Impact of triptolide during ex vivo lung perfusion on grafts after transplantation in a rat model

Check for updates

Sarah Burki, MD,[a] Kentaro Noda, PhD,[a] Brian J. Philips, PhD,[a] Murugesan Velayutham, PhD,[b,c] Sruti Shiva, PhD,[b] Pablo G. Sanchez, MD, PhD,[a] Ajay Kumar, PhD,[a] and Jonathan D'Cunha, MD, PhD[d]

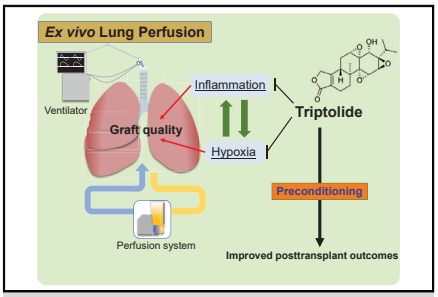
ABSTRACT

Objective: Ex vivo lung perfusion creates a proinflammatory environment leading to deterioration in graft quality that may contribute to post-transplant graft dysfunction. Triptolide has been shown to have a therapeutic potential in various disease states because of its anti-inflammatory properties. On this basis, we investigated the impact of triptolide on graft preservation during ex vivo lung perfusion and associated post-transplant outcomes in a rat transplant model.

Methods: We performed rat normothermic ex vivo lung perfusion with acellular Steen solution containing 100 nM triptolide for 4 hours and compared the data with untreated lungs. Orthotopic single lung transplantation after ex vivo lung perfusion was performed.

Results: Physiologic and functional parameters of lung grafts on ex vivo lung perfusion with triptolide were better than those without treatment. Graft glucose consumption was significantly attenuated on ex vivo lung perfusion with triptolide via inhibition of hypoxia signaling resulting in improved mitochondrial function and reduced oxidative stress. Also, intragraft inflammation was markedly lower in triptolide-treated lungs because of inhibition of nuclear factor-κB signaling. Furthermore, post-transplant graft function and inflammatory events were significantly improved in the triptolide group compared with the untreated group.

Conclusions: Treatment of lung grafts with triptolide during ex vivo lung perfusion may serve to enhance graft preservation and improve graft protection resulting in better post-transplant outcomes. (J Thorac Cardiovasc Surg 2021;161:e65-74)



TL preconditioning improved the grafts during EVLP and subsequent transplantation.

CENTRAL MESSAGE

Treatment of lung grafts with TL during EVLP may serve to enhance graft preservation and improve graft protection resulting in better post-transplant outcomes.

PERSPECTIVE

Although EVLP is a highly valued technique for assessing and reconditioning marginal donor lungs for transplantation, its disadvantages, including the development of inflammation, need to be addressed to maintain organ quality during reevaluation. TL can effectively attenuate these adverse events, thus improving clinical outcomes after transplantation.

See Commentaries on pages e75, e77, and e79.

Video clip is available online.

For patients with end-stage lung disease for whom medical management has been exhausted, lung transplantation remains the ultimate lifesaving therapy. The past several years have witnessed a steady increase in the number of

From the [a]Division of Lung Transplant and Lung Failure, Department of Cardiothoracic Surgery; [b]Department of Medicine, Vascular Medicine Institute; [c]Department of Surgery, Thomas E. Starzl Transplantation Institute, University of Pittsburgh, Pittsburgh, Pa; and [d]Department of Cardiothoracic Surgery, Mayo Clinic Arizona, Pheonix, Ariz.

This study was supported by grants from the research funds of the Department of Cardiothoracic Surgery, University of Pittsburgh Medical Center.

Received for publication Sept 23, 2019; revisions received Oct 30, 2019; accepted for publication Dec 19, 2019; available ahead of print Jan 23, 2020.

Address for reprints: Jonathan D'Cunha, MD, PhD, Cardiothoracic Surgery, 5777 E Mayo Blvd, Phoenix, AZ 85054 (E-mail: DCunha.Jonathan@mayo.edu).

0022-5223/$36.00

Copyright © 2020 Published by Elsevier Inc. on behalf of The American Association for Thoracic Surgery

https://doi.org/10.1016/j.jtcvs.2019.12.104

Abbreviations and Acronyms

Cdyn = dynamic lung compliance
ChIP = chromatin immunoprecipitation
EPR = electron paramagnetic resonance
EVLP = ex vivo lung perfusion
HIF = hypoxia-inducible factor
IκB = inhibitor of κB
IL = interleukin
MCP = monocyte chemoattractant protein
mRNA = messenger RNA
NF-κB = nuclear factor-κB
P/F ratio = Pa_{O_2}/F_{IO_2} ratio
PGD = primary graft dysfunction
PVR = pulmonary vascular resistance
TL = triptolide
TNF = tumor necrosis factor

lung transplantations performed annually, yet the supply of donor organs has remained relatively unchanged, creating a demand–supply mismatch.

Ex vivo lung perfusion (EVLP) contributes to an expanded donor pool by creating an increased time frame between graft procurement and transplantation allowing for additional evaluation under the dynamic platform for marginal lungs previously deemed untransplantable.[1] Despite these benefits, the accumulation of proinflammatory cytokines in the perfusate over time has been reported in preclinical and clinical EVLP.[2-4] Also, some reports have indicated that graft metabolism, specifically glucose consumption and lactate production, during EVLP is associated with graft quality and edema development.[5] In addition, our previous studies have demonstrated that EVLP creates a proinflammatory environment resulting from activated hypoxic signaling in the lungs and causing subsequent graft damage to the mitochondria and microvasculature.[6,7] Despite EVLP use, significant improvement regarding clinical outcomes in lung transplantation has not been reported, with primary graft dysfunction (PGD) and chronic lung allograft dysfunction still affecting patient survival.[8] In terms of PGD, biomarker studies have revealed that mRNA upregulation of multiple proinflammatory cytokine expression in preimplant lung grafts is associated with severe PGD.[9] More important, the recipients' PGD incidence is an important risk factor for the development of chronic lung allograft dysfunction and overall survival.[10] Consequently, the preconditioning of grafts with compounds that inhibit inflammation on EVLP would be desired to potentially improve post-transplant outcomes.[11]

Triptolide (TL) is a diterpenoid triepoxide with a broad array of biological effects. It is extracted from *Tripterygium wilfordii*, a medicinal plant that has been extensively investigated because of its antitumorigenic, immunosuppressant, and anti-inflammatory properties. Of note, TL has also been shown to attenuate lung injury and obliterative airway disease in rodent models.[12] Additionally, recent evidence suggests that Minnelide (Minneamrita Therapeutics, Moline, Ill), a water-soluble form of TL, may improve oxygen status and decrease in metabolic dysregulation caused by hypoxia.[13] In light of these insights, we hypothesized that TL administration during EVLP may improve overall graft preservation and function, ultimately leading to better post-transplant outcomes.

In this study, we aimed to suppress or reduce hypoxia-induced graft inflammation in lung grafts during EVLP by assessing the effectiveness of TL as an EVLP preconditioning agent.

