Stress-activated protein kinase inhibition to ameliorate lung ischemia reperfusion injury

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Objective: Inhibition of cytokines offers modest protection from injury in animal models of lung ischemia–reperfusion. Improved strategies would selectively inhibit the transcriptional activation response to oxidative stress. Mitogen-activated protein kinases (p38, c-jun N-terminal kinase, extracellular signal–regulated kinase) have been shown to be activated after oxidative stress and in animal models of acute inflammatory lung injury. We hypothesized that mitogen-activated protein kinase inhibition would block downstream transcriptional activation, providing robust protection from lung ischemia–reperfusion injury.

Methods: Experimental rats received inhibitors of p38, c-jun kinase, or extracellular signal–regulated kinase before in situ left lung ischemia–reperfusion. Immunohistochemistry localized cellular sites of mitogen-activated protein kinase activation. Several markers of lung injury were assessed. Enzyme-linked immunosorbent assay measured soluble cytokine and chemokine contents. Western blotting assessed mitogen-activated protein kinase phosphorylation. Electromobility shift assays measured transcription factor nuclear translocation.

Results: Immunohistochemistry localized p38 and c-jun kinase activations in positive controls to alveolar macrophages. Extracellular signal–regulated kinase was activated in endothelial and epithelial cells. Animals treated with p38 or c-jun kinase inhibitor demonstrated significant reductions in transcription factor activation and markers of lung injury. Extracellular signal–regulated kinase inhibition was not protective. Western blotting confirmed inhibitor specificity.

Conclusion: Inhibition of p38 and c-jun kinase provided significant protection from injury. The alveolar macrophage appears to be the key coordinator of injury in response to oxidative stress. Therapeutically targeting specific cell population (macrophage) responses to oxidative stress has the potential benefit of reducing lung reperfusion injury severity while leaving host immune responses intact.

A significant barrier to successful outcomes after lung transplantation is the development of lung ischemia–reperfusion injury (LIRI). Clinically known as primary graft dysfunction, LIRI is thought to affect as many as 25% of recipients.¹ LIRI is strongly associated with the warm ischemic phase of implantation and persists despite improvements in donor management, organ preservation, and postoperative recipient care.^{2,3} LIRI is known to increase both early mortality and contribute to late graft failure through the development of bronchiolitis obliterans.^{4,5}

LIRI is dependent on the activation of inflammatory signaling cascades within resident cells of the lung. The alveolar macrophage (AM) is a key coordinator of these

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Abbreviations and Acronyms

AM	= alveolar macrophage
AP-1	= activator protein 1
BSA	= bovine serum albumin
CINC	= cytokine-induced neutrophil chemoattractant
DMSO	= dimethylsulfoxide
ERK1/2	= extracellular signal-regulated kinase
IL-1 β	= interleukin 1 β
JNK	= c-jun N-terminal kinase
LIRI	= lung ischemia-reperfusion injury
MAPK	= mitogen-activated protein kinase
MPO	= myeloperoxidase
NF- κ B	= nuclear factor κ B
PBS	= phosphate-buffered saline solution
SAPK	= stress-activated protein kinase

responses.⁶ The inflammatory responses of other resident cell types, such as the endothelial and epithelial cells, are likely amplified by the early secretory products of the AM.⁷ Inhibition of individual cytokines and chemokines, such as tumor necrosis factor α (TNF- α) and interleukin 1 β (IL-1 β), provides only modest protection from lung injury in response to ischemia-reperfusion.⁷ This is explained by the significant redundancy in the inflammatory response at the posttranscriptional level. Targeted inhibition of pretranscriptional signaling events would therefore potentially provide more robust protection from injury.

Transcriptional activation through nuclear factor κ B (NF- κ B) and activator protein 1 (AP-1) is centrally important to the development of lung injury in response to oxidative stress.^{8,9} It has been suggested that transcription factor activation is regulated by mitogen-activated protein kinases (MAPKs).^{10,11} MAPKs are a group of intracellular signaling proteins activated by multiple stimuli, including inflammatory cytokines (tumor necrosis factor α), lipopolysaccharide, radiation, and ischemic injury.^{12,13} They are highly conserved serine/threonine kinases that require dual phosphorylation to become activated.¹⁴ Three MAPKs have been characterized in ischemia reperfusion injury: the two stress-activated protein kinases (SAPKs), p38 and c-jun N-terminal kinase (JNK), and extracellular signal-regulated kinase (ERK1/2). The functional significance of MAPK activation in response to oxidative stress has been described in various organ systems, though MAPK roles vary. In the heart, p38 appears to augment reperfusion injury, whereas ERK1/2 plays a protective role.^{15,16} In contrast, both p38 and JNK activation have been shown to promote liver reperfusion injury.^{17,18}

