Functional Roles of Tumor Necrosis Factor-Alpha and Interleukin 1-Beta in Hypoxia and Reoxygenation

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Background. **Intercellular signaling plays an important role in the development of lung ischemia–reperfusion injury. However, the role of specific mediators remains poorly characterized. Alveolar macrophages (AM) produce soluble mediators early in reperfusion, which modulate the responses of endothelial and epithelial cells to oxidative stress. There is a burst of proinflammatory cytokine production in a variety of cells; however, interleukin 1-beta (IL-1β) and tumor necrosis factor-alpha (TNF-α) localize to the AM. We hypothesized that these cytokines account for the costimulatory effects that AM exert on endothelial and epithelial cells.**

Methods. **Activated AM media was placed on cultured rat type 2 pneumocytes and pulmonary artery endothelial cells, which were then subjected to hypoxia and reoxygenation. To assess the contributions of IL-1β and TNF-α, the cells were treated with control media or media that had been depleted of IL-1β or TNF-α. To deplete specific cytokines, activated media was passed through a column with immobilized IL-1β or TNF-α antibodies. Nuclear translocation of transcription factors, mitogen-activated protein kinase activation, and cytokine and chemokine production were assessed.**

Results. **Depletion of IL-1β or TNF-α effectively eliminated the ability of AM media to enhance the response of endothelial and epithelial cells to oxidative stress. There were significant reductions in monocyte chemotactic protein 1 and cytokine-induced neutrophil chemoattractant (CINC) production ($p < 0.05$) at 4 hours of reperfusion. Additionally there was decreased nuclear translocation of nuclear factor-kappa B, and extracellular signal-regulated kinase phosphorylation.**

Conclusions. **Interleukin 1-beta and TNF-α are critical mediators in the intercellular communication pathways that allow the AM to enhance the response of surrounding cells to oxidative stress.**

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Lung ischemia–reperfusion injury (LIRI) remains a significant complication after lung transplantation, leading to early graft dysfunction and the development of bronchiolitis obliterans [1, 2]. Previous work has demonstrated that the pathogenesis of LIRI is biphasic in nature [3]. The late phase is dependent on neutrophil recruitment and activation, and is characterized by increased vascular permeability and a heterogeneous chemokine and cytokine milieu, whereas the early phase is neutrophil independent and characterized by a predominance of tumor necrosis factor-alpha (TNF-α) and interleukin 1-beta (IL-1β).

Both TNF-α and IL-1β are proinflammatory cytokines that lead to leukocyte chemoattraction, phagocyte stimulation, enhancement of downstream cytokine and chemokine production, and variable effects on cell growth and death. Previous work has demonstrated a functional role for these cytokines in multiple models of ischemia–reperfusion injury, including liver [4, 5], heart [6, 7], kidney [8], brain [9], gut [10], limb [11], and lung [12] ischemia and reperfusion. In an in vivo experimental model of LIRI, blockade of TNF-α or IL-1β was associated with effective, but incomplete, protection. Furthermore, simultaneous blockade of TNF-α and IL1-ß was additive in providing a protective benefit [12]. This suggests that these cytokines are working through independent parallel pathways to drive the early phase of LIRI.

Within 15 minutes of reperfusion there is a burst of TNF-α and IL-1β, which localizes to the alveolar macrophage (AM) [12]. Although clinically impractical, depletion of AM with gadolinium chloride or clondronate leads to significant protection in an experimental model of LIRI [13, 14]. This suggests that the AM plays a key coordinating role in this early phase of LIRI and that likely this is through production of TNF-α and IL-1β.

This series of experiments is designed to characterize the role of the AM in amplifying the early response in LIRI. Previously we have shown that oxidative stress triggers mitogen-activated protein kinase activation leading to upregulation of proinflammatory transcription factors (such as nuclear factor-kappa B [NFκB], early growth response protein 1 [EGR-1], and activator protein 1 [AP-1]), which in turn causes elaboration of proinflammatory mediators in three primary lung cell types: pulmonary artery endothelial cells (PAEC), type 2 pneumocytes (T2P), and AM [15–18]. We hypothesize that in addition to the direct effect of oxidative stress, the AM plays a key role in priming surrounding cells to the

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Abbreviations and Acronyms

AM = alveolar macrophages
AP-1 = activator protein 1
EGR-1 = early growth response protein 1
EMSA = electromobility shift assay
FBS = fetal bovine serum
IL-1β = interleukin 1-beta
LIRI = lung ischemia–reperfusion injury
MCP-1 = monocyte chemotactic protein 1
NFκB = nuclear factor-kappa B
PAEC = pulmonary artery endothelial cells
PBS = phosphate-buffered saline
PBSt = phosphate-buffered saline with Tween
TNF-α = tumor necrosis factor-alpha
T2P = type 2 pneumocytes

effects of oxidative stress and amplifying their response. Furthermore, we hypothesize that the AM does this through two parallel pathways, one TNF-α–dependent and one IL-1β–dependent. To elaborate these pathways and determine the downstream effects of these cytokines, we used a series of media transfer experiments with specific cytokine depletion.