MATERIALS AND METHODS

Animals

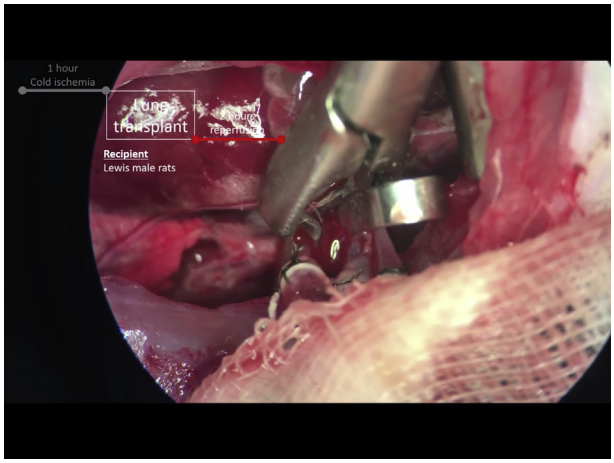
Inbred male Lewis (RT-1^{l}) rats weighing 250 to 300 g were purchased from Envigo RMS, Inc (Indianapolis, Ind). Animals were maintained in laminar flow cages in a specific pathogen-free animal facility at the University of Pittsburgh and given a standard diet and water ad libitum. All procedures were 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

EVLP was performed using a commercial available rodent EVLP system (IL-2 Isolated Perfused Rat or Guinea Pig Lung System, Harvard Apparatus, Holliston, Mass) as described previously.[3] Acellular Steen solution (XVIVO Perfusion AB, Göteborg, Sweden) was used for perfusate, and TL (MilliporeSigma, St Louis, Mo) was administered into perfusate at a final concentration at 100 nM in the treatment group. Control group animals received Steen solution containing methylprednisolone and cephalosporin only. Perfusion flow was started at 10% of target flow and gradually increased for 1 hour toward a target flow rate that was calculated as 20% of cardiac output (75 mL/min/250 g donor body weight). Pulmonary artery pressure, peak airway pressure, and airway flow were monitored continuously, and dynamic lung compliance (Cdyn) and pulmonary vascular resistance (PVR) were analyzed. Each hour for a total of 4 hours, the ex vivo perfused lung was ventilated with 100% O_2 for 5 minutes, and then the perfusate was sampled for obtaining Pa_{O_2}/F_{IO_2} ratio (P/F ratio) and electrolyte analysis. Sham-operated animals underwent anesthesia, tracheotomy, and mechanical ventilation with 100% O_2, and then the lungs were immediately removed for analysis (Video 1).

Rat Orthotopic Left Lung Transplantation After Ex Vivo Lung Perfusion

Orthotopic, single-lung transplantation of the left lung was performed using the cuff method as described previously.[6] After EVLP for 4 hours, the lungs were precooled with 4°C Perfadex on the EVLP system and stored at 4°C for 1 hour before transplantation. The recipient animals were killed 2 hours after reperfusion. At the time of analysis of graft function, the naïve lung was clamped, 100% O_2 was administered for 5 minutes through a ventilator, and recipient's blood was sampled from the graft pulmonary vein for blood gas analysis (Video 1).



VIDEO 1. Experimental procedure of EVLP followed by single left lung transplantation using 3-cuff technique in rats. Video available at: https://www.jtcvs.org/article/S0022-5223(20)30191-4/fulltext.

Real-Time Reverse Transcription-Polymerase Chain Reaction

The levels of messenger RNA (mRNA) were assessed by SYBR Green 2-step, real-time, reverse transcription-polymerase chain reaction as previously described.[6] Graft tissues collected after 4 hours of EVLP and 2 hours after transplantation were examined for interleukin (IL)-6, IL-1β, tumor necrosis factor (TNF)-α, monocyte chemoattractant protein (MCP)-1, and glyceraldehyde-3-phosphate dehydrogenase. Also, hypoxia-related genes such as hypoxia-inducible factor (HIF)-1α, glucose transporter 1, heme oxygenase-1, and lysyl oxidase were also detected in lung tissues after 4 hours EVLP. All gene expressions were normalized with glyceraldehyde-3-phosphate dehydrogenase mRNA content.

Western Blot Analysis

Western blot analysis was performed on 30 μg of whole cell protein from each lung graft after 4 hours EVLP as described previously.[14] The following primary antibodies were used: anti-inhibitor of κB (IκB)-α/p-IκB-α (Novus Biologicals, LLC, Centennial, Colo) and anti–β-actin (MilliporeSigma). After incubation with horseradish-peroxidase conjugated goat anti-rabbit or anti-mouse secondary antibodies (Pierce Chemical, Rockford, Ill), the membranes were developed with the SuperSignal detection system (Pierce Chemical), and signals were detected by Imaging System (ChemiDoc, Bio-Rad, Hercules, Calif). Expression of the listed proteins was quantified by ImageJ (National Institutes of Health) and normalized with β-actin level.

Chromatin Immunoprecipitation

The chromatin immunoprecipitation (ChIP) assay was carried out per the manufacturer's instructions of the ChIP Assay Kit (MilliporeSigma). Briefly, tissues after 4 hours EVLP were treated with 1% formaldehyde for 10 minutes at 37°C to crosslink protein and associated chromatin. Lysate samples were prepared by homogenizing the tissues with sodium dodecyl sulfate buffer and sonication to shear DNA-protein complex. The DNA-protein complexes were then immunoprecipitated by incubating with mice anti–nuclear factor-κB (NF-κB) p65 monoclonal antibody (Santa Cruz Biotechnology, Inc, Dallas, Tex) overnight at 4°C followed by incubation with protein A agarose beads for 1 hour at 4°C. After reverse cross-linking, the associated DNA fragments were eluted. Conventional polymerase chain reaction was performed on input DNA and ChIP DNA samples using the following primer pairs: distal NF-κB motif (223 bp), 5′-AGCATCTGGAGCTCATATTCCAGC-3′ and 5′-CAGTTAGCATAC GATGCAACACAGT-3′ and proximal NF-κB motif (200 bp), 5′-GCAG CTTCATTTGCTCCCAGTAGT-3′ and 5′-TTATTGTAAGCCAGAGGG TGGAGTCAGG-3′.[15]

Mitochondrial Complex I Enzyme Activity Assay

Mitochondria were isolated from the lung tissues after 4 hours of EVLP using a mitochondria isolation kit (Thermo Fisher Scientific, Rockford, Ill). Mitochondrial proteins then were extracted and enzyme activities of complex I were measured by spectrophotometric kinetic assay as described earlier.[14]

Electron Paramagnetic Resonance Spectroscopy

The perfusate samples obtained at 1 hour and 4 hours during EVLP, as well as homogenate of lung tissues 2 hours after transplantation, were mixed with 0.2 mmol/L CMH electron paramagnetic resonance (EPR) spin probe (Enzo Life Sciences Inc, Farmingdale, NY) and incubated for 20 minutes at 37℃. EPR spectra were recorded at room temperature using a Bruker X-band EMX premiumX spectrometer (Bruker BioSciences, Billerica, Mass). The following EPR instrument settings were used: microwave frequency, 9.85 GHz; sweep width, 60 G; microwave power, 20 mW; modulation amplitude, 0.5 G; receiver gain, 30 dB; time constant, 5 ms; conversion time, 25 ms, sweep time, 30 s; number of scans, 10.