The potential role of MAPK activation in LIRI has been examined in several reports. JNK inhibition was found to reduce AP-1 activation and apoptosis in an ex vivo buffer-perfused model, whereas the p38 inhibitor FR167653 was found to be protective when added to cold lung-preservation

solution used in a model of canine single-lung transplantation.^{19,20} No studies examining the functional role of ERK1/2 in LIRI exist to date, though recently both ERK1/2 and JNK phosphorylation were demonstrated in transplanted human lungs.²¹

Although MAPK activation has been demonstrated in a variety of vascular beds, including the lung, MAPK differential roles in mediating injury in response to oxidative stress are not well understood. This is particularly true in the lung. In these studies, we determined the functional significance of MAPK activation in our warm ischemic in vivo model of LIRI. Furthermore, we determined the specific cellular sites of activation of each MAPK and elucidated the downstream transcription factors activated by the MAPKs.

Materials and Methods**Reagents**

SP600125 and U0126 were purchased from Sigma-Aldrich Corporation (St Louis, Mo). SP600125 was solubilized in 3 mL phosphate-buffered saline solution (PBS) containing 10% dimethylsulfoxide (DMSO). U0126 was solubilized in 1 mL PBS containing 1% DMSO. FR167653 was a generous gift from Akira Shimamoto and was solubilized in 1 mL PBS. All other reagents were purchased from Sigma-Aldrich unless otherwise noted.

Animal Model

Pathogen-free Long-Evans rats (Harlan Sprague Dawley, Inc, Indianapolis, Ind) weighing between 275 and 300 g were used for all experiments. Approval for all experimental protocols was granted by the University of Washington Animal Care Committee. All animals received humane care and treatment in accordance with the "Guide for the Care and Use of Laboratory Animals" (www.nap.edu/catalog/5140.html) and the "Principles of Laboratory Animal Care" established by the National Society for Medical Research.

A well-established warm, in situ, ischemia-reperfusion model was used as previously described.^{6,7} In brief, rodents were anesthetized with 30 mg intraperitoneal pentobarbital, after which a 14-gauge angiocatheter serving as an endotracheal tube was placed under direct visual guidance into the trachea through a midline neck incision. The catheter was secured with a single suture, and the animal was hooked up to a Harvard Rodent Ventilator (Harvard Apparatus, Inc, Holliston, Mass). Ventilator settings were standardized throughout the experimental protocol, with an inspired oxygen content of 60%, 2 cm H₂O positive end-expiratory pressure, and a respiratory rate of 80 breaths/min. Peak pressures were monitored and did not exceed 10 cm H₂O. With the animal in the right lateral decubitus position, a left thoracotomy was performed, and the pleural space was entered sharply through the fifth interspace. The left lung was mobilized atraumatically, with subsequent sharp division of the inferior pulmonary ligament. After mobilization, 50 units heparin in a volume of 500 μ L was then administered through the penile vein and allowed to circulate for 5 minutes. A noncrushing clamp was then placed across the left lung hilum, occluding the pulmonary artery, pulmonary vein, and main stem bronchus, for 90 minutes. This period of ischemia and hypoxia was held constant for all experimental groups. At the end of this period, the clamp was removed and the left lung was allowed to reventilate and reperfuse for 4

hours, during which time 500 μ L warm saline solution was injected subcutaneously every hour to maintain hydration. At the end of the reperfusion period, midline laparotomy and sternotomy were performed. Blood samples were taken from the inferior vena cava, and the animals were killed by aortic transection. The heart–lung block was excised, and the pulmonary circulation was flushed with 20 mL saline solution. The left lung was then separated from other mediastinal structures.

