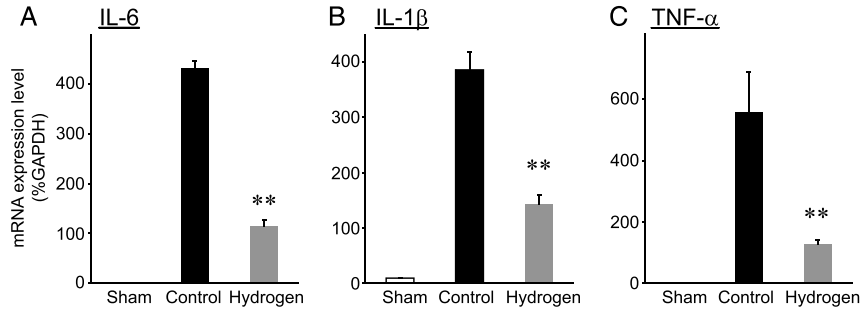

FIGURE 2. Effects of exposure to hydrogen on lung graft inflammation during EVLP. Real-time RT-PCR for proinflammatory mediators in lung graft tissue after 4 hr EVLP for (A). IL-6. B, IL-1A. C, TNF-α. Relative levels of the mRNAs for IL-6, IL-1β, and TNF-α were normalized to GAPDH mRNAs. n=5 for each group; $^{**}P<0.01$ vs. control EVLP. EVLP, ex vivo lung perfusion; RT-PCR, reverse-transcriptase polymerase chain reaction; TNF, tumor necrosis factor; IL, interleukin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; mRNA, messenger RNA.

with hydrogen during EVLP as compared with control grafts ventilated with air during EVLP (Fig. 4E–G).

Posttransplant Graft Function: With Hydrogen Precondition Versus Without Hydrogen Precondition

Finally, we examined posttransplant graft function, comparing lung grafts subjected to standard cold storage, lung grafts subjected to EVLP using air for ventilation, and lung grafts subjected to EVLP with hydrogen-supplemented ventilation. Posttransplant graft function, as evaluated by P:F ratio, was better in the lung grafts that underwent EVLP without hydrogen (control) than in grafts that underwent standard static cold preservation (without EVLP); however, lung grafts that were hydrogen-preconditioned while on EVLP exhibited even better graft function than those that received EVLP without hydrogen (Fig. 5A).

Histologically, it was noted that cellular infiltration and edema formation were remarkable in lung grafts preserved with cold static preservation. In contrast, cellular infiltration and edema formation were attenuated in lungs on EVLP both with and without hydrogen (Fig. 5B). After