Wet-to-Dry Weight Ratio

The weight of lung tissues 2 hours after transplantation was measured immediately after collection, and the tissues were placed in a 60°C oven to dry for 72 hours. Tissues were weighed to determine the wet-to-dry lung weight.

Statistical Analysis

All data were analyzed using SPSS Version 25 statistical software (SPSS Inc, Chicago, Ill). Results are presented as a box and whisker plot, showing the lower quartile, median and upper quartile as a box, and minimum and maximum values in the data set as whisker plots. Multiple observations over time were analyzed using 2-way repeated-measures analysis of variance. The other data with multiple groups were analyzed with 1-way analysis of variance followed by post hoc analysis with the Bonferroni correction for multiple comparisons.

RESULTS

Triptolide Improved Graft Quality and Inhibited Inflammatory Cascade During Ex Vivo Lung Perfusion

As a representation of lung physiologic and functional status, the graft's P/F ratio, Cdyn, and PVR showed an overall trend of declining performance over the duration of EVLP in control lungs, with an exacerbation in the last hour of perfusion. Conversely, the same parameters were significantly improved in the TL-treated lungs. Furthermore, both the Cdyn and PVR values remained steady throughout 4 hours of EVLP (Figure 1, *A-C*). No marked difference in lung tissue architecture was observed between the groups with hematoxylin–eosin staining for the lung tissues after 4 hours EVLP (Figure 1, *D*).

Triptolide Blocked Nuclear Factor-κB Signaling Pathway in Lung Grafts During Ex Vivo Lung Perfusion

Lung tissue after 4 hours EVLP showed significantly higher expression levels of TNF-α, IL-1β, IL-6, and MCP-1

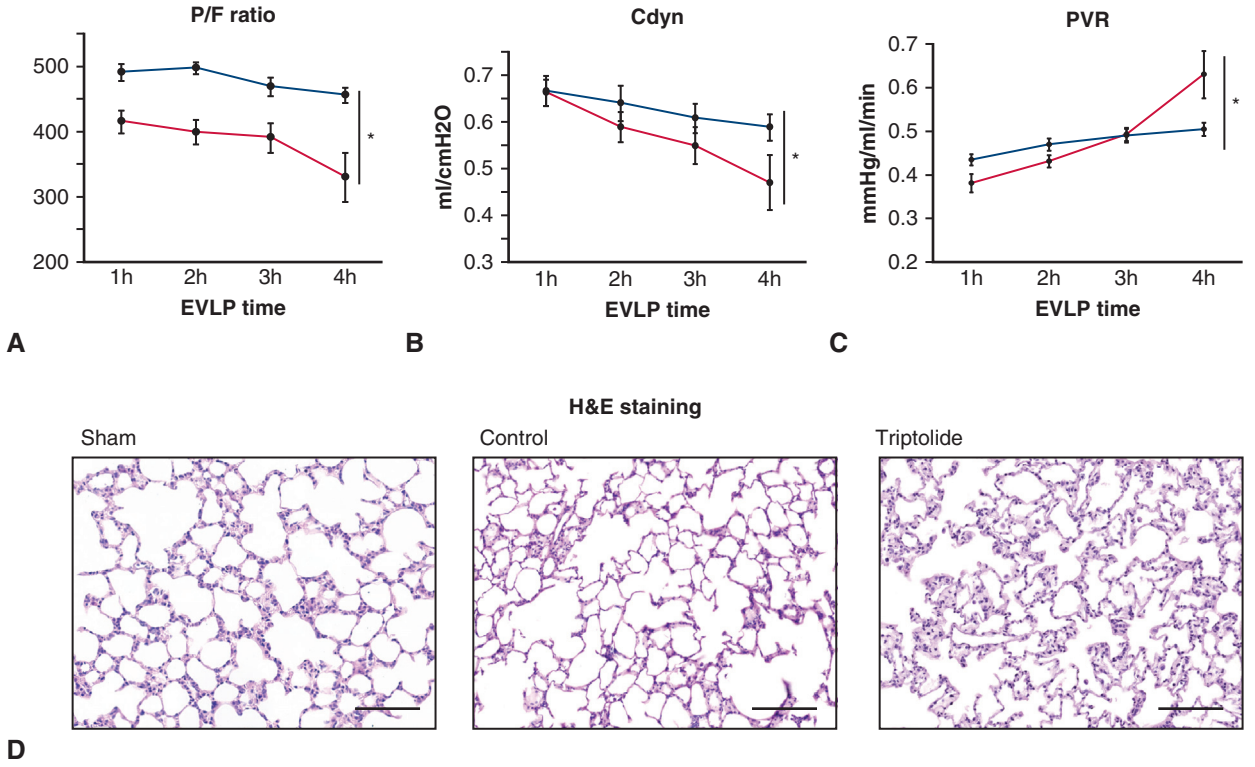


FIGURE 1. TL improved EVLP parameters and graft quality. TL administration improved functional and physiologic graft parameters measured during EVLP including (A) P/F ratio, (B) Cdyn, and (C) PVR. n = 9 for each group. *Red line*: control, *blue line*: TL. *$P < .05$. D, Representative images of histopathologic analysis of the lungs after 4 hours EVLP. Scale bar = 100 μm. *P/F*, Pao_2/Fio_2 ratio; *EVLP*, ex vivo lung perfusion; *Cdyn*, dynamic lung compliance; *PVR*, pulmonary vascular resistance; *H&E*, hematoxylin–eosin.

mRNA compared with the sham group. TL treatment significantly suppressed gene expression of these inflammatory cytokines in lung tissue after 4 hours EVLP except for IL-1β, which exhibited a similar expression profile as sham group (Figure 2, *A*). To examine TL's inhibitory effect on proinflammatory pathways, we focused on NF-kB signaling after EVLP. ChIP analysis showed the binding of p65 NF-κB to the promoters of *MCP-1* gene in control-treated lungs; however, TL inhibited p65 NF-κB binding in the lung tissue after 4 hours of EVLP (Figure 2, *B*). In addition, the proportion of phosphorylated to total IκBα expressed in the TL group was significantly lower than in the control group (Figure 2, *C*).

Triptolide Attenuated Oxidative Stress and Improved Mitochondrial Function Through Metabolic Modulation in Lungs Exposed to Ex Vivo Lung Perfusion

Hypoxia is associated with cellular glucose metabolism and simultaneously creates a proinflammatory microenvironment through increased reactive oxygen species and mitochondrial dysfunction/damage, as well as NF-κB activation.[16-18] Accordingly, we assessed whether TL could improve mitochondrial function of lung grafts on EVLP. As shown in Figure 3, *A* and *B*, glucose levels declined and ionized calcium increased over time in perfusate during EVLP in control lungs, whereas TL significantly attenuated the change of these metabolic parameters of the lungs during EVLP. EPR spectroscopy, a technique used to detect free radicals, and thus used as a measurement of oxidative stress, showed the EPR signal amplitudes in perfusate were augmented between 1 hour and 4 hours during EVLP in control lungs; however, TL treatment during EVLP significantly attenuated this signal augmentation (Figure 3, *C*). Also, mitochondrial complex I activity from lung tissues treated with TL after EVLP was significantly higher compared with control lung tissues (Figure 3, *D*). Furthermore, mRNA expression profiles of *HIF-1α*, glucose transporter *1*, heme oxygenase-*1* (both HIF-1α-regulated), and lysyl oxidase were significantly upregulated in control tissues compared with TL-treated tissues after 4 hours EVLP (Figure 3, *E*).

