Synergistic Protection in Lung Ischemia-Reperfusion Injury With Calcineurin and Thrombin Inhibition

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Background. **Ischemia-reperfusion injury impairs lung transplant outcomes. The transcription factors, activator protein-1, and nuclear factor kappa B, are activated early in reperfusion and drive the development of injury. Thrombin inhibition with hirudin, and calcineurin inhibition with tacrolimus have independently been shown to ameliorate lung ischemia-reperfusion injury by reducing activator protein-1 and nuclear factor kappa B activation, respectively. However, high doses were required to achieve protection using individual agents, raising concerns about potential toxicities. We sought to determine if low-dose combination therapy reduced injury through synergistic inhibition of pretranscriptional signaling events.**

Methods. **Rats were pretreated with either intravenous hirudin or tacrolimus at low doses or high doses, or both at low doses, prior to undergoing left lung ischemia and reperfusion. Lungs were assessed for markers of lung injury, including bronchoalveolar lavage cytokine-chemokine content and transcription factor transactivation of activator protein-1 and nuclear factor kappa B.**

Results. **High-dose monotherapy with hirudin or tacrolimus reduced lung injury and transactivation of activator protein-1 and nuclear factor kappa B activation, respectively, whereas low-dose monotherapy with either agent did not alter transcription factor activation or lung injury compared with positive controls. Low-dose combination therapy was more protective than high-dose monotherapy with either drug, and correlated with a reduction in activation of both transcription factors and their associated cytokines.**

Conclusions. **The significant decrease in lung injury severity and transcription factor activation with combined pathway inhibition suggests pretranscriptional signaling redundancy between the calcineurin and thrombin dependent pathways in lung reperfusion injury.**

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Lung transplantation is a life-saving treatment option for select patients with end-stage lung disease. Unfortunately, the inevitable period of ischemia and subsequent reperfusion can lead to significant lung injury in up to 20% of patients [1]. The development of lung ischemia-reperfusion injury (LIRI) is dependent upon transactivation of the proinflammatory transcription factors nuclear factor kappa B (NFκB) and activator protein-1(AP-1) and the subsequent cytokine-chemokine secretion by resident pulmonary cell populations [2–4]. Ultimately, significant pulmonary edema, inflammatory cell infiltration, and impaired compliance and gas exchange develop as full injury manifests.

Thrombin, a serine protease involved in the coagulation cascade, is also known to activate multiple inflammatory cell populations. Through activation of proteinase-activated receptors on such cell types as endothelial cells, macrophages, and platelets, thrombin promotes loss of endothelial integrity, leukocyte migration, and proinflammatory cytokine release [5]. Utilizing an in vivo model of lung reperfusion injury, we have previously demonstrated thrombin localization to alveolar macrophages as well as pulmonary endothelial and epithelial cells within 15 minutes of reperfusion [6]. Hirudin, a direct thrombin inhibitor, was shown to ameliorate lung injury in our model by reducing AP-1 dependent proinflammatory transcriptional activation, including cytokine-induced neutrophil chemoattractant (CINC), but did not alter tumor necrosis factor-alpha (TNF-α) secretion [6].

Calcineurin inhibition with tacrolimus is a cornerstone of current immunosuppressive regimens in solid organ transplantation. By limiting calcineurin-dependent activation of the transcription factor, nuclear factor of activated T cell, tacrolimus effectively modulates the cellular immune response responsible for acute rejection. Calcineurin is also known to promote acute nonalloimmune specific proinflammatory signaling through NFκB dependent transcriptional activation. We have shown that calcineurin inhibition with either cyclosporine or tacrolimus diminished the transactivation of NFκB in LIRI, and subsequently reduced pulmonary capillary leak, leukocyte infiltration, as well as CINC and TNF-α secretion without altering interleukin-1 beta (IL-1β) secretion [7, 8].