Six experimental groups of animals were studied. The negative control group did not undergo surgical manipulation. The positive control group received PBS vehicle only, either with or without DMSO, 45 minutes before left hilar occlusion and subsequently underwent the full ischemia–reperfusion protocol. Vehicle was administered in the equivalent volume with equivalent percentage DMSO concentration by the same route as described previously for each inhibitor. Each of the three remaining groups was allocated to receive one of the specific MAPK inhibitors 45 minutes before the onset of ischemia; all three groups were then subjected to the ischemia–reperfusion protocol of 90 minutes of ischemia followed by up to 4 hours of reperfusion. Dosages for all three pharmacologic inhibitors were determined with dose–response curves and Western blotting of injured lung specimens for the phosphorylated MAPKs. For each inhibitor, the most suppressive dose for each targeted MAPK without nonspecific inhibition of other MAPKs was used. FR167653, a specific p38 inhibitor, was given intravenously at a dose of 6 mg/kg. JNK inhibition was achieved with SP600125 given through the intraperitoneal route at a dose of 25 mg/kg. U0126, an ERK1/2 inhibitor, was administered intravenously at 200 μ g/kg. Lung injury and molecular analysis of tissue samples were determined with the following protocols.

Immunohistochemical Examination

Whole left lung specimens subjected to 90 minutes of ischemia followed by 15 minutes of reperfusion were prepared for immunohistochemical analysis as previously described.^{7,9} In brief, left lung tissue specimens were fixed in 10% formalin. Samples were dehydrated through graded alcohol baths and embedded in paraffin. Specimens were cut in 5- μ m sections, baked overnight at 50°C, and rehydrated through graded baths to a final distilled water wash. Specimens were blocked with 5% serum for 30 minutes at room temperature. Primary antibodies for the phosphorylated MAPK isoforms (Cell Signaling Technology, Inc, Beverly, Mass) were applied, and specimens were incubated overnight at 4°C. After primary antibody incubation, manufacturer stock secondary antibody (Cell Signaling Technology) was applied and incubated at room temperature for 30 minutes. After the secondary antibody incubation, avidin–biotin–peroxidase complex conjugate (Vector Laboratories, Inc, Burlingame, Calif) was applied. DAB reagent was then applied, and the sections were developed. Sections were rinsed in running tap water for 10 minutes, dehydrated, cleared, and mounted with permanent mounting media. Stained sections were examined with the image analysis software Image Pro Plus (Media Cybernetics, LP, Silver Spring, Md).

Lung Permeability Index

Changes in lung vascular permeability were assessed as a ratio of iodine 125–radiolabeled bovine serum albumin (¹²⁵I-BSA) in the left lung to that of 1 mL of blood. ¹²⁵I-BSA was purchased from NEN

Life Science Products, Inc (Boston, Mass) and was serially diluted to obtain an activity of approximately 800,000 cpm. A 1% BSA/PBS solution was added to make a final volume of 500 μ L, which was injected intravenously 5 minutes before removal of the left hilar clamp. The lung was allowed to reperfuse for 4 hours. Immediately before the animals was killed, 1 mL blood was aspirated from the inferior vena cava. The left lung was subsequently harvested as described previously. The activities for the left lung and the blood were then quantified in a gamma counter. The permeability index was then calculated as follows: Permeability index = left lung activity (in cpm)/1 mL blood activity (in cpm). This ratio corrected for any variation in the systemic distribution of ¹²⁵I-BSA.

Myeloperoxidase Assay

Left lung neutrophil accumulation was measured after 4 hours of reperfusion by determining myeloperoxidase (MPO) content in lung specimens as previous described.^{6,7,9} Lung specimens were homogenized for 1 minute in a solution of 0.5% hexadecyltrimethylammonium bromide and 5-mmol/L EDTA in 50-mmol/L potassium phosphate buffer (pH 6.0). Samples were then sonicated in three 15-second bursts. Samples were centrifuged at 3000g for 30 minutes at 4°C, and the supernatants were recovered. The change in absorbance over 1 minute at 460-nm wavelength was recorded after mixing 50 μ L of each sample with 1.45 mL of assay buffer (0.0005% hydrogen peroxide and 0.167-mol/L O-dianisidine dihydrochloride in 100-mmol/L potassium phosphate buffer, pH 6.0).

Bronchoalveolar Lavage and Inflammatory Cell Counts

After 4 hours of reperfusion, the left lung was selectively lavaged with 5 mL sterile saline solution through the endotracheal tube. A clamp was placed across the right hilum to achieve selective left lung lavage. The recovered lavage fluid was centrifuged at 1800 rpm for 10 minutes at 4°C to pellet the cells. The supernatant was frozen at –80°C for later cytokine analysis, and the pellet was resuspended in 10 mL sterile PBS. A 1-mL portion of resuspended cells was subsequently stained with Gill solution and counted with a hemacytometer (Hausser Scientific, Horsham, Pa). Differential cell counts were not performed, because previous work has found nearly all cells to be neutrophils.