Pretreatment With Triptolide on Ex Vivo Lung Perfusion Improved Post-Transplant Graft Function and Reduced Graft Inflammation

The P/F ratio, as a marker of graft function, was significantly higher in the TL-pretreated lungs versus

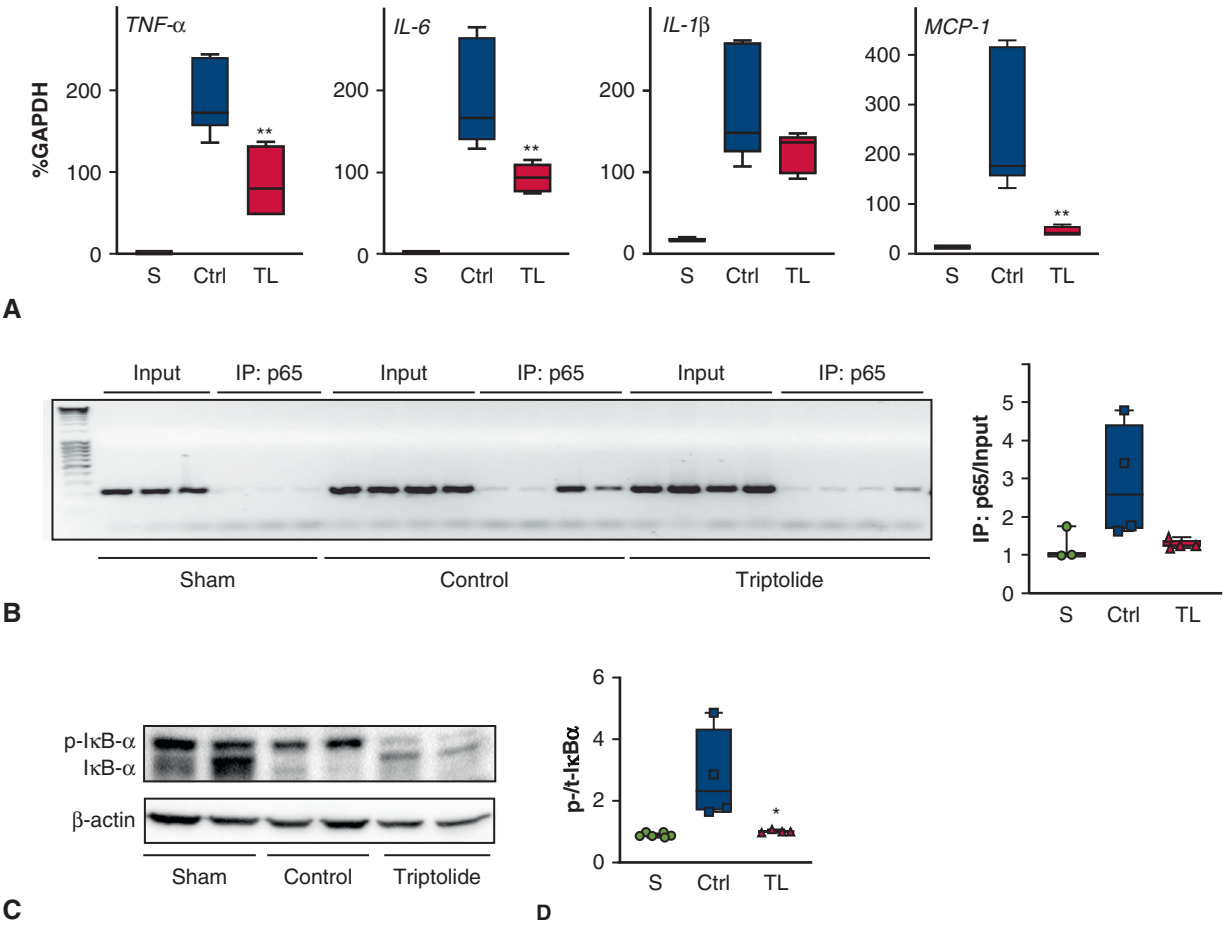


FIGURE 2. Analysis of proinflammatory profile and NF-κB signaling in the lung grafts after EVLP. TL treatment during EVLP decreased the mRNA levels of proinflammatory cytokines including (A) TNF-α, IL-6, IL-1β, and MCP-1 in lung tissue after 4 hours of EVLP. n = 5 for each group. $^{**}P < .01$. B, ChIP assay showed TL inhibited NF-κB DNA binding to *MCP-1* gene promoters. The different samples were loaded into each lane, and the samples were lined on the gel as the same order of input and IP:p65 for each group. Quantified data for polymerase chain reaction normalized by input are shown on the right of picture and shown as a box-whisker plot. n = 3-4 for each group. Input: Input DNA samples, IP:p65: samples after ChIP. C, Representative image of western blotting for IκB-α and β-actin and (D) quantified data for IκB-α indicated TL inhibited the κB activation in lung tissue after EVLP. The different samples were loaded in each lane and IκB-α and β-actin were imaged on the same gel. n = 4 for each group. $^{*}P < .05$, $^{**}P < .01$ versus control. *GAPDH*, glyceraldehyde–phosphate dehydrogenase; *TNF*, tumor necrosis factor; *S*, sham; *Ctrl*, control; *TL*, triptolide; *IL*, interleukin; *MCP*, monocyte chemoattractant protein; *IκB-α*, inhibitor of κB-α.

control lungs 2 hours after transplantation (Figure 4, *A*). TL-pretreated lungs also showed significantly lower mRNA expression levels of proinflammatory cytokines *TNF-α, IL-6, IL-1β*, and *MCP-1* after transplantation compared with lungs in the control group (Figure 4, *C-F*). In addition, wet to dry ratio of lung tissues 2 hours after transplant was significantly higher in the control group compared with the sham group. However, pretreatment with TL during EVLP markedly decreased wet to dry ratio compared with control lungs 2 hours post-transplant (Figure 4, *G*). In agreement with wet to dry ratio data, histopathologic findings indicated increased congested alveolar wall and alveoli edema fluid in control group transplanted grafts compared with sham group lungs, whereas these characteristics presented less frequently in the TL group transplanted grafts (Figure 4, *B*). Also, intragraft EPR signal amplitudes of control-treated transplant grafts were significantly higher than those of sham group lungs, whereas TL-treated transplanted grafts demonstrated significantly attenuated EPR signal amplitudes versus control-treated lung transplant tissues (Figure 4, *H*).