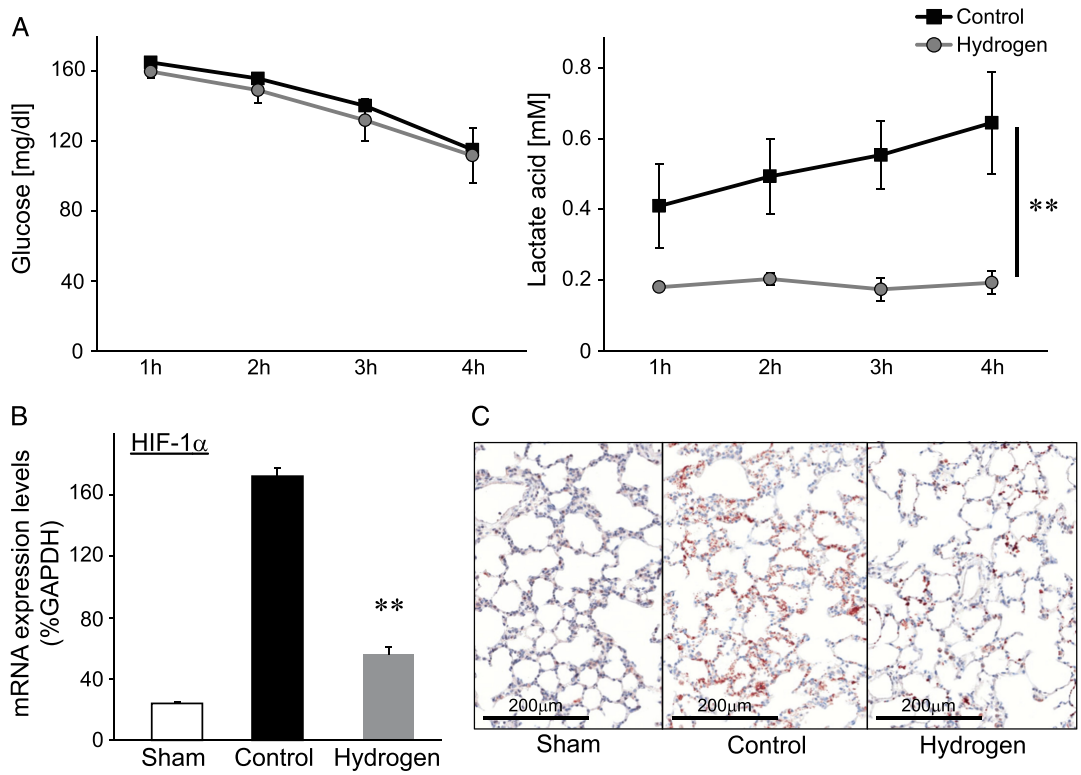

FIGURE 3. Assessment of metabolism during EVLP and graft hypoxia after EVLP. A, Glucose and lactate levels in perfusate over time (n=5 for each group; $^{**}P<0.01$). B, The expression levels of HIF-1α mRNA in lung tissue 4 hr after R=EVLP (n=5 for each group; $^{**}P<0.01$ vs. control EVLP). C, Reproducible images for HIF-1α assessment by immunohistochemistry. HIF-1α protein was stained red. EVLP, ex vivo lung perfusion; mRNA, messenger RNA; HIF, hypoxia-inducible factor.