Western Blot Analysis

After 15 minutes of reperfusion, total protein was harvested from whole left lung samples as previously described.⁷ Protein concentrations from each harvest were quantitated with the bicinchoninic assay (Thermo Fisher Scientific, Inc, Waltham, Mass). Protein samples (60 μ g) were loaded on 12% sodium dodecylsulfate–polyacrylamide gels and electrophoresed at 90 V for 1 to 2 hours. After transfer to a polyvinylidene difluoride membrane, the membranes were incubated overnight either with anti–total p38, JNK, or ERK1/2, or with anti–phosphorylated p38, JNK, or ERK1/2 (Cell Signaling Technology, Inc, Beverly, Mass). A horseradish peroxidase–conjugated secondary antibody was applied for 1 hour, and the proteins were subsequently visualized with Pierce Supersignal Reagents (Thermo Fisher Scientific) and autoradiography. Densitometry was performed to assess relative differences in activation between groups with Image J software (version 1.2; Media Cybernetics, Inc, Silver Spring, Md).

Electrophoretic Mobility Shift Assay

Whole lung samples allowed to reperfuse for 15 minutes had nuclear protein harvested as previously described.^{6,9} Protein concentrations were again determined with the bicinchoninic assay. A 5- μ g sample of nuclear protein was incubated with biotin-labeled NF- κ B or AP-1 oligonucleotide probe (Panomics, Inc, Fremont, Calif) for 20 minutes at room temperature. Running unlabeled probe in a cold competition reaction allowed assessment of the specificity of each probe. Samples were resolved on a 6% dodecylsulfate-polyacrylamide gel run at 80 V for 1 to 2 hours. After transfer to a nylon membrane (Amersham Biosciences, Piscataway, NJ), membranes were incubated for 15 minutes in a streptavidin-horseradish peroxidase conjugate solution and subsequently visualized with Pierce Enhancer Solution (Thermo Fisher Scientific) and autoradiography. Densitometry was performed as described previously.

Enzyme-Linked Immunosorbent Assay

IL-1 β and cytokine-induced neutrophil chemoattractant (CINC) concentrations were analyzed in recovered bronchoalveolar lavage fluid with sandwich enzyme-linked immunosorbent assays as previously described (R&D Systems, Minneapolis, Minn).^{6,9}

Statistical Analysis

All data are presented as mean $\pm$ SEM unless otherwise designated. Statistical differences between groups were assessed with the 2-tailed Student *t* test (Microsoft Excel 2002; Microsoft Corporation, Redmond, Wash), with post hoc Bonferroni adjustment for multiple comparisons. *P* values refer to comparisons between vehicle-treated positive control animals and animals that received a specific MAPK inhibitor.

Results

Immunohistochemical Examination

Immunohistochemical examination of left lung specimens localized cell-specific sites of MAPK phosphorylation (Figure 1). Unmanipulated negative control animals demonstrated no staining for the phosphorylated isoforms of the

Figure 1. Electromobility shift assay for activator protein 1. Vehicle-treated positive control animals (phosphate-buffered saline solution *lane 3*, phosphate-buffered saline solution plus dimethylsulfoxide *lane 4*) exhibited marked activator protein 1 nuclear translocation relative to negative control animals (*lane 2*). Activator protein 1 translocation was decreased significantly with p38 inhibition (*lanes 5 and 6*, *p* = .03) and c-jun N-terminal kinase inhibition (*lanes 7 and 8*, *p* = .008). Cold competition lane (*lane 1*) verified band as activator protein 1.

MAPKs. Vehicle-treated positive control animals showed marked staining for both phosphorylated p38 and JNK, which localized exclusively to the AM. Conversely, phosphorylated ERK1/2 was evident only in the endothelial and epithelial cells of positive control lungs.

IR15=90 minutes ischemia and 15 minutes reperfusion

Figure 2. Western blots for phosphorylated p38 (*p-p38*), c-jun N-terminal kinase (*pJNK*), and extracellular signal-regulated kinase (*pERK1/2*) demonstrating inhibitor specificity. Target mitogen-activated protein kinases for each inhibitor are indicated with heavy outlines. Specific p38, c-jun N-terminal kinase, and extracellular signal-regulated kinase inhibitions were achieved with FR167653 (*FR*, 6 mg/kg), SP600125 (*SP*, 25 mg/kg), and U0126 (*U0*, 200 μ g/kg), respectively, after 90 minutes of ischemia and 15 minutes of reperfusion (*IR 15*).

