In vitro modeling of nonhypoxic cold ischemia–reperfusion simulating lung transplantation

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Objective: Although anoxia/reoxygenation of cultured cells has been used to model lung ischemia–reperfusion injury, this does not accurately mimic events experienced by lung cells while a lung is retrieved from a donor, stored, and transplanted. We developed an in vitro model of nonhypoxic ischemia–reperfusion injury to simulate these events.

Methods: Human umbilical vein endothelial cells underwent simulated cold ischemia by replacing 37°C culture media with 4°C Perfadex (Vitrolife, Kungsbäcka, Sweden) solution for 5 hours in 100% O₂. Culture dishes were allowed to warm to room temperature for 1 hour (implantation), and then Perfadex solution was replaced with 37°C culture media (reperfusion).

Results: During cold ischemia, the human umbilical vein endothelial cell filamentous actin cytoskeleton quickly became rearranged, and gaps developed in the previously confluent monolayer occupying 20% of the surface area. Simulated reperfusion resulted in reorganization to a confluent monolayer. Development of gaps was not due to enhanced necrosis based on lactate dehydrogenase retention assay. Endothelial cytoskeletal rearrangement could account for early edema caused by ischemia–reperfusion injury with reperfusion. Mitogen-activated protein kinase and nuclear factor κ B activation occurred with simulated reperfusion despite normoxia. Levels of the proinflammatory cytokines interleukin 6 and interleukin 8 were significantly increased in media at the end of reperfusion.

Conclusions: Exposing human umbilical vein endothelial cells to simulated cold ischemia without hypoxia causes reversible cytoskeletal alterations, activation of inflammatory pathways, and elaboration of cytokines. Because this model accurately depicts events occurring during lung transplantation, it will be useful to explore mechanisms regulating lung cell response to this unique form of ischemia–reperfusion injury.

Lung transplantation (LTX) is appropriate for patients with end-stage lung disease. However, transplant success is limited by a shortage of suitable donor lungs, early posttransplantation lung dysfunction, and later bronchiolitis obliterans syndrome (BOS). A potential solution to the lung donor shortage is the use of lungs recovered from non-heart-beating donors (patients who have died suddenly of cardiac arrest outside the hospital or in the emergency department),^{1–3} but

this strategy is associated with development of pulmonary edema and impaired gas exchange in recipient animals.⁴ Early graft failure is a major cause of morbidity and the leading cause of early mortality after LTX.⁵ Early graft dysfunction is a risk for development of BOS.⁶ Thus improved understanding of lung ischemia–reperfusion injury (IRI) would benefit recipients of conventional LTX and facilitate transplantation of lungs recovered from non-heart-beating donors and might reduce the risk of BOS.

IRI is a coordinated series of events involving components of the innate and adaptive immune systems.⁷ A hallmark of lung IRI is pulmonary edema caused by increased permeability of endothelial cells⁸ associated with inflammation and elaboration of proinflammatory cytokines. The cellular and molecular mechanisms responsible for the phenotype of the injury remain unknown.

The goal of our study was to develop an in vitro cell culture model of IRI that realistically mimics the sequence of events experienced by human endothelial cells during lung retrieval, cold storage, and transplantation.

MATERIALS AND METHODS

Cell Culture

Human umbilical vein endothelial cells (HUVECs; Cambrex Bio Science Walkersville, Inc, Walkersville, Md) were seeded at a density

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Abbreviations and Acronyms

AM	= alveolar macrophage
BOS	= bronchiolitis obliterans syndrome
ERK	= extracellular signal-regulated kinase
HMVEC	= human pulmonary microvascular endothelial cell
HUVEC	= human umbilical vein endothelial cell
I κ B α	= inhibitor of nuclear factor κ B, α
IL	= interleukin
IRI	= ischemia–reperfusion injury
JNK	= Janus kinase
LDH	= lactate dehydrogenase
LTX	= lung transplantation
MAPK	= mitogen-activated protein kinase
NF	= nuclear factor
PBS	= phosphate-buffered saline
TLR	= Toll-like receptor

of 2500 cells/cm² on collagen-coated (Invitrogen, San Diego, Calif) 100-mm-diameter cell culture dishes; maintained in Clonetics EGM-2 BulletKits medium (Cambrex) containing 1.8% fetal bovine serum, growth factors, heparin, hydrocortisone, and gentamicin/amphotericin B at 37°C in a humidified incubator in 95% room air/5% CO₂; and grown to confluence. Cells were subcultured on similar dishes at 2500 cells/cm² after enzymatic digestion with 0.1% trypsin in 1 mmol/L ethylenediamine tetra-acetic acid for 5 minutes at 37°C and used between the third and tenth passages.