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Abbreviations and Acronyms

AP-1 = activator protein-1
aPTT = activated partial thromboplastin time
BAL = bronchoalveolar lavage
BSA = bovine serum albumin
CINC = cytokine-induced neutrophil chemoattractant
CPM = counts per minute
IL-1β = interleukin-1 beta
LIRI = lung ischemia-reperfusion injury
NFκB = nuclear factor kappa B
TNF-α = tumor necrosis factor alpha

Given individually at the doses we employed, both hirudin and tacrolimus provided significant protection in our in vivo model of LIRI, through modulation of AP-1 and NFκB dependent mechanisms respectively. Secondary effects of each agent, when given to a donor in the setting of multiorgan procurement, must be taken into account. High-dose thrombin inhibition will clinically alter the coagulation profile, potentially causing significant intraoperative bleeding. Intratracheal administration of tacrolimus decreases, but does not eliminate, system absorption at the doses previously used [8]. To circumvent these potential side effects, we proposed using low-dose combination therapy with intratracheal tacrolimus and intravenous hirudin. We hypothesized that the low-dose combination therapy of hirudin and tacrolimus would provide improved protection from lung injury when compared with either agent alone through a synergistic effect on the inhibition of AP-1 and NFκB and the subsequent production of proinflammatory mediators.

Material and Methods

Reagents

All reagents were purchased from Sigma-Aldrich Chemical (St. Louis, MO) unless otherwise specified. Recombinant hirudin (Bayer Healthcare Pharmaceuticals, Wayne, NJ) and the calcineurin inhibitor tacrolimus (Astellas Pharma Inc, Deerfield, IL) were obtained from the pharmacy at the University of Washington Medical Center.

Animal Model

Pathogen-free Long-Evans rats (Harlan Sprague Dawley, Indianapolis, IN), weighing between 275 grams and 300 grams, 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 in compliance with the "Principles of Laboratory Animal Care" established by the National Society for Medical Research and the "Guide for the Care and Use of Laboratory Animals" developed by the Institute of Laboratory Animal Resources and published by the National Institute of Health (NIH Publication No. 86-23, revised 1996).

A well-established, warm, in situ, ischemia-reperfusion model was used as previously described [4, 9]. In brief, animals were anesthetized with 30 mg of intraperitoneal pentobarbital after which a 14G angiocatheter was placed under direct vision into the trachea through a midline neck incision. The catheter was secured with a suture and the animal placed on a Harvard Rodent Ventilator (Harvard Apparatus, Boston, MA). Ventilator settings were standardized with an inspired oxygen content of 60%, 2 cm H_2O of positive end-expiratory pressure, and a respiratory rate of 80. Peak pressures were monitored and did not exceed 10 cm H_2O. A left thoracotomy was performed and the pleural space entered sharply through the fifth interspace. The left lung was mobilized atraumatically and the inferior pulmonary ligament sharply divided. After mobilization, 50 units of heparin in a volume of 500 μL was then administered through the penile vein and allowed to circulate for five minutes. A noncrushing clamp was subsequently placed across the left lung hilum, occluding the pulmonary artery, vein, and mainstem bronchus, for a period of 90 minutes. This period of ischemia and hypoxia was held constant for all experimental groups, after which the clamp was removed and the left lung allowed to reventilate and reperfuse for up to four hours. During reperfusion 500 μL of warm saline was injected subcutaneously every hour to maintain hydration. At the end of the reperfusion period, a midline laparotomy and sternotomy were performed. Blood samples were taken from the inferior vena cava and the animals sacrificed by aortic transection. The heart-lung block was excised and the pulmonary circulation flushed with 20 mL of saline. The left lung was separated from other mediastinal structures.

Rats were treated with 0.1 mg/kg (high dose) or 0.0125 mg/kg (low dose) tacrolimus through their endotracheal tube 1 hour prior to the onset of ischemia as previously described [8]. Recombinant hirudin was given at a bolus dose of 1 mg/kg (high dose) or 0.125 mg/kg (low dose) intravenously 30 minutes before ischemia. The higher doses of hirudin and tacrolimus had previously been shown to reduce LIRI severity [6–8] while preliminary studies showed that treatment with low-dose (one-eighth of the high dose) therapy with either hirudin or tacrolimus was not protective. Tacrolimus and hirudin were administered 1 hour and 30 minutes prior to ischemia, respectively, based on prior studies showing that administration prior to ischemia, and specifically at these time points, was most beneficial in comparison to at initiation of reperfusion or after 2 hours of reperfusion [6, 7]. In addition, pretreatment allows for translation to clinical lung transplantation given that the timing of the onset of ischemia is predictable.

Seven different experimental groups were studied in vivo. Negative controls were sacrificed without undergoing surgical manipulation. The other six groups underwent 90 minutes of ischemia followed by 4 hours of reperfusion. Positive control animals received an equivalent volume of intravenous or intratracheal vehicle prior to ischemia. Treated groups were then either given high-dose tacrolimus or hirudin, low-dose tacrolimus or hirudin or combined low-dose therapy. Combination high-dose