Figure 3. Immunohistochemical staining for phosphorylated p38 (A), c-jun N-terminal kinase (B), and extracellular signal-regulated kinase (C). *Left column* represents lung sections from unmanipulated negative control animals, with no staining for any phosphorylated mitogen-activated protein kinase isoforms. *Right column* represents lung sections from positive control animals subjected to 90 minutes of ischemia followed by 15 minutes of reperfusion (IR 15). Phosphorylated p38 and c-jun N-terminal kinase localized exclusively to alveolar macrophages (*thin arrows*). Phosphorylated extracellular signal-regulated kinase localized to endothelial (*thick outline arrow*) and epithelial cells (*thick dark arrow*).

MAPK Phosphorylation

Western blotting was performed on whole left lung homogenates to detect MAPK phosphorylation patterns. Preliminary work demonstrated that p38 and JNK phosphorylation peaked at 15 minutes of reperfusion, with somewhat delayed ERK1/2 phosphorylation that, although detectable at 15 min-

utes of reperfusion, did not peak until 60 minutes of reperfusion. Whereas p38 and JNK phosphorylation did not persist beyond 1 hour of reperfusion, ERK1/2 phosphorylation was still evident at 4 hours of reperfusion. Preliminary work determined appropriate inhibitor dosing to achieve specific MAPK inhibition (Figure 2). Representative blots for

TABLE 1. Results of left lung injury parameters

Variable	Negative control (n = 4)	Vehicle-treated positive control (n = 8)	Treatment groups (n = 4/group)		
			FR167653	SP600125	U0126
Permeability index					
Value	0.09 ± 0.006	0.89 ± 0.06	0.42 ± 0.05	0.58 ± 0.05	0.87 ± 0.07
Decrease from positive control (%)			53%	35%	NA
P vs positive control			.002	.03	>.2
Myeloperoxidase					
Value	0.09 ± 0.009	0.83 ± 0.04	0.46 ± 0.02	0.66 ± 0.013	0.76 ± 0.06
Decrease from positive control (%)			45%	20%	NA
P vs positive control			<.001	.05	>0.2
Bronchoalveolar lavage (×10 ⁵ cells)					
Value	4.5 ± 0.5	176 ± 10	62 ± 7	96 ± 5	
Decrease from positive control (%)			65%	46%	
P vs positive control			<.001	.001	

Values are mean ± SEM. NA, Not applicable.

phosphorylated p38, JNK, and ERK1/2 for control and treated animals are shown (Figure 3), demonstrating the degree of inhibitor efficacy for each targeted MAPK.

Markers of left lung injury, including permeability index, MPO content, and bronchoalveolar lavage cell counts, are summarized in Table 1. Across all markers of lung injury, no difference was detected for PBS-treated positive control samples compared with PBS and DMSO—treated positive control samples, thus all control animals were pooled for comparisons with the individual MAPK inhibitor—treated animals.

Permeability Index

Vehicle-treated positive control animals had a significantly increased permeability index relative to negative control animals, demonstrating a nearly 10-fold increase ($P < .001$). FR167653 (p38 inhibitor) and SP600125 (JNK inhibitor) decreased the permeability index by 53% and 35%, respectively, both of which were statistically significantly different from positive control animals. ERK1/2 inhibition with U0126 did not decrease left lung vascular permeability.

MPO Content

Negative control animals had an MPO content of 0.09 ± 0.009 . This was significantly increased among vehicle-treated positive control animals after 4 hours of reperfusion ($P < .001$). Both p38 and JNK inhibitions showed significantly decreased left lung MPO content relative to positive control animals. Conversely, MPO content was not significantly reduced by ERK1/2 inhibition. Although U0126 has been shown to inhibit ERK1/2 phosphorylation (Figure 2), it was not functionally protective in our model of LIRI; further animal studies with U0126 were therefore not performed.

Bronchoalveolar Lavage Inflammatory Cell Counts

Positive control animals had significantly more inflammatory cells in bronchoalveolar lavage fluid than did negative control animals ($P < .001$). FR167653-treated animals had 65% fewer inflammatory cells than did positive control animals, whereas SP600125 treatment reduced inflammatory cell counts by 46%.

Transcription Factor Nuclear Translocation

Electrophoretic mobility shift assays for NF- κ B and AP-1 were performed after 15 minutes of reperfusion to detect the nuclear translocation of these relevant transcription factors. Representative shift assays are shown for NF- κ B (Figure 4) and AP-1 (Figure 5). Both p38 and JNK inhibitions significantly limited the nuclear translocation of each transcription factor.