Model of Simulated IRI

HUVECs on 35-mm-diameter glass-bottom dishes with integral coverslips (MatTek Corp, Ashland, Mass) grown until 100% confluent were placed inside a sealed Plexiglas chamber modified to simulate the rapid changes that occur during the course of lung recovery, storage, implantation, and reperfusion, as depicted in Figure 1, A. Cell culture medium (37°C) was suddenly replaced with cold Perfadex (Vitrolife, Kungsbacka, Sweden) buffered with Tham (Abbott Pharmaceuticals, Chicago, Ill) to pH 7.2. The Plexiglas chamber was maintained at 4°C on ice, and the box was ventilated with 100% O₂ at 1 L/min. It took 1 hour for the temperature to reach 4°C after replacement of media with Perfadex. After 4 additional hours of simulated cold ischemia, the Plexiglas chamber was allowed to warm to room temperature for 1 hour to simulate implantation. To simulate reperfusion, Perfadex was suddenly replaced with 37°C culture media, and the Plexiglas chamber was ventilated with 95% room air/5% CO₂ in a 37°C oven. Dishes were removed from the chamber in triplicate at different time points throughout the experiment and immediately fixed in 4% paraformaldehyde. Cells maintained in EGM2- medium at 37°C in a humidified 5% CO₂ incubator were used as 24-hour controls; medium was changed at the same time points when medium or Perfadex was changed in experimental dishes. The time line of the experiment is depicted in Figure 1, B. Probes inserted through sealed ports continuously recorded temperature, pH, and PO₂ values in 1 representative cell culture dish on a PC by using Pico Software (Pico, St Neots, United Kingdom).

Phalloidin Staining and Image Analysis

HUVECs were fixed in 4% paraformaldehyde for 10 minutes at room temperature and washed 3 times with phosphate-buffered saline (PBS). Dishes were stored at 4°C and stained the following day for F-actin filaments by using Alexa Fluor 568 phalloidin (Invitrogen, Eugene, Ore). Cells were incubated for 1 hour at room temperature in the dark with a 1:100 dilution

of phalloidin in 1% bovine serum albumin/PBS/0.05% Tween 20 and then rinsed 3 times with PBS. Coverslips were immediately examined with a Leica DM IRB Inverted Fluorescence/DIC microscope (Leica Microsystems, Wetzlar, Germany) at 200 $\times$ and 400 $\times$ magnification and a confocal microscope at 400 $\times$ magnification, and 3 photographs were taken to evaluate cell shape and F-actin cytoskeleton. The gap surface area was quantified on grayscale images with MetaMorph software (Universal Imaging Corp, Downingtown, Pa). Each cell was labeled by a masked observer as “normal” or “abnormal” with respect to actin filaments. Normal cells had actin fibers at the periphery and crossing the cell, whereas abnormal cells had no central actin filaments; actin was either not apparent or visualized only in the cell periphery. Labeled cells were quantified by using MetaMorph software. Features of the actin cytoskeleton were evaluated in groups by a masked observer (KB, an actin cytoskeletal expert).

Cell Viability Assay

In separate experiments at different time points during simulated IRI, cells and cell culture media were assessed for lactate dehydrogenase (LDH) activity by using the CytoTox96 Non-Radioactive Cytotoxicity Assay (Promega, Madison, Wis) per the manufacturer’s instructions. Cell viability was expressed based on cytoplasmic LDH as a percentage of total LDH. Culture media and Perfadex were used as background controls to normalize the absorbance value.