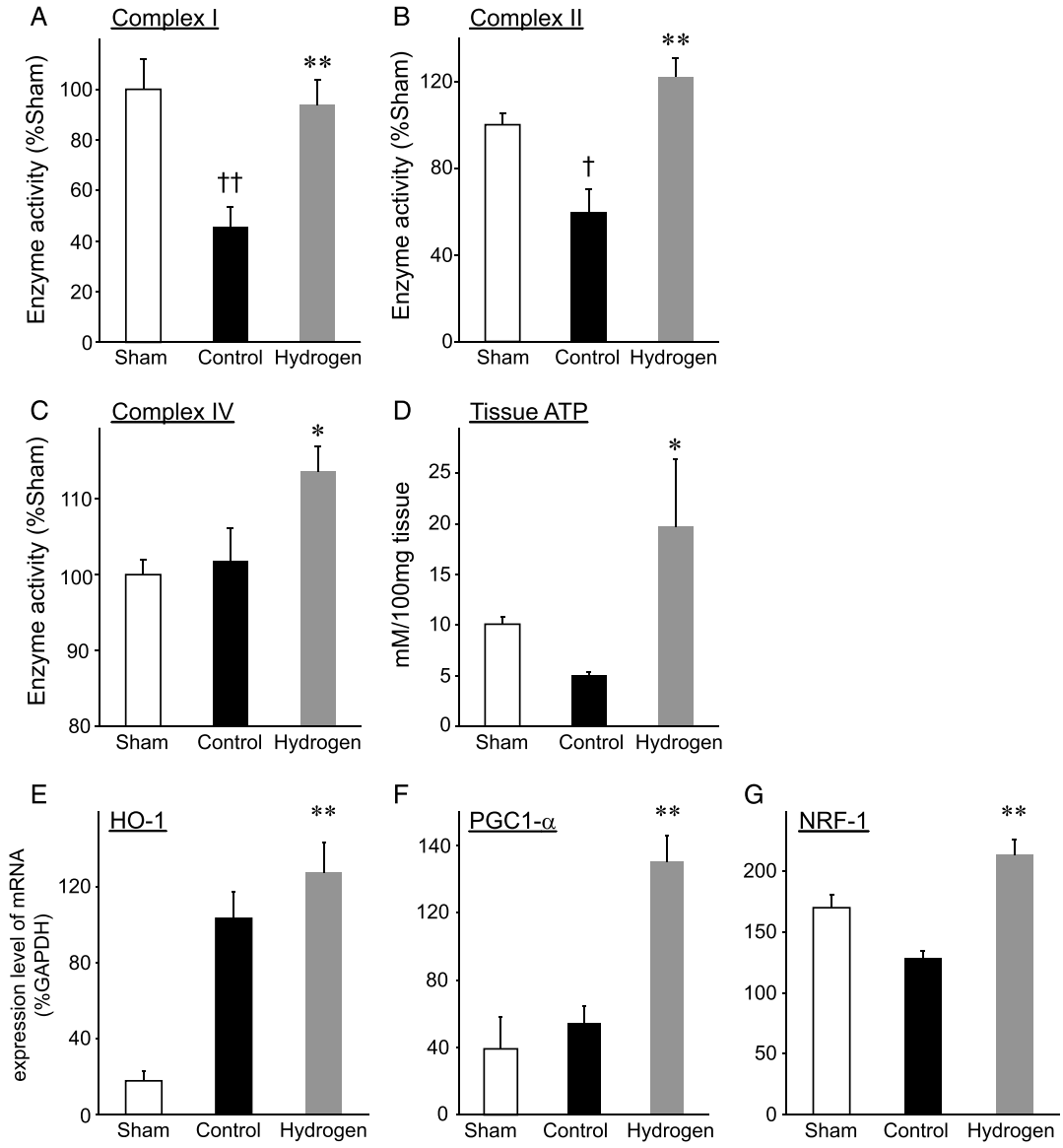

FIGURE 4. Mitochondrial complex enzyme activities, ATP maintenance and mitochondrial biogenesis-related molecules in lung tissue after 4 hr of EVLP. Mitochondrial complex enzyme activities in lung grafts after 4-hr EVLP. A, Mitochondrial complex enzyme I activity. B, Mitochondrial complex enzyme II activity. C, Mitochondrial complex enzyme IV activity. D, Tissue ATP content after EVLP. n=5 for each group, †P<0.05 versus Sham; ††P<0.01 versus Sham; *P<0.05 versus control EVLP; **P<0.01 versus control EVLP. E–G, Expression of mitochondrial biogenesis-related mRNAs in lung tissue stored on EVLP for 4 hr. E, HO-1. F, PGC-1α. G, NRF-1. Gene expression was normalized to GAPDH mRNA levels. n=5 for each group, **P<0.01 versus control EVLP. ATP, adenosine triphosphate; EVLP, ex vivo lung perfusion; HO, heme oxygenase; PGC, peroxisome proliferator-activated receptor gamma coactivator; NRF, nuclear respiratory factor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

transplantation, the mRNAs for IL-6, IL-1β, and TNF-α remained significantly decreased in the grafts that received hydrogen treatment during EVLP as compared with lungs subjected to EVLP with air, suggesting that the hydrogen-induced anti-inflammatory effects lasted not only throughout EVLP but also after transplantation (Fig. 5C–E).

DISCUSSION

Accumulating evidence supports increasing the role of EVLP in clinical lung transplantation, and expectations are high that EVLP will improve current lung transplant outcomes (*18*). However, EVLP is not a "magic bullet." Without an understanding of the limitations of EVLP, it could even cause significant problems in valuable organs. In this study, we focused on the adverse effects of EVLP on the quality of lung grafts, suggested how to resolve potential problems by preconditioning the lung grafts during the EVLP procedure, and demonstrated that hydrogen-supplemented preconditioning contributed to better posttransplant outcomes. Flammability limit of hydrogen is 4%, and the dose of 2% in this study was defined based on the safety reasons for