Left Lung Bronchoalveolar Lavage Cytokine/Chemokine Content

Table 2 demonstrates the concentrations of IL-1 β and CINC within the left lung bronchoalveolar lavage, as measured by enzyme-linked immunosorbent assay. Relative to negative control animals, positive control animals had significantly elevated levels of IL-1 β and CINC. Treatment with FR167653 and SP600125 significantly reduced the levels of both IL-1 β and CINC relative to positive control animals after 4 hours of reperfusion.

Discussion

These studies demonstrate an important functional role for the SAPKs in our in vivo model of LIRI. Immunohistochemical examination localized SAPK activation exclusively to AMs. Inhibition of either p38 or JNK led to marked protection from lung injury, as measured by pulmonary endothelial

Figure 4. Electromobility shift assay for nuclear factor κ B. Unmanipulated negative control animals demonstrated minimal nuclear translocation (*lane 2*). After 90 minutes of ischemia and 15 minutes of reperfusion, vehicle treated-positive control animals (with phosphate buffered saline solution *lane 3*, with phosphate-buffered saline solution plus dimethylsulfoxide *lane 4*) demonstrated marked translocation of nuclear factor κ B. FR167653 (*lanes 5 and 6*) and SP600125 (*lanes 7 and 8*) significantly reduced transactivation of nuclear factor κ B relative to that in positive control animals (FR167653 $P = .02$, SP600125 $P = .007$). Cold competition lane (*lane 1*) verified band as nuclear factor κ B.

cell dysfunction, lung inflammatory cell infiltration, and soluble cytokine production. Amelioration of injury occurred through a reduction in NF- κ B and AP-1 nuclear translocation, with a subsequent decrease in cytokine production. We thus have demonstrated that pretranscriptional inhibition of the inflammatory signaling cascade can reduce lung injury severity in response to oxidative stress. Conversely, inhibition of ERK1/2, which was localized to endothelial and epithelial cells but not the AM, was not found to be protective, suggesting that ERK1/2 does not significantly contribute to lung injury in our LIRI model. A limitation of this study was our use of pharmacologic inhibition. Although these agents provided specific MAPK inhibition, it was unfortunately incomplete. MAPK inhibition occurs in a dose-dependent manner with these agents, but at the escalating doses necessary to afford more robust inhibition, specificity is lost. We therefore chose to concentrate on specificity, enabling us to determine the contribution of each individual MAPK to the generation of LIRI. Modest inhibition of the SAPK clearly reduced the degree of lung injury. Although we were unable to show protection with ERK1/2 inhibition, it however remains possible that a greater degree of inhibition would afford protection.

Figure 5. Western blots for phosphorylated p38 (A), c-jun N-terminal kinase (B), and extracellular signal-regulated kinase (C). Positive control animals (phosphate-buffered saline solution in *lane 2*, phosphate-buffered saline solution plus dimethylsulfoxide in *lane 3*) exhibited significantly more mitogen-activated protein kinase phosphorylation than did unmanipulated negative control animals (*lane 1*, p38 $P = .04$, c-jun N-terminal kinase $P = .04$, extracellular signal-regulated kinase $P = .01$). FR167653-treated animals (*lane 4*) demonstrated significantly reduced p38 phosphorylation relative to positive control animals ($P = .03$), whereas c-jun N-terminal kinase and extracellular signal-regulated kinase phosphorylation were unaffected. SP600125 treatment (*lane 5*) inhibited c-jun N-terminal kinase phosphorylation ($P = .04$) and did not significantly reduce extracellular signal-regulated kinase or p38 phosphorylation. Relative to positive control animals, U0126-treated animals (*lane 6*) showed reduced extracellular signal-regulated kinase phosphorylation ($P = .03$) but no significant inhibition of either p38 or c-jun N-terminal kinase.