Protein Extraction and Immunoblotting

At various time points, HUVECs cultured on 60-mm collagen-coated culture dishes until 100% confluent were subjected to simulated IRI, protein was extracted, and Western blots were performed, as previously described.⁹ Monoclonal antibody to phosphorylated p38 was from BD Biosciences PharMingen (San Jose, Calif). Polyclonal antibody to p38 and monoclonal β -actin antibodies were from Sigma–Aldrich (St Louis, Mo). Polyclonal antibodies to Janus kinase (JNK) 1/2/3, extracellular signal-regulated kinase (ERK) 1/2, and inhibitor of nuclear factor (NF) κ B, α (I κ B α) were from Cell Signaling Technology (Danvers, Mass). Secondary antibody was from Jackson ImmunoResearch Laboratories (West Grove, Pa). Immobilon Western Chemiluminescence horseradish peroxidase (HRP) substrate was from Millipore (Billerica, Mass). Films were scanned on an Epson Precision 4180 flatbed scanner (Epson, Long Beach, Calif) at 600 dpi in 16-bit grayscale with the Epson Scan software and saved in TIFF format. The densities of bands obtained from the same membrane were measured by using the 1-D Gel Scan function in the MetaMorph Software package and expressed as relative optical density to a blank lane loaded with lysis sample buffer. The phospho/total ratio for mitogen-activated protein kinases (MAPKs) and I κ B α / β -actin at each time point on each blot was normalized to the corresponding ratio of control samples on each blot by dividing each value by the control ratio. Thus the ratios of control samples for each blot were arbitrarily set to 1. Expression at each time point was compared with control 24-hour samples, cells that were maintained at 37°C in an incubator with media changed at the same times as media was changed in experimental dishes.

Interleukin 6 and Interleukin 8 Measurements

After 24 hours of “reperfusion,” cell culture media were collected from samples in triplicate, briefly centrifuged, and stored at –20°C before interleukin (IL) 6 and IL-8 levels were measured by means of enzyme-linked immunosorbent assay (ELISA), according to the manufacturer’s instructions (DuoSet ELISA Development kit; R&D Systems, Inc, Minneapolis, Minn).

Statistical Analysis

All data are reported as means $\pm$ standard error of the mean. Statistical analysis was performed with Statistica software (Statsoft, Tulsa, Okla). Analysis of variance with Tukey’s honest significant difference for post hoc corrections or the Student’s *t* test was used for data analysis. Because of large variances that

FIGURE 1. A, Time course of events that occur during lung transplantation modeled by our nonhypoxic cell culture model. Warm blood is suddenly replaced with cold preservation solution (Perfadex) flushed through the pulmonary artery after crossclamping to cool the lung to 4°C. At the time of circulatory arrest, lungs are still ventilated with 100% oxygen, and the trachea is clamped with lungs fully inflated with oxygen. After several hours of cold storage, the lung gradually rewarms to room temperature while the anastomoses are performed in the recipient (approximate time, 1 hour). Suddenly, circulation is restored, and temperature is rapidly increased to 37°C. Reprinted with permission from Koukoulis and colleagues.¹³ B, Time course of experiment. In preliminary experiments, despite the replacement of warm media with cold Perfadex, it takes approximately 1 hour for culture dishes in Plexiglas boxes to reach 4°C. C, Data from a representative experiment showing measured change in temperature, pH, and Po₂ values during simulated cold ischemia–reperfusion.

were proportional to means, normalized intensity data of Western blots were log₁₀ transformed.¹⁰ A *P* value <.05 was considered significant.

RESULTS

Cell Culture Model Reflects the Environment of Lung Tissue During IRI

The experimental plan is detailed in Figure 1, which shows that acidosis occurs but that hypoxia is not a feature of the model.

Simulated Cold Nonhypoxic Ischemia Causes Actin Cytoskeletal Rearrangement and Formation of Gaps in the Monolayer

HUVECs maintained inside the incubator at 37°C and ventilated with 95% O₂/5% CO₂ were 100% confluent and showed the typical cobblestone appearance. The filamentous actin network in untreated HUVECs consisted of peripheral and cell-spanning stress fibers. Replacement of culture media with Perfadex at 4°C resulted in rear-

range of the actin cytoskeleton, with most of the HUVECs demonstrating disappearance of F-actin stress fibers, peripheral location of stress fibers, or both (Figure 2). By using an actin cytoskeleton normality index, nearly 60% of cells were observed to be abnormal within 15 minutes of the onset of cold ischemia. F-actin rearrangement persisted through the early phase of reperfusion (15 minutes) but appeared normal after 4 hours of reperfusion, when the actin cytoskeleton normality index returned to pre-IRI levels (Figure 3, A). An observer masked to group identity noted dramatic changes in the actin stress fibers during cold ischemia and early after reperfusion (6 photos per group, 3 at 200× and 3 at 400× magnification, selected randomly), with return to a normal appearance after 4 hours of reperfusion (Table 1).