Material and Methods

Reagents

All reagents were purchased from Sigma Chemical Company (St. Louis, MO) unless otherwise specified.

Alveolar Macrophage Harvest

Pathogen-free adult male Long-Evans rats (Simonsen Labs, Gilroy, CA) weighing 250 to 300 g were used for all experiments. The University of Washington Animal Care Committee approved all experimental protocols. Animals received humane care in compliance with the "Principles of Laboratory Animal Care" formulated by the National Society for Medical Research and the "Guide for the Care and Use of Laboratory Animals" prepared by the Institute of Laboratory Animal Resources and published by the National Institutes of Health (NIH Publication No. 86-23, revised 1996).

Animals were euthanized with 120 mg/kg of intraperitoneal pentobarbital. A 14-gauge angiocatheter was inserted into the trachea through a midline neck incision and secured with a 4-0 braided silk suture. The heart-lung block was rapidly excised through a median sternotomy. Intratracheal lavage with 50 mL of cold phosphate-buffered saline (PBS) was performed as previously described [18]. The collected fluid was centrifuged at 1,500*g* for 10 minutes, and the cell pellet was resuspended in serum-free RPMI (Gibco BRL, Grand Island, NY). Cell counts and viability were assessed by standard trypan blue exclusion methods. Cells were then plated at a density of 500,000 cells per well in a 12-well culture plate (Fisher Scientific, Pittsburgh, PA). The AM were incubated at 37°C for 60 minutes to allow adherence to the culture plates. Media was then substituted with fresh RPMI containing 5% heat-inactivated fetal bovine serum (FBS).

Pulmonary Artery Endothelial Cell Culture

Heart-lung blocks were rapidly excised as described above. Endotracheal lavage of the lungs was performed 15 times with 6 to 9 mL of PBS containing 0.25 mmol/L EDTA to deplete alveolar macrophages. Strips of peripheral lungs (2 mm) were excised from all lung lobes. The peripheral tissue was minced, rinsed in RPMI, transferred to a dispase (10 mg/mL) solution, and incubated for 60 minutes at 37°C [15, 16]. The cell suspension was homogenized and incubated for an additional 5 minutes. Complete media with 10% FBS was added to terminate the reaction, and the cellular suspension was filtered through a 100-μm mesh. The filtrate was centrifuged at 800*g* for 8 minutes, and the cell pellet was resuspended in supplemented RPMI media and plated on gelatin-coated culture dishes. Media was changed every 48 hours until the cells were confluent. Once confluent, the cells were labeled for 8 hours with 4 μg/mL acetylated low-density lipoproteins, which bind selectively to endothelial cells. Cells were separated using flow cytometry (FAC STAR Plus, San Jose, CA). The pure cultures of endothelial cells were then maintained in RPMI with 10% FBS. All cells used in these experiments were from passages 4 through 10.

Type 2 Pneumocyte Culture

A rat type 2 pneumocyte cell line, RLE-6TN [19] (American Tissue Cell Company, Manassas, VA) was maintained in Ham's F-12 culture media containing 10% heat-inactivated FBS. Cells were cultured in 12-well plates at a density of 10^5 cells/mL. Culture media was replenished every 48 hours until 95% confluence was reached. Cell counts and viability were assessed by standard trypan blue exclusion techniques [20].

Hypoxia and Reoxygenation

Plated AM were placed in a humidified hypoxic chamber (Coy Lab Products, Grass Lake, MI) with a partial percentage of oxygen of 0.5% for 2 hours. After exposure to hypoxia they were transferred to a normoxic incubator for 15 minutes, at the end of which the supernatant was collected and stored at −70°C until used as experimental media.

Plated T2P and PAEC were placed in a humidified hypoxic chamber at a partial pressure of 0.5% for 2 hours with either control media (F-12 or RPMI, respectively, with 5% FBS), activated AM media, TNF-α–depleted media, or IL-1β–depleted media. At the end of the hypoxic period, the cells were transferred to a normoxic humidified incubator for up to 4 hours. Media achieves atmospheric P_{O_2} within 5 minutes [16, 17]. Negative controls remained in the normoxic incubator for up to 6 hours.

After 15 minutes of reoxygenation (or 2 hours 15 minutes of normoxia for negative controls), nuclear protein was harvested for electromobility shift assay analysis.