Currently, cold ischemia, although injurious, is modifiable and is being safely extended with improvements in storage techniques.²² Warm ischemia, however, is another matter. Our warm ischemia model recapitulates the most injurious phase of clinical lung transplantation. The period of lung implantation leads to the inevitable consequence of warm atelectatic ischemia, the duration of which correlates with acute graft dysfunction. Clinically, this injury is manifest within several hours of lung implantation. Although cold ischemic

TABLE 2. Left lung bronchoalveolar lavage cytokine and chemokine contents by enzyme-linked immunosorbent assay

Variable	Negative control (n = 4)	Vehicle-treated positive control (n = 8)	Treatment groups (n = 4/group)	
			FR167653	SP600125
Interleukin 1β (pg/mL)				
Value	70.8 ± 6.6	561.8 ± 118.2	122.5 ± 18.5	178.4 ± 28.1
Decrease from positive control (%)			78%	68%
P vs positive control			.01	.03
Cytokine-induced neutrophil chemoattractant (pg/mL)				
Value	42.5 ± 13.9	1361.7 ± 144	392 ± 24	384.7 ± 45.4
Decrease from positive control (%)			71%	72%
P vs positive control			<.001	<.001

Values are mean ± SEM.

transplant models can be used to study the effects of cold ischemic storage, they introduce the variables of allogeneic disparity and technical complications. Our warm hilar occlusion model is more specific and reliable for the study of mechanisms of injury related to warm atelectatic ischemia and reperfusion.

Treatment with inhibitors before the onset of ischemia was used to resemble what would occur in the clinical transplant setting. Ideally, donors would receive treatment before organ removal, thus inhibiting SAPK phosphorylation before the onset of reperfusion. This is important, as we have demonstrated that SAPK phosphorylation occurs early in reperfusion within the lung. Because the effects of SAPK inhibition on other organ systems have not been fully elucidated, the intravenous or intraperitoneal administration of specific inhibitors is as yet not practical in the clinical setting of multiorgan retrieval. The potential exists, however, for the development of inhibitors that can be delivered transbronchially, limiting systemic absorption and thus unwanted effects on other organ systems.

The AM has been shown to be a key mediator in multiple models of acute inflammatory lung injury.²³ A central role for the AM in the development of LIRI was determined from previous work in our laboratory, which found that AM depletion with clodronate disodium reduced proinflammatory mediator secretion and LIRI severity.⁶ The data from these studies suggest the SAPKs are centrally important in initiating the transcriptional activation of proinflammatory mediators in response to oxidative stress in AMs. Conversely, modulation of the inflammatory responses of endothelial and epithelial cells through ERK1/2 inhibition was not found to reduce acute LIRI.

The importance of the vascular endothelium in mediating inflammatory tissue injury is well known. Multiple models have shown that in response to various stimuli, the endothelium secretes multiple inflammatory mediators, including chemoattractants and cell adhesion molecules.^{24,25} Previously, with an in vitro model of hypoxia and reoxygenation, we demonstrated significant increases in secretions of CINC

and monocyte chemoattractant protein 1 by pulmonary endothelial cells.²⁶ The inflammatory responses of type 2 pneumocytes have also been characterized. In response to oxidative stress, type 2 cells showed increased CINC, IL-1β, and monocyte chemoattractant protein 1 secretion after as long as 6 hours of reperfusion.²⁷ Furthermore, this response was found to be dependent on ERK1/2 but not SAPK. Although oxidative stress directly activates both endothelial and epithelial cells, our current in vivo data indicate that inflammatory signaling within both cell types is highly dependent on secondary amplification by soluble mediators secreted by AMs. This is supported by our finding that SAPK phosphorylation peaks earlier than does ERK1/2 phosphorylation, suggesting that the initial cell population in the lung activated in response to oxidative stress consists of AMs. As such, to obtain durable protection from lung reperfusion injury, strategies should focus on the AM, which appears to be the key effector cell driving the injurious response to oxidative stress.

This work is the first to describe SAPK involvement in the AM in response to lung ischemia–reperfusion. It is also appreciated that SAPK-dependent inflammatory signaling is also activated in other models of injury to organs other than lung and injury not related to ischemia–reperfusion, with activation occurring in response to multiple inflammatory triggers, including oxidative stress, trauma, bacterial products, and inflammatory cytokines.^{12,13,28} Ideally, selective deletion of the SAPK response to oxidative stress would reduce LIRI severity while leaving their response to other stimuli intact. This would protect from reperfusion injury while maintaining host immunity.

In conclusion, p38 and JNK are critically important in mediating the inflammatory response of the AM to LIRI. Pretranscriptional inhibition of proinflammatory mediator production in the AM is vital to limit the secondary amplification of both pulmonary endothelial and epithelial responses. This targeted therapy, aimed at a proximal step in the signaling cascade, provides more robust protection

from injury than does inhibition of individual cytokines, which appear to be produced late and display some functional redundancy. Selective deletion of the SAPK response in the lung to oxidative stress could provide protection from primary graft dysfunction. The exclusive expression of SAPK in the AM (a resident cell) suggests that pretreatment of potential lung donors could be an effective strategy to limit LIRI clinically.