Cytoskeletal alterations were associated with formation of gaps in the confluent monolayer. After 15 minutes and 4 hours at 4°C (simulated cold ischemia), gaps were obvious and accounted for approximately 20% of the surface area

FIGURE 2. Control cells maintained in media at 37°C were 100% confluent, and actin stress fibers were evident in the cytoskeleton, with focal adhesion between cells. After 15 minutes of cold “ischemia” at 4°C (replacement of warm media with cold Perfadex), human umbilical vein endothelial cells quickly shrank, losing their junctions and disrupting the confluent monolayer with disassembly of actin filaments. This resulted in the formation of gaps in the monolayer (*white arrows*). After 4 hours of cold storage, monolayers looked the same (not shown). Early after simulated reperfusion, when Perfadex was replaced with media at 37°C, most of the stress fibers were not apparent and actin was visualized at the periphery of cells, leaving only fragments of cortical actin. After 4 hours of reperfusion, actin filaments appeared to reassemble with reorganization of the actin network. Fluorescence (*top*) and confocal microscope (*bottom*) images are shown.

(*Figure 3, B*). After 1 hour of simulated rewarming (when Plexiglas boxes were removed from the ice and allowed to rewarm to room temperature, simulating implantation time), gap area was reduced to approximately 4% of the surface area, although actin filaments were still abnormal. After simulated reperfusion (when Perfadex was replaced with 37°C cell culture media), the gaps became smaller (approximately 3.5%), and after 1 hour of reperfusion, the gap area was normal, and actin stress fibers began to reassemble with complete reorganization of the monolayer (100% confluent) after 4 hours of reperfusion. This was maintained through 24 hours after reperfusion (*Figure 3, A*).

To ensure that gap formation was not a result of cell death or loss of cellular adherence, we measured cell viability at various times throughout cold ischemia, rewarming, and reperfusion. Cell viability was maintained at approximately 90% for all times assessed during simulated IRI out to 3 hours of reperfusion (*Figure 3, C*), despite cytoskeletal alteration and formation of gaps indicating that cold ischemia–induced gap formation was not due to cell death or detachment. Detached cells would be in the supernatant and lysed, resulting in increased LDH levels. Interestingly, after 24 hours of simulated reperfusion, cell viability decreased to approximately 82% of the 24-hour control value, suggesting either that IRI might limit the proliferative capacity of HUVECs relative to the 24-hour control value or that delayed cell death, perhaps caused by apoptosis, was occurring as a consequence of simulated cold IRI.

Simulated IRI Causes MAPK Activation and I κ B α Degradation

Simulated IRI was associated with activation of MAPKs (ERK 1/2, stress-activated protein kinase/JNK 1/2, and p38) and NF- κ B pathways (*Figure 4*), almost exclusively during reperfusion. p38 was activated weakly during

simulated cold ischemia and more during warming before reperfusion, with maximal phosphorylation after 5 and 15 minutes of simulated reperfusion. Baseline phospho-ERK was present in control HUVECs (in media with no manipulation), which is not unexpected because the media was not growth factor depleted.⁹ ERK and JNK activation was dramatic after 5 minutes of simulated reperfusion. I κ B α degradation was evident after 5 minutes of simulated reperfusion. Activation of NF- κ B is controlled by inducible phosphorylation and degradation of the inhibitory proteins I κ B (I κ B α , I κ B β , and I κ B ϵ) that bind and inhibit activation of NF- κ B by masking its nuclear localization sequence. Thus degradation of I κ B α indicates NF- κ B activation. Because of slightly reduced intensity and variability in 24-hour control samples, these reductions in I κ B α / β -actin were not statistically significant. Although p38 activation begins to some extent during simulated cold storage and warming, the major stimulus for MAPK and NF- κ B activation in our model is simulated reperfusion. Taken together, these data suggest that MAPK and NF- κ B activation is preceded by, and not the cause of, endothelial intercellular gap formation.