DISCUSSION

With a well-established rat EVLP transplant model based on current clinical EVLP settings requiring 3 to 6 hours for graft evaluation,[6,19] we first used TL as a preconditioning agent in the setting of organ transplant and found that 4 hours of graft EVLP treatment with TL contributed to improved lung graft quality with attenuated tissue inflammation. Also, we demonstrated that TL reduced anaerobic

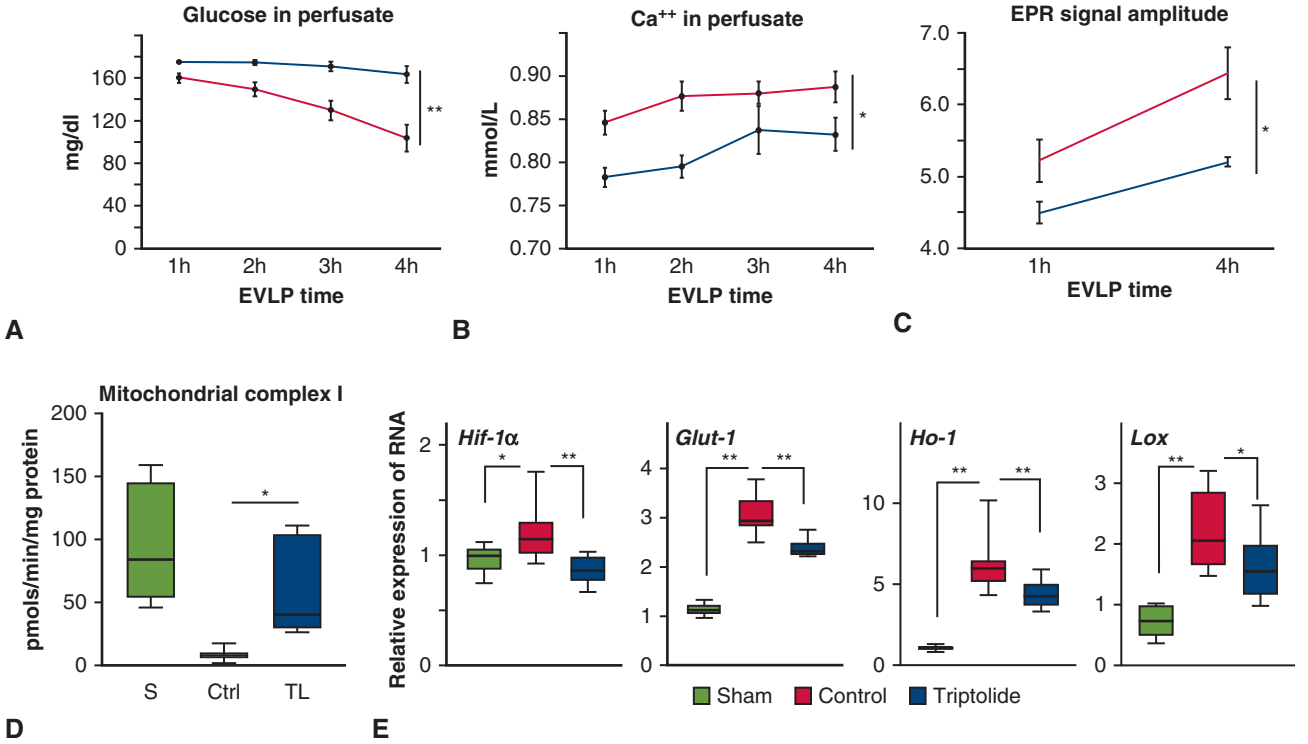


FIGURE 3. Metabolic/hypoxic alteration and oxidative stress alleviation by TL. Time course of (A) glucose and (B) ionized calcium and (C) the levels of free radical detected as EPR signal amplitude in perfusate during EVLP. n = 5 for each. *Red line*: Control, *blue line*: TL. *$P < .05$, **$P < .01$. D, Complex I activity in mitochondria isolated from tissue after 4 hours EVLP. n = 5 for each. *$P < .05$. E, The mRNA levels of hypoxia related genes including HIF-1α, glucose transporter 1, heme oxygenase-1, and lysyl oxidase in lung tissue after 4 hours of EVLP. n = 5 for each group. *$P < .05$, **$P < .01$. *EVLP*, Ex vivo lung perfusion; *EPR*, electron paramagnetic resonance; *S*, sham; *Ctrl*, control; *TL*, triptolide.

metabolism and oxidative stress in lung grafts during EVLP via inhibition of NF-κB signaling, potentially through downregulation of hypoxia signaling. Moreover, these favorable graft preconditioning effects by TL during EVLP were sustained, resulting in superior post-transplant graft function and quality.

Pharmacologic Ex Vivo Lung Perfusion Approaches Targeting Graft Inflammation to Prevent Post-Transplant–Related Adverse Events

Donor/graft intervention is well acknowledged to improve post-transplant outcomes using various strategies, including gene transfection, regenerative medicine, and surgery; however, each of these strategies comes with inherent limitations and challenges.[20] A promising interventional approach that has gained popularity is the delivery of pharmacologic agents to the lungs before transplantation. Treatment with pharmacologic intervention on EVLP is a highly feasible option in the clinical setting and allows the ability to move forward from bench to bedside in a relatively straightforward manner. Proposed reasons for the enthusiasm underlying pharmacologic reconditioning on EVLP include (1) ease of administration; (2) ability to titrate doses to maximize desired effects; (3) the option to retain or wash out the reagent/drug of interest; and (4) for drugs with narrow therapeutic indices, the benefit of shielding other organs from adverse consequences.[21] Various drugs/prodrugs have been tested on EVLP to treat/control inflammation, a key factor reported in preclinical EVLP.[1] Nevertheless, EVLP is not without its challenges because ex vivo creates a proinflammatory environment, suboptimal for lung quality and performance. For example, grafts undergoing EVLP have been found to demonstrate a heightened proinflammatory state. It was reported that perfusate cytokine levels such as IL-6, IL-8, and MCP-1 significantly increased with time during EVLP.[2] Also, the DEVELOP-UK trial found that increased IL-1β perfusate levels were found to be a potential predictive biomarker for post-transplant outcomes.[22] In addition, our previous EVLP work in rats demonstrated that the graft itself was a major source of inflammatory cytokines and that graft/perfusate cytokine levels were significantly increased, suggesting marginal lungs may be particularly susceptible on EVLP and may express higher cytokine levels than those normal-quality lungs.[3] On the basis of these findings, targeting inflammation during pharmacologic preconditioning could contribute to better transplant outcomes. In this