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Discussion

Dr David A. Fullerton (Denver, Colo). Dr Wolf, I congratulate you on both a phenomenal presentation and a really superb article. For those in the audience who do not think about this on a daily basis, I would like to point out a couple of things. Number one, there is a tremendous amount of important information in your study. Number two, the techniques and the model that you have used are quite sophisticated, so I think you are to be congratulated again on that point.

I learned a couple of things from your study. One is that it is possible to—well, let me back up and say what I learned is that MAP kinases are not uniformly distributed among different tissues, and so I found one of the novel findings in your study to be the dissociation of P38 and JNK from ERK1/2 in one organ. Of course, as you highlighted, P38 and JNK are found in the macrophages, whereas ERK1/2 are found in the endothelial and epithelial cells. That turns out to be important, because there is a tremendous amount of interest in the role of the endothelial and epithelial cells in acute lung injury. This is of course an important form of acute lung injury, and these two cell lines have kind of heretofore been ignored in the pathogenesis of many of these things.

Could you speculate as to whether your findings suggest that strategies to effect the response of the endothelial and epithelial cells are really not very important and that all focus should be on the macrophages? Or perhaps different strategies, other than those you studied, might be effective in addressing the endothelial and epithelial cells.

Dr Wolf. I think that the roles of the endothelial and epithelial cells in the generation of LIRI are extremely important. Unfortunately, with pharmacologic inhibition in this model, we were not able to fully suppress ERK1/2 in these cell types because of the collateral effects of higher drug dosing, which would have suppressed JNK and P38 as well. We are currently starting to examine the role of targeted molecular deletion in our cell culture model to try and delineate the cellular signaling mechanisms in response to oxidative stress within these cell types, and we will try in the future to extrapolate these findings to our animal model.

Dr Fullerton. Another question occurred to me. Given the fact that macrophages are ubiquitous from one organ to the next, and given the fact that one thing that all solid organ transplants must endure are the injuries of ischemia and reperfusion—and it is thought of course that macrophages are operative, and I think you have shown us that in spades—could you speculate a little bit as to whether not macrophages behave in a similar manner from one organ to the next? For instance, lung versus liver versus heart? Have your studies addressed any of that?

Dr Wolf. Our studies did not specifically address the role of macrophages in other organ systems. I think that the Kupffer cell within the liver probably does have a role similar to that of the AM. They are both a myeloid-derived lineage. The heart is a little bit tougher to speculate on, because as far as I know it does not have the same degree of myeloid-derived cells. I believe that work in one of the collaborative laboratories at our institution is examining the role of circulating leukocytes in generating cardiac ischemia–reperfusion injury.

Unidentified Discussant. I just had one question. It seems that your model is purely one of warm ischemia. Have you gone further to simulate a clinical situation of cold ischemia followed by warm ischemia followed by reperfusion? And have you looked at any of

the readily available pulmonoplegic solutions to see how that affects the whole injury phenomenon and your model?

Dr Wolf. Our goal in this study was to recapitulate what we consider the most injurious phase of the reperfusion injury process, the warm ischemic period. From my understanding with the new preservation techniques, we can carry cold ischemia out to about 12 hours. In this study we have not done that because as I stated, we are most interested in the warm ischemic phase.

Dr David Follette (*Sacramento, Calif*). This was a great study, beautifully presented and carried out. As Dr Halloran pointed out and we have looked at, a major component will be the ischemic injury to the organ, because that affects how the host immune system will approach the organ once it is implanted. As Dr Cohen pointed out, you have a fairly severe model, because in this whole area there are three components: what happens either before or during the ischemic phase, the storage phase, and then the reperfusion phase. My colleague and our member Dr Ardehali showed how modification of lung reperfusion can make significant differences.

Are there ways that this knowledge, which is fantastic, can be applied before the ischemic phase to give added protection against ischemia, looking more as you have done at a more molecular way rather than a global way as we would do with either cardioplegia or pulmonoplegia?

Dr Wolf. Unfortunately, I do not think that these specific inhibitors would lend themselves to the clinical setting, because they lose specificity at higher doses. We have recently begun to use short interfering RNA, which we have found localized exclusively to the AM within the lung. I think that potentially in the future, if we can reliably continue to show promise, this technique could provide clinical benefits. This technology is still in its infancy, however, so we will have to see where this goes.