Simulated IRI Results in Elaboration of the Proinflammatory Cytokines IL-6 and IL-8

Because MAPK and NF- κ B activation is commonly implicated in transcription of certain proinflammatory chemokines and cytokines, we measured IL-6 and IL-8 levels in the media of HUVECs after simulated IRI. IL-6 and IL-8 levels were significantly increased 24 hours after reperfusion (*Figure 5*).

DISCUSSION

Our model of simulated IRI exposed HUVECs to an environment similar to that experienced by lung endothelial cells

FIGURE 3. A, Actin stress fibers disappear or are oriented peripherally (*abnormal*) in human umbilical vein endothelial cells exposed to simulated ischemia–reperfusion injury. The cytoskeleton of each cell was graded by a masked observer as normal or abnormal. ‡*P* < .001 and **P* < .05, analysis of variance with Tukey’s post hoc correction. B, Area of gaps (in percentages) in the monolayer of human umbilical vein endothelial cells subjected to simulated cold ischemia without hypoxia. Even though there is considerable recovery of the monolayer during 1 hour of warming (*WI*), there are still significant gaps at the onset of reperfusion and during early reperfusion, which resolve within 1 hour. ‡*P* < .001, analysis of variance with Tukey’s post hoc correction. Because of profound differences during cold ischemia, gap surface areas at warm ischemia and 15 minutes of reperfusion do not reach significance with Tukey’s post hoc correction. **P* < .01 compared with control by using the unpaired *t* test. C, Viability is maintained in human umbilical vein endothelial cell monolayers despite gap formation. Thus gap formation is not due to cell death or shedding. Late death (24-hour reperfusion) might be due to apoptosis. *CI*, Cold ischemia; *WI*, warm ischemia; *rep*, reperfusion. **P* < .05, analysis of variance with Tukey’s post hoc correction.

during recovery for transplantation and implantation into a recipient. Although some investigators have used hypoxia/reoxygenation to model IRI in cell culture,^{11,12} this does not accurately simulate the environment experienced by lung cells during transplantation. Conventional organ do-

TABLE 1. Photographs of groups were viewed by a cytoskeletal expert (KB) masked to group identity, who evaluated actin cytoskeleton

Time	Comments
Control	Stress fibers going across the cells.
15 min CI	Major gaps between cells. Actin stress fibers not very prominent.
4 h CI	Gaps between the cells are obvious. Actin is peripheral and weak.
End WI	Actin is peripheral. Some gaps are evident.
15 min rep	Actin organized around cell periphery, clearly marking the cell border. Few gaps.
1 h rep	Actin is organized in prominent stress fibers, some peripheral, some crossing cells. A few gaps?
4 h rep	Monolayer looks intact with no gaps. Actin is enriched at the borders but dispersed throughout the cells.
24 h rep	Actin stress fibers crossing the cells.

CI, Cold ischemia; *WI*, warm ischemia; *rep*, reperfusion.

nors are almost always ventilated with 100% oxygen. In rats breathing room air before death, we showed that PO_2 values did not decrease to less than 70 mm Hg in lungs maintained at 37°C for 4 hours, although many of the cells died, presumably from substrate depletion and acidosis.¹³ During cold storage, aerobic metabolism persists, presumably because of the availability of alveolar oxygen.¹⁴

Replacing cell culture media with buffered cold Perfadex, a commonly used pulmonary preservation solution, causes rapid and sustained changes in endothelial cell actin cytoskeleton associated with formation of gaps in the monolayer. The endothelial cytoskeleton, particularly the F-actin stress fibers, is responsible for maintaining cell shape and connections between cells and plays a critical role in the regulation of pulmonary vascular permeability.¹⁵ The cytoskeleton may also function as an intracellular communication system or signaling scaffold. Although these changes begin to reverse with rewarming (simulating lung implantation), endothelial monolayers were still abnormal at the time of simulated reperfusion. Attachment of endothelial cells to the basement membrane of alveolar capillaries in vivo might limit movement more than attachment to collagen-coated culture dishes in vitro. Nevertheless, if this in vitro phenotype reflects changes that occur in vivo, then it is not surprising that early pulmonary edema is a feature of lung IRI. This might also explain why controlled reperfusion (the practice of restricting flow to a lung graft for the first 5 minutes) results in better gas exchange.¹⁶ Controlled reperfusion might result in less edema as gaps in the monolayer resolve in the early minutes after reperfusion. Indeed, our data suggest that 5 minutes might not be enough controlled reperfusion time. Delaying implantation of the second lung for longer than 15 minutes might not be practical but could be an area for future study. Our model could uncover a strategy to reduce endothelial ultrastructural abnormalities during ischemia that can be clinically tested.