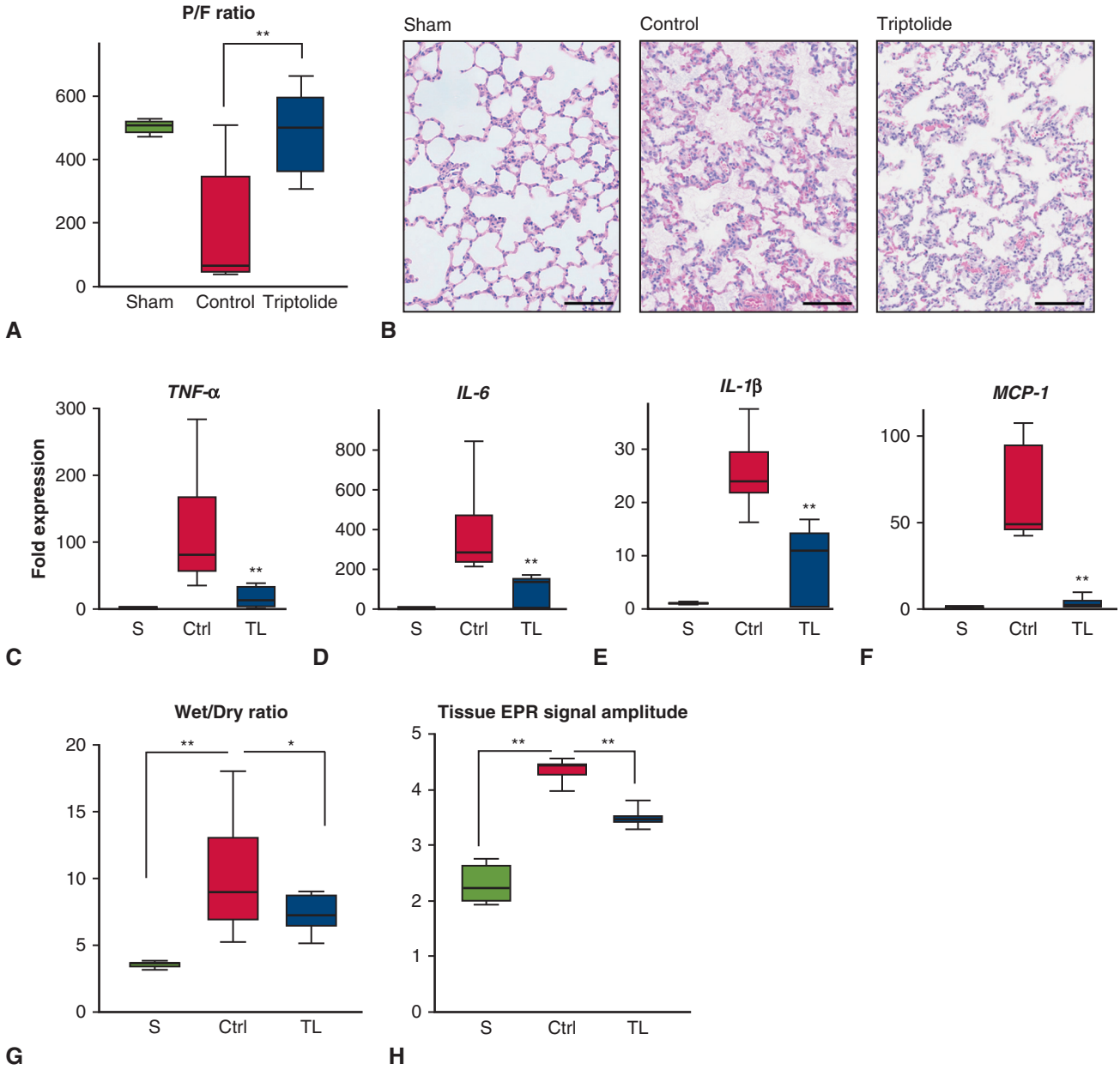


FIGURE 4. Assessment of lung grafts after transplantation. A, Improved lung function shown as P/F ratio in grafts 2 hours post-transplantation after 4 hours of EVLP with TL preconditioning. n = 5 for each group. $^{**}P < .01$ versus control. B, Representative images of hematoxylin-eosin–stained lung grafts 2 hours post-transplantation. Scale bar = 100 μm. C-F, Real-time reverse transcription-polymerase chain reaction for proinflammatory mediators in lung grafts 2 hours post-transplantation after 4 hours of EVLP, including (C) TNF-α, (D) IL-6, (E) IL-1β, and (F) MCP-1. n = 5 for each group. $^{**}P < .01$ versus control. G, Wet-to-dry (Wet/Dry) weight ratio of graft tissue 2 hours post-transplant after 4 hours of EVLP was improved by TL preconditioning. n = 5 for each group. H, Preconditioning of TL decreased EPR signal amplitude indicating free radical level in post-transplant lung grafts. n = 4 for each group. $^{*}P < .05$, $^{**}P < .01$. *P/F*, Pao_2/Fio_2 ratio; *TNF*, tumor necrosis factor; *S*, sham; *Ctrl*, control; *TL*, triptolide; *IL*, interleukin; *MCP*, monocyte chemoattractant protein; *EPR*, electron paramagnetic resonance.

study, we examined the efficacy of TL as a preconditioning EVLP agent and successfully demonstrated that TL decreases EVLP-related inflammatory responses in lung grafts, resulting in enhanced early post-transplant graft function and quality. Additionally, we showed post-transplant graft dysfunction as a primary outcome indicator resulted directly from multiple adverse factors, including reactive oxygen species generation and edema

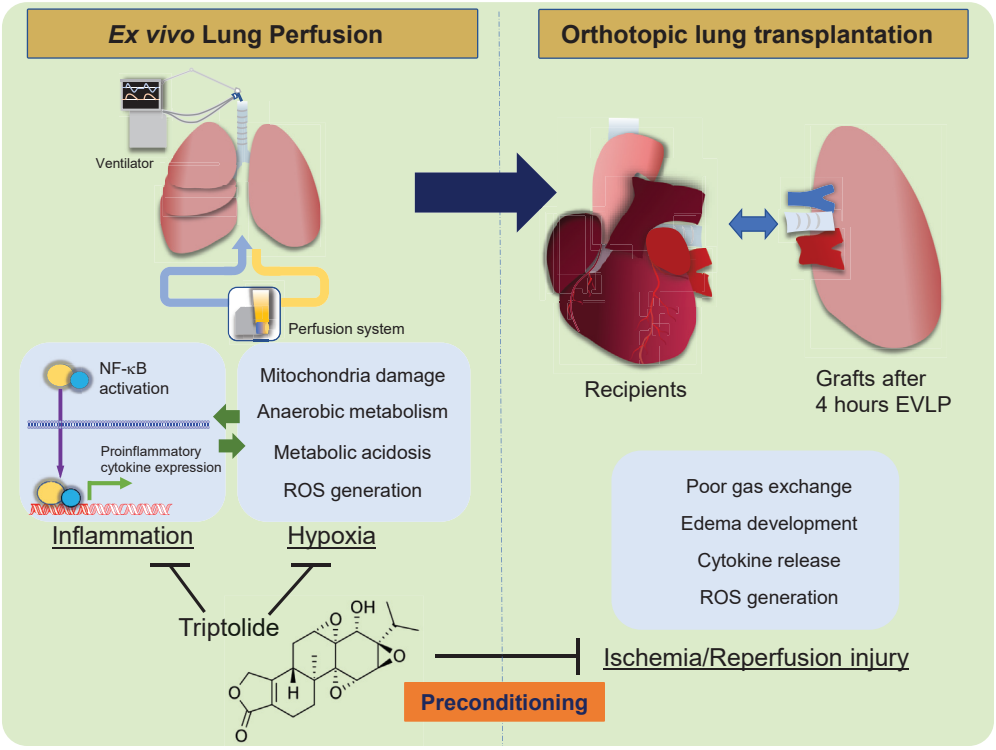


FIGURE 5. EVLP creates a proinflammatory and hypoxic environment in lung grafts potentially resulting in poor graft quality after transplantation. TL can effectively attenuate these adverse events, thus improving clinical outcomes after transplantation. *NF-κB*, Nuclear factor-κB; *ROS*, reactive oxygen species; *EVLP*, ex vivo lung perfusion.

development. Our findings suggest that TL can be used to reduce localized lung graft inflammation, thereby resulting in better transplant outcomes.