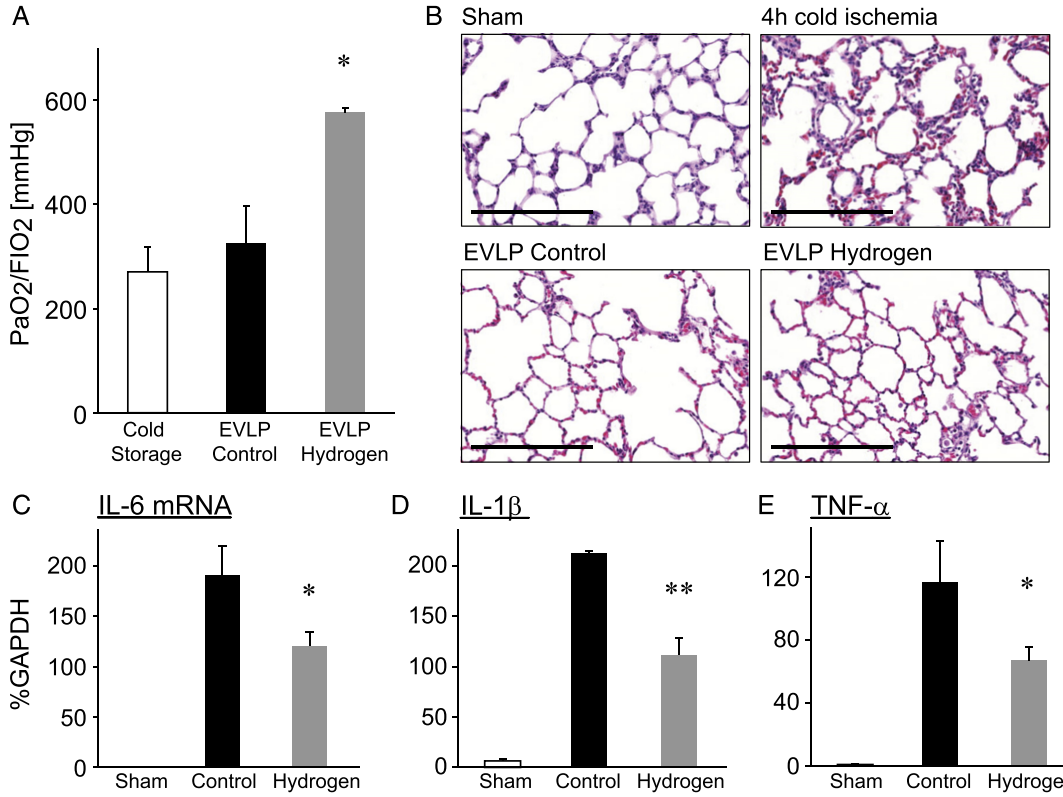

FIGURE 5. Effect of hydrogen preconditioning during EVLP on lung grafts after transplantation. A, Lung function in grafts 2 hr after reperfusion (n=4 for each group; *P<0.05). B, Representative images of H&E staining of lungs grafts 2 hr after reperfusion (scale bar=200 μm). (C–E) Real-time reverse-transcription-polymerase chain reaction for proinflammatory mediators in lung graft tissue 2 hr after reperfusion. C, IL-6. D, IL-1β. E, TNF-α. n=4 for each group; *P<0.05 versus control EVLP, **P<0.01 versus control EVLP. Cold ischemia, lungs transplanted after 4 hr of cold preservation; EVLP control, lungs transplanted after EVLP with air ventilation; EVLP hydrogen, lungs transplanted after EVLP with hydrogen inhalation. H&E, hematoxylin-eosin; EVLP, ex vivo lung perfusion; IL, interleukin; TNF, tumor necrosis factor.

priority, whereas our previous studies also confirmed the effectiveness with 2% (*15*). The hydrogen concentration can be monitored and maintained with commercially available tools. Therefore, hydrogen inhalation therapy is easily applicable for clinical setting.