FIGURE 4. A, Representative Western blots show some phosphorylation of extracellular signal-regulated kinase (ERK) present in all samples but increased phosphorylation after reperfusion. Janus kinase (JNK) activation is apparent with reperfusion only. p38 activation begins to become apparent during cold ischemia, increases during warming, and is most intense after reperfusion. Inhibitor of nuclear factor κ B ($I\kappa B\alpha$) degradation is apparent after reperfusion. Representative of triplicate experiments. CI, Cold ischemia; WI, warm ischemia. B, To compare expression of phospho/total or $I\kappa B\alpha/\beta$ -actin, ratios of intensity by laser scanning of controls was normalized to 1. Phosphorylation of ERK and JNK occurs with simulated reperfusion. p38 activation begins to become apparent during cold ischemia and is significant with warming and maximal after reperfusion. $I\kappa B\alpha$ degradation occurs after reperfusion. Ratios of control sample were arbitrarily set to 1, and all other ratios on the same blots were adjusted so that intensities of 3 experiments could be compared across time points, and therefore comparison to control samples could not be made. Control 24-hour samples were used to establish whether differences in intensity were significant. Because of large variances that were proportional to the means, data were $\log_{10}$ transformed. For mitogen-activated protein kinases (MAPKs), ratios of phospho/total for control 24-hour samples was similar to that for control samples. For $I\kappa B\alpha/\beta$ -actin, control 24-hour intensities were less than control intensities with more variability, and therefore decreased $I\kappa B\alpha$ levels after reperfusion obvious on all blots was not statistically significant. * $P < .05$, † $P < .01$, and ‡ $P < .001$ by using analysis of variance with Tukey's post hoc correction for multiple comparisons compared with control 24-hour results.

FIGURE 5. Interleukin 8 (IL-8) and interleukin 6 (IL-6) levels are both increased in media after simulated ischemia–reperfusion injury (IRI). Cytokines were measured in culture media by means of enzyme-linked immunosorbent assay (ELISA) from unstimulated human umbilical vein endothelial cells (HUVECs) (control) and from HUVEC dishes 24 hours after the initiation of the experiment. ELISA was performed in triplicate from 2 culture dishes ($n = 6$ assessments; mean $\pm$ standard error of the mean). * $P < .000005$ and † $P < .006$, unpaired t test.

We simulated reperfusion by means of sudden replacement of Perfadex with warm cell culture media. In vivo, Perfadex in the lung vasculature warms slowly during lung implantation and then is suddenly replaced with warm blood, resulting in an abrupt change in temperature and pH. Our simulated reperfusion caused activation of MAPKs and NF- κ B associated with elaboration of inflammatory cytokines. Although there was some activation of p38 during cold ischemia and warming, simulated reperfusion was responsible for most of the activation of the signaling pathways we studied, although actin cytoskeletal rearrangement and gap formation occurred during simulated ischemia. It is possible that MAPK and NF- κ B activation might play a role in re-establishment of a normal cytoskeleton and closure of intercellular gaps, but this process begins during rewarming before substantial pathway activation. Thus we suspect the 2 processes are unrelated. We measured inflammatory cytokines only after 24 hours of reperfusion. In this in vitro system the cytokines are stable; additional studies to document the time course of cytokine production would be useful.

This pattern of signaling pathway activation is identical to that seen in whole lung tissue after warm lung IRI in mice and conventional LTX in rats (unpublished ongoing work in our laboratory). Although we did not assess cell proliferation or apoptosis, apoptosis might account for our observed late increase in cell death assessed by using the LDH assay (Figure 3, C). Apoptosis occurs after LTX¹⁷ and is associ-

ated with activation of p38 and JNK MAPKs,¹⁸ but the clinical significance of apoptosis is unknown.