Potential Mechanism for Anti-Inflammatory Effect of Triptolide

The anti-inflammatory effects of TL have been well documented both in vitro and in vivo; however, the precise mechanism has not been clearly elucidated.[23,24] Various intracellular molecules have been identified as targets of TL, suggesting TL predominantly exerts its pleiotropic effects via binding intracellular molecular targets including NF-κB. TL-induced inhibition of the NF-κB signaling pathway via blocking the translocation of NF-κB into the nucleus, obstructing NF-κB DNA binding, and inhibiting IκB phosphorylation has been demonstrated.[24-26] Similar findings were obtained in this study as we showed that TL decreased NF-κB p65 DNA binding activity and IκB phosphorylation in the lungs after 4 hours of EVLP. As a result of these findings, we focused our attention on the activator of NF-kB signaling pathway and investigated hypoxia-related events of lung grafts on EVLP.

Graft hypoxia–related events such as hyperglycolysis, mitochondrial damage, and reactive oxygen species generation can be associated with de novo graft inflammation on EVLP.[6,7] Glucose consumption has been reported as a risk factor for the development of graft edema development, ultimately affecting clinical decision-making regarding graft use for transplantability after EVLP.[5] Accordingly, we have reported that this inflammatory state may be associated with glucose consumption of lung grafts during EVLP.[27] Additionally, we have previously demonstrated that this elevated graft glucose consumption requires lower perfusate pO_2 (deoxygenation in pulmonary artery flow) within an acellular perfusate setting,[6] suggesting current clinical protocol for acellular EVLP could lead to graft hypoxia. Although metabolic change, hypoxia, and inflammation are often discussed together and well linked to each other, hypoxia is known activate NF-kB signaling and create a proinflammatory environment.[16,17] TL has been shown to inhibit hypoxia signaling or HIF-1α accumulation and even improve hypoxia status.[13] In this study, we demonstrated that TL diminished the hypoxic response in healthy rat lungs. Specifically, TL decreased glucose metabolism, hypoxia-related gene expression, and the overall lung proinflammatory environment during EVLP, suggesting that TL may improve oxygen status in lung grafts during EVLP. Furthermore, we showed that TL treatment improved lung mitochondrial complex I activity after 4 hours EVLP. Mitochondria are critical organelles in which the biochemical processes of respiration and energy production occur, and thus are

extremely sensitive to cellular oxygenation status. It is recognized that mitochondrial protection during transplant process is a key factor to obtain better post-transplant outcomes.[28-30] Our findings in this study suggest that TL helps maintain mitochondrial function and overall improved graft quality and viability.

Related to mitochondrial damage, we found that TL attenuated calcium elevation in lung perfusate during EVLP. This elevation is a reflection of intracellular calcium influx, which controls homeostasis and is an indicator of cellular stress. We initially investigated endoplasmic reticulum stress in the lungs after EVLP, although no significant protein expression changes were observed for C/EBP homologous protein and phosphorylated eukaryotic translation initiation factor 2A in lungs ± TL treatment as well as gene expression for C/EBP homologous protein, protein phosphatase 1 regulatory subunit 15A (GADD34), and Trib3 (data are not shown). Consequently, we considered this perfusate calcium increase as a direct response of the mitochondria to hypoxia-induced stress as we previously reported,[31] and TL decreased their stress, which may have resulted in reduced oxidative stress in the grafts during EVLP. On the basis of our findings, it appears that, mechanistically, the anti-inflammatory effects of TL are mediated through the attenuation of NF-kB/hypoxia signaling, resulting in enhanced oxygenation status in lung tissue during EVLP.

Dual Action of Triptolide: Anti-Cancer and Anti-Inflammatory Properties

Although TL has therapeutic effects against inflammatory diseases as discussed, it is also well known for its proapoptotic effects both in vitro and in vivo.[32] We previously demonstrated in A549 lung cancer cells that TL exerts its anticancer activity through a mitochondrial-related pathway in a p53-dependent manner.[14] In the absence of p53, we found that TL interrupts mitochondrial function via Sirtuin 3 modulation. However, in this study, we found that TL improved mitochondrial function in normal lung tissue on EVLP, suggesting that TL-induced cell death could be targeted in a cancer setting, and that TL may switch between anticancer and anti-inflammation effects depending on p53 expression. The role of p53 regarding inflammation has been well investigated,[33] and it is known that both hypoxia and inflammation increase p53 transcriptional activity.[34] Future investigation into a potential switching mechanism of TL may be important to understanding its efficacy against cancer or inflammation and to enhancing those effects with combination therapy.

Study Limitations

In this study, we have little information regarding the pharmacokinetics of TL during EVLP and did not investigate pharmaceutical drug delivery or cellular internalization of TL. Because TL is more soluble in dimethyl sulfoxide than aqueous solvents, treatment with Minnelide, a water-soluble form of TL, may maximize the impact in the lung grafts on EVLP. In addition, our ex vivo studies did not include a TL dose-response evaluation, because our single dose of TL (100 nM) was based on our previous in vitro experiments.[14] Perhaps pharmacologic preconditioning during EVLP with lower doses of TL may produce similar effects as demonstrated in this article, potentially reducing off-target cellular apoptosis. Finally, our rat model does have notable limitations as described previously.[7] In this study, we focused specifically on acute phase changes after transplantation, although studies involving chronic changes of graft function will likely require a smaller (mouse) animal model. Clearly, further studies are warranted to address these study limitations.

CONCLUSIONS

TL may serve as a therapeutic compound for enhanced lung graft preservation and viability during EVLP to increase donor availability for lung transplantation. Specifically, we found that TL attenuated graft hypoxia and inflammation during 4 hours of EVLP and that (more important) these preconditioning effects were maintained 2 hours post-transplantation. (Figure 5). Through pharmacologic preconditioning, we hope that an increased number of marginal lungs may be sufficiently recovered, leading to improved long-term outcomes after transplantation.

Conflict of Interest Statement

Authors have nothing to disclose with regard to commercial support.

The authors thank Dr John Ryan of the Department of Cardiothoracic Surgery, University of Pittsburgh, for supporting statistical analysis of our data, and Drs Ashok Saluja and Vikas Dudeja, and their research groups for their collaboration as part of this work.

References

1. Tane S, Noda K, Shigemura N. Ex vivo lung perfusion: a key tool for translational science in the lungs. *Chest*. 2017;151:1220-8.
2. Sadaria MR, Smith PD, Fullerton DA, Justison GA, Lee JH, Puskas F, et al. Cytokine expression profile in human lungs undergoing normothermic ex-vivo lung perfusion. *Ann Thorac Surg*. 2011;92:478-84.
3. Noda K, Tane S, Haam SJ, D'Cunha J, Hayanga AJ, Luketich JD, Shigemura N, et al. Targeting circulating leukocytes and pyroptosis during ex vivo lung perfusion improves lung preservation. *Transplantation*. 2017;101:2841-9.
4. Andreasson AS, Karamanou DM, Gillespie CS, Özalp F, Butt T, Hill P, et al. Profiling inflammation and tissue injury markers in perfusate and bronchoalveolar lavage fluid during human ex vivo lung perfusion. *Eur J Cardiothorac Surg*. 2017;51:577-86.
5. Valenza F, Rosso L, Pizzocri M, Salice V, Umbrello M, Conte G, et al. The consumption of glucose during ex vivo lung perfusion correlates with lung edema. *Transplant Proc*. 2011;43:993-6.