Preconditioning Lung Grafts on EVLP—Importance and Approaches

Normothermic EVLP seems to be an ideal, isolated environment for many therapeutic strategies, allowing specific, targeted treatment of the organ without systemic side effects. Therefore, the concept of treatment intervention during EVLP has long been advocated, and many attempts have been made to treat pulmonary edema, pulmonary emboli, pneumonia, or lung inflammation using pharmacologic, gene-based, and cell-based therapies (*2, 19, 20*). The therapeutic concept that we applied in this study is a bit different from previously reported therapeutic strategies using EVLP because we focused on the procedure-related adverse effects of conventional EVLP (activated proinflammatory responses and compromised metabolic circumstances), and aimed to precondition the lung grafts to overcome these inherent insufficiencies, which could develop into potentially critical problems.

A group from Toronto recently reported a highly effective gene therapy method with an IL-10-expressing adenoviral vector and demonstrated anti-inflammatory changes in lung grafts on EVLP and improved posttransplant outcomes similar to those seen in our study (*2, 21*). However, vector-associated inflammation remains a concern in particular in the clinical setting of posttransplant immunocompromised lungs (*2*). In contrast, hydrogen can be easily delivered by means of inhalation through a ventilator circuit, which is simple and straightforward and without adverse effects. Therapeutic use of hydrogen as a medical gas has increasingly gained acceptance in many fields, not only for lung pathologies (*15*) but also for kidney, liver, brain, and heart diseases (*22, 23*). This robust evidence supports our strategy of hydrogen preconditioning lung grafts on EVLP.

Activation of Proinflammatory Mediators in the Lungs During EVLP

Interpreting the proinflammatory responses in lung grafts on EVLP requires caution. In animal and human models of kidney, liver, heart, and lung transplantation, ischemia-reperfusion injury results in the release of proinflammatory cytokines and chemokines including IL-8, IL-11, and interferon-γ in addition to IL-1β, IL-6, and TNF-α (the

cytokines examined in this study) (*24–27*). More importantly in lung transplantation, elevated levels of these proinflammatory mediators, in the graft tissue or in the blood, seem to be associated with subsequent critical events, such as severe primary graft dysfunction (*28, 29*). Similar findings of elevated levels of proinflammatory mediators have been clearly shown in the lungs on EVLP (*30*). However, after careful review of the literature on EVLP, it seems that most studies consider these mild elevations in cytokine levels to be well tolerated and without adverse consequences in lung grafts (*30*). The pitfalls of this assumption are that: (1) cytokine levels were typically measured only in the perfusate, not in the lung tissue (*31*); and (2) the evidence of no "adverse consequences" has been limited (*32*). Although a report from Japan demonstrated that removal of cytokines in the perfusate using an adsorbent membrane device did not improve the quality of lung grafts on EVLP (*31*), the clearance in the perfusate alone would not necessarily eliminate the proinflammatory responses in lung grafts on EVLP. Therefore, this finding should not be translated into the conclusion that the proinflammatory responses in lung grafts on EVLP are not relevant.

The study from Toronto supports our findings. Using gene transfection of IL-10, which inhibits proinflammatory cytokine secretion, Cypel and colleagues showed that IL-10 successfully blocked inflammatory processes in lung grafts on EVLP, promoted recovery of the alveolar-blood barrier, and led to significantly better preserved graft function (*21*). This is valuable evidence supporting the critical need to control inflammatory responses in lung grafts on EVLP for successful lung graft preservation. In our study, we demonstrated that inhaled hydrogen decreased inflammatory responses in lung grafts on EVLP, and this effect persisted even after transplantation. In addition, hydrogen increased the expression of HO-1, a well-known antioxidant key enzyme, in the lung grafts (*33*). The strategy of HO-1 induction for better organ graft protection has been well studied in kidney, heart, and liver (*34*), and we have elaborated the beneficial effects of HO-1 induction in other lung injury models (*35*). Hydrogen-induced cytoprotective and anti-inflammatory effects may be the underlying mechanisms leading to improved posttransplant graft function in lung grafts on EVLP that have been exposed to hydrogen.