Our study complements a study by Hall and colleagues,¹⁹ who showed similar actin cytoskeletal derangements in cultured porcine pulmonary artery endothelial cells cooled to 4°C for 2 or 4 hours and similar gaps in the monolayer, but these were not quantified. Endothelial cell shape recovered quickly with rewarming, but actin stress fibers were still poorly organized 1 hour after rewarming. In another study the effect of different organ preservation solutions was assessed, and although there were some differences, cooling alone by replacing the cell culture media with media at 4°C had a considerable effect on the actin cytoskeleton.²⁰

Cardella and colleagues²¹ subjected human pulmonary epithelial cancer cells (A549 cells) to simulated IRI using a similar model, replacing cell culture media with cold low potassium dextran–glucose solution or variations of this solution. In their model prolonged ischemia (≥ 12 hours) led to increasing amounts of epithelial cell death and separation from the culture plate, worsened by simulated reperfusion. Inoue and colleagues²² used A549 cells to show that NF- κ B activation in cells cooled to 4°C occurred after rewarming and IL-8 mRNA levels were increased 1 hour after rewarming. This is consistent with our observation that simulated reperfusion appears to be the main stimulus for NF- κ B and MAPK activation and not the antecedent period of hypothermia. However, malignant cells might not be ideal to study, and bronchial epithelial cells in vivo might not contact preservation solution flushed through the vasculature; therefore the relevance of these models to LTX is unclear.

MAPKs are activated in cell culture in response to a variety of stimuli, including mechanical stretch¹⁸ and tumor necrosis factor α or stimulation of Nod-like and Toll-like receptors (TLRs).²³ Recently, we showed that TLR4 on pulmonary parenchymal cells, not bone marrow–derived macrophages, mediates early edema in a murine model of in situ normothermic lung IRI.²⁴ Why replacing Perfadex after a period of hypothermia with 37°C cell culture media should stimulate signaling pathways associated with inflammation is unclear and is an area for future study. However, because activation occurs primarily with reperfusion, it might be practical to provide inhibitors of some of these signaling pathways either to the donor before organ recovery or in the preservation solution.

There is no doubt that other cell types, such as alveolar macrophages (AMs), participate in lung IRI,²⁵ but we have shown that TLR4 on parenchymal cells plays a more important role in early edema formation caused by IRI than TLR4 on AMs using chimeric TLR4 mice.²⁴ Accordingly, we chose to establish our model with endothelial cells. Furthermore, although AMs clearly contribute to the MAPK activation that occurs with IRI and some have argued that the AM is a “key coordinator” in the pathophysiology of lung IRI,²⁶ our data clearly show that endothelial cells also

activate MAPKs and elaborate inflammatory cytokines caused by IRI in the absence of AMs. In addition, this study suggests a mechanism for IRI-induced edema independent of AMs and offers an explanation for the observation that the practice of controlled reperfusion results in less edema.

This study validates a realistic model of simulated IRI that can be used to expose a variety of pulmonary cell types to simulated IRI and investigate a number of interventions to reduce IRI, such as inhibitors of MAPK or NF- κ B activation. Limitations of the model include the absence of mechanical shear stress and hydraulic pressure across the cell layer. Endothelial cell attachment to the extracellular matrix in vivo might be more robust than that to collagen-coated Petri dishes. Although our model suggests a role for endothelial cells as major mediators of edema caused by IRI, impaired alveolar fluid clearance by alveolar epithelial cells might also contribute.²⁷ A potential criticism is our use of HUVECs instead of human pulmonary microvascular endothelial cells (HMVECs). We chose to create our model with HUVECs because they are inexpensive, readily available, and well characterized and have been used to demonstrate the deleterious effects of pulmonary preservation storage solutions.²⁸ Now that we have a well-characterized model of simulated IRI, we plan to use it to study the response of HMVECs, alveolar type II cells, and bronchial epithelial cells in air-liquid interface cultures to simulated IRI. In preliminary experiments with HMVECs, similar changes in actin rearrangement and gap formation occur. There is no doubt that in vivo, different cell populations interact with each other and the extracellular matrix in response to stresses such as IRI. The role of intravascular thrombosis and coagulation is ignored in in vitro cell culture models, as well as the contribution of leukocytes and AMs.

In conclusion, exposing human endothelial cells to simulated cold ischemia without hypoxia causes reversible cytoskeletal alterations associated with significant gaps in the otherwise confluent monolayer and activation of inflammatory signaling pathways. Because this model accurately depicts events occurring during lung recovery and transplantation, it might be a valuable tool to explore mechanisms regulating endothelial cell response to IRI and therapeutic interventions.

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