6. Noda K, Tane S, Haam SJ, Hayanga AJ, D'Cunha J, Luketich JD, Shigemura N, et al. Optimal ex vivo lung perfusion techniques with oxygenated perfusate. *J Heart Lung Transplant*. 2017;36:466-74.
7. Tanaka Y, Noda K, Isse K, Tobita K, Maniwa Y, Bhama JK, et al. A novel dual ex vivo lung perfusion technique improves immediate outcomes in an experimental model of lung transplantation. *Am J Transplant*. 2015;15:1219-30.
8. Tikkanen JM, Cypel M, Machuca TN, Azad S, Binnie M, Chow CW, et al. Functional outcomes and quality of life after normothermic ex vivo lung perfusion lung transplantation. *J Heart Lung Transplant*. 2015;34:547-56.
9. Saito T, Takahashi H, Kaneda H, Binnie M, Azad S, Sato M, Waddell TK, et al. Impact of cytokine expression in the pre-implanted donor lung on the development of chronic lung allograft dysfunction subtypes. *Am J Transplant*. 2013;13:3192-201.
10. Huang HJ, Yusen RD, Meyers BF, Walter MJ, Mohanakumar T, Patterson GA, et al. Late primary graft dysfunction after lung transplantation and bronchiolitis obliterans syndrome. *Am J Transplant*. 2008;8:2454-62.
11. Cypel M, Liu M, Rubacha M, Yeung JC, Hirayama S, Anraku M, Sato M, et al. Functional repair of human donor lungs by IL-10 gene therapy. *Sci Transl Med*. 2009;1:4ra9.
12. Ziaei S, Halaby R. Immunosuppressive, anti-inflammatory and anti-cancer properties of triptolide: a mini review. *Avicenna J Phytomed*. 2016;6:149-64.
13. McGinn O, Gupta VK, Dauer P, Arora N, Sharma N, Nomura A, et al. Inhibition of hypoxic response decreases stemness and reduces tumorigenic signaling due to impaired assembly of HIF1 transcription complex in pancreatic cancer. *Sci Rep*. 2017;7:7872.
14. Kumar A, Corey C, Scott I, Shiva S, D'Cunha J. Minnelide/Triptolide impairs mitochondrial function by regulating SIRT3 in P53-dependent manner in non-small cell lung cancer. *PLoS One*. 2016;11:e0160783.
15. Woo J-I, Kil S-H, Oh S, Lee YJ, Park R, Lim DJ, Moon SK, et al. IL-10/HMOX1 signaling modulates cochlear inflammation via negative regulation of MCP-1/CCL2 expression in cochlear fibrocytes. *J Immunol*. 2015;194:3953-61.
16. Culver C, Sundqvist A, Mudie S, Melvin A, Xirodimas D, Rocha S. Mechanism of hypoxia-induced NF-κB. *Mol Cell Biol*. 2010;30:4901-21.
17. Eltzschig HK, Carmeliet P. Hypoxia and inflammation. *N Engl J Med*. 2011;364:656-65.
18. McGarry T, Biniecka M, Veale DJ, Fearon U. Hypoxia, oxidative stress and inflammation. *Free Radic Biol Med*. 2018;125:15-24.
19. Machuca TN, Cypel M. Ex vivo lung perfusion. *J Thorac Dis*. 2014;6:1054-62.
20. Feng S. Donor intervention and organ preservation: where is the science and what are the obstacles? *Am J Transplant*. 2010;10:1155-62.
21. Karimian N, Yeh H. Opportunities for therapeutic intervention during machine perfusion. *Curr Transplant Rep*. 2017;4:141-8.
22. Andreasson ASI, Borthwick LA, Gillespie C, Jiwa K, Scott J, Henderson P, et al; DEVELOP-UK Investigators. The role of interleukin-1β as a predictive biomarker and potential therapeutic target during clinical ex vivo lung perfusion. *J Heart Lung Transplant*. 2017;36:985-95.
23. Zhao G, Vaszar LT, Qiu D, Shi L, Kao PN. Anti-inflammatory effects of triptolide in human bronchial epithelial cells. *Am J Physiol Lung Cell Mol Physiol*. 2000;279:L958-66.
24. Zheng L, Jia J, Dai H, Wan L, Liu J, Hu L, et al. Triptolide-assisted phosphorylation of p53 suppresses inflammation-induced NF-κB survival pathways in cancer cells. *Mol Cell Biol*. 2017;37. e00149-17.
25. Qiu D, Zhao G, Aoki Y, Shi L, Uyei A, Nazarian S, et al. Immunosuppressant PG490 (triptolide) inhibits T-cell interleukin-2 expression at the level of purine-box/nuclear factor of activated T-cells and NF-κB transcriptional activation. *J Biol Chem*. 1999;274:13443-50.
26. Nomura A, Majumder K, Giri B, Dauer P, Dudeja V, Roy S, et al. Inhibition of NF-kappa B pathway leads to deregulation of epithelial–mesenchymal transition and neural invasion in pancreatic cancer. *Lab Invest*. 2016;96:1268.
27. Noda K, Haam S, D'Cunha J, Luketich JD, Shigemura N. Relationship between grafts' quality, glucose consumption and IL-6 production during ex vivo lung perfusion. *J Heart Lung Transplant*. 2016;35:S190.
28. Noda K, Burki S, Kumar A, D'Cunha J. Cyclosporin A: teaching an old drug new tricks? *Transplantation*. 2019;103:1084-5.
29. Sommer SP, Sommer S, Sinha B, Wiedemann J, Otto C, Aleksic I, et al. Ischemia-reperfusion injury-induced pulmonary mitochondrial damage. *J Heart Lung Transplant*. 2011;30:811-8.
30. Jassem W, Fuggle SV, Rela M, Koo DDH, Heaton ND. The role of mitochondria in ischemia/reperfusion injury. *Transplantation*. 2002;73:493-9.
31. Noda K, Haam S, D'Cunha J, Luketich JD, Bermudez CA, Shigemura N. Mitochondrial calcium: the missing link between hypoxia and quality in lung grafts during ex vivo lung perfusion. *J Heart Lung Transplant*. 2015;34:S268.
32. Noel P, Von Hoff DD, Saluja AK, Velagapudi M, Borazanci E, Han H. Triptolide and its derivatives as cancer therapies. *Trends Pharmacol Sci*. 2019;40:327-41.
33. Gudkov AV, Gurova KV, Komarova EA. Inflammation and p53: a tale of two stresses. *Genes Cancer*. 2011;2:503-16.
34. Sermeus A, Michiels C. Reciprocal influence of the p53 and the hypoxic pathways. *Cell Death Dis*. 2011;2:e164.

Key Words: lung transplantation, ex vivo lung perfusion, graft preconditioning, triptolide, inflammation