Compromised Cellular Metabolic Activities in the Lungs During EVLP

The metabolic circumstances in lung grafts on EVLP are compromised as compared with those in lungs in vivo. Robust evidence suggests that the loss of bronchial artery circulation results in tissue hypoxia, in particular airway hypoxia, leading to the development of bronchial obliterans syndrome (*7*). Although the fact that bronchial artery circulation is disrupted in lung grafts on EVLP has rarely been discussed, significant glucose consumption using anaerobic metabolic process in lung grafts on EVLP, as seen in this study, has also been observed in other studies (*13, 36*), and can in part be attributed to this disruption of bronchial artery circulation.

The mechanisms by which ischemia, hypoxia, and proinflammatory changes facilitate fibrosis are not established, but the clustering of hypoxia, ischemia, inflammation, and fibrosis is routinely observed in several clinical situations including pulmonary fibrosis, normal skin wound healing, and chronic kidney diseases (*8, 37, 38*). Notably, hydrogen inhalation promoted mitochondrial biogenesis through activated expression of key enzymes including PGC-1α, NRF-1 and HO-1, and contributed to establishing a high-energy system with accelerated ATP production in lung grafts on EVLP. Hydrogen inhalation ultimately led to decreased expression of HIF-1 in the lung graft tissue, indicating an enhanced lung graft quality from a metabolic standpoint because HIF-1 is a master regulator of O_2 homeostasis and a global marker of tissue hypoxia.

Mitochondrial quality control is regulated in part by the HO-1 system through the redox-regulated NF-E2-related factor-2 (Nrf2) transcription factor (*36*). We have recently demonstrated that hydrogen can ameliorate hyperoxic lung injury through induction of the Nrf2-mediated HO-1 system using Nrf2-deficient mice (*15*). This Nrf2-mediated HO-1 system could provide a mechanistic explanation for hydrogen-induced mitochondrial biogenesis (*39*).

Given the syngeneic transplant models, the effects of hydrogen supplement on immune response were not demonstrated in the present study, although we are aware of their importance. We are currently conducting this investigation specifically using allogeneic transplant models.

In conclusion, the present study demonstrated that lung grafts on EVLP exhibited significant proinflammatory responses and compromised cellular metabolic activity as a result of EVLP. Preconditioning the lung grafts using inhaled hydrogen on EVLP resolved these EVLP-related adverse effects through inhibiting proinflammatory responses and reinforcing mitochondrial biogenesis, leading to improved posttransplant graft function after lung transplantation.

MATERIALS AND METHODS

Animals

Inbred male Lewis (RT-1^l) rats (Harlan Sprague Dawley, Indianapolis, IN) weighing 250 to 300 g were purchased. Animals were maintained in laminar flow cages in a specific pathogen-free animal facility at the University of Pittsburgh and fed a standard diet and water ad libitum. All procedures were performed according to the guidelines of the Institutional Animal Care and Use Committee at the University of Pittsburgh and the National Research Council's Guide for the Humane Care and Use of Laboratory Animals.

Ex Vivo Lung Perfusion in Rats

Ex vivo lung perfusion was performed using a commercially available rodent EVLP system (IL-2 Isolated Perfused Rat or Guinea Pig Lung System; Harvard Apparatus, Holliston, MA) as described previously (*40*). The rats were anesthetized and underwent tracheotomy, mechanical ventilation with 100% O_2, and heparinized with 300 IU intravenous heparin, before procurement of heart-lung blocks that were flushed with cold low potassium dextran solution (Perfadex; Vitrolife AB, Göteborg, Sweden) and then stored in low potassium dextran solution at 4°C for 1 hr. The trachea, pulmonary artery, and pulmonary vein were cannulated during cold ischemia, and then the heart-lung blocks were applied to EVLP system for 4 hr. During EVLP, the lungs were ventilated with air or air supplemented with 2% hydrogen at 37°C and perfused with Steen solution (XVIVO Perfusion AB, Göteborg, Sweden) that was supplemented with 50 mg of methylprednisolone (Solu-Medrol; Pfizer, Inc., New York, NY) and 50 mg of cephalosporin (Cefazolin; APP pharmaceuticals LLC, Schaumburg, IL) and deoxygenated with 6% O_2, 8% CO_2, and balanced N_2. Perfusion was started in a gentle pace at 10% of target flow and gradually increased for 1 hr toward whole target flow rate that was calculated as 20% of cardio output. Pulmonary artery pressure, peak airway pressure, and airway flow were monitored continuously, and dynamic lung

compliance and pulmonary vascular resistance were analyzed. Every hour, the ex vivo perfused lung was ventilated with 100% O_2 for 5 min, and then the perfusate was sampled for pulmonary oxygenation and electrolytes analysis. Sham-operated animals underwent anesthesia, tracheotomy, and mechanical ventilation with 100% O_2. The lungs of the sham animals were then immediately removed for analysis.

Rat Orthotopic Lung Transplantation

To investigate the effect of hydrogen preconditioning on transplanted lung grafts, orthotopic lung transplantation after EVLP for 2 hr was performed using the cuff method as previously described (*32*). After 2 hr of EVLP, the lungs were precooling with 4°C Steen solution on the EVLP system and stored at 4°C for 1 hr. After cold storage, the grafts were orthotopically implanted into recipient rats. No steroid was given to the recipient rats postoperatively. Two hours after reperfusion, the naive lung was clamped, 100% O_2 was inhaled for 5 min through a ventilator, and then recipient blood was sampled from the graft pulmonary vein and used for blood gas analysis. The posttransplant functional data were compared between the grafts preserved with a basic cold preservation method for 4 hr, grafts perfused on EVLP and ventilated with air, and grafts perfused on EVLP and ventilated with hydrogen.

Determination of Lactate Levels in Perfusate

Lactate levels in perfusate sampled hourly during EVLP were measured using a Lactate Assay Kit II (BioVision, Mountain View, CA).

Real-Time Reverse Transcription-Polymerase Chain Reaction

Messenger RNA levels for IL-6, IL-1β, TNF-α, HIF-1α, HO-1, PGC-1α, NRF-1, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in graft tissue after 4 hr of EVLP were assessed by SYBR Green 2-step, real-time reverse transcription-polymerase chain reaction as previously described (*41*). Also, IL-6, IL-1β, TNF-α, and GAPDH mRNAs were measured in lung grafts 2 hr after reperfusion.

Mitochondrial Enzyme Activities Assay

Mitochondria were isolated from the lung tissue after 4 hr of EVLP using a mitochondria isolation kit (Thermo Fisher Scientific, Rockford, IL). The lung tissue was finely minced and then homogenized with cold mitochondrial isolation buffer. After the mitochondria were collected, the protein concentration was determined using bicinchoninic acid assay (Pierce Chemical Co., Rockford, IL). Mitochondrial proteins were extracted and used for microplate assays to determine the enzyme activities of mitochondrial complexes I, II, and IV (Abcam, Cambridge, MA).

ATP Contents Measurement

For quantification of ATP in lung tissue after 4 hr of EVLP, an ENLITEN ATP assay system bioluminescence kit (Promega, Madison, WI) was used as previously described (*16, 42*).

Histopathologic Analysis

After EVLP for 4 hr, lung graft tissue was formalin-fixed, paraffin-embedded, sectioned to 4-μm thickness, and stained with hematoxylin-eosin. For immunohistochemical staining, anti-HIF-1α protein antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA; dilution, 1:500) were used, as described previously (*16*).

Statistical Analysis

The results are expressed as mean with standard error of the mean. All data were analyzed using SPSS Version 12 statistical software package (SPSS Inc., Chicago, IL). When analysis of variance indicated a significant overall effect, differences among individual means were assessed using the Bonferroni post hoc test for multiple comparisons. *P* value less than 0.05 was considered statistically significant.

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