Shear stress-related mechanosignaling with lung ischemia: lessons from basic research can inform lung transplantation

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Submitted 17 July 2014; accepted in final form 15 September 2014

Chatterjee S, Nieman GF, Christie JD, Fisher AB. Shear stress-related mechanosignaling with lung ischemia: lessons from basic research can inform lung transplantation. Am J Physiol Lung Cell Mol Physiol 307: L668–L680, 2014. First published September 19, 2014; doi:10.1152/ajplung.00198.2014.—Cessation of blood flow represents a physical event that is sensed by the pulmonary endothelium leading to a signaling cascade that has been termed “mechanotransduction.” This paradigm has clinical relevance for conditions such as pulmonary embolism, lung bypass surgery, and organ procurement and storage during lung transplantation. On the basis of our findings with stop of flow, we postulate that normal blood flow is “sensed” by the endothelium by virtue of its location at the interface of the blood and vessel wall and that this signal is necessary to maintain the endothelial cell membrane potential. Stop of flow is sensed by a “mechanosome” consisting of PECAM-VEGF receptor-VE cadherin that is located in the endothelial cell caveolae. Activation of the mechanosome results in endothelial cell membrane depolarization that in turn leads to activation of NADPH oxidase (NOX2) to generate reactive oxygen species (ROS). Endothelial depolarization additionally results in opening of T-type voltage-gated Ca2+ channels, increased intracellular Ca2+, and activation of nitric oxide (NO) synthase with resultant generation of NO. Increased NO causes vasodilatation whereas ROS provide a signal for neovascularization; however, with lung transplantation overproduction of ROS and NO can cause oxidative injury and/or activation of proteins that drive inflammation and cell death. Understanding the key events in the mechanosignaling cascade has important lessons for the design of strategies or interventions that may reduce injury during storage of donor lungs with the goal to increase the availability of lungs suitable for donation and thus improving access to lung transplantation.

THE RELATED TERMS mechanosensing, mechanosignaling, and mechanotransduction refer to the sensing of physical forces by cells and their translation into a biochemical response, analogous to the processes termed chemotransduction. Among the various physical forces to which cells in vivo are constantly exposed, mechanical stimuli associated with blood flow are particularly important. Indeed, fluid shear stress and distending pressure that act on the vessel wall play a major role in vascular homeostasis. The vascular endothelium, by virtue of its location, serves as an interface between the blood and tissue for hemodynamic changes. Recent work demonstrates that the endothelium senses and integrates hemodynamic stimuli including shear to effect maintenance of vascular function and possibly modulate the onset of vascular disease. The currently accepted paradigm is that “sensing” of a change (either increase or decrease) in the shear force occurs via elements on the endothelial cell membrane that activate intracellular signaling pathways to modify cell structure, metabolism, and gene expression.

Most studies of shear-related signaling have utilized endothelial cells in culture that experimentally are exposed to shear associated with onset of perfusate flow. For reasons described below, we consider this to be an inadequate model for study of physiological events. Our laboratory during the past two decades has evaluated endothelial mechanotransduction using intact lungs as well as isolated cells that are exposed to abrupt cessation of blood flow (3, 15, 19, 20, 25, 79, 94, 125). Thus these studies have utilized a physiological model that has relevance for understanding the effects of lung ischemia followed by reperfusion (I/R) as occurs clinically following pulmonary embolism, during cardiopulmonary bypass (CPB), and also during lung transplantation. Lung injury from I/R, termed primary graft dysfunction, is a major complication after lung transplantation and is considered as a major factor for transplant failure (27, 36, 64, 65, 102). In the case of CPB, lung I/R...
has been reported as responsible for ~20% of the complications associated with cardiac surgery (78, 111). Thus a thorough understanding of the physiological, cellular, and molecular changes that occur during lung I/R is critically important with direct relevance to the practice of pulmonary medicine. The insights gained from the cell and animal studies could potentially be useful to design strategies to combat tissue injury with I/R.

Although endothelial mechanotransduction has been reviewed by us and others in great detail in the past (22, 24, 33, 103), our focus here is novel. This article is not limited merely to discussing the physiological events that occur in the pulmonary endothelium secondary to altered shear stress during I/R but extends to using our understanding of flow sensing and signaling to derive lessons for application to clinical situations. These lessons, in the form of interventions that prevent mechanosignaling, are particularly relevant in lung transplantation, where several episodes of stop and stop of perfusion occur with procurement and storage and later reattachment of the donor lung. We will consider in detail the relationship of these changes to the lung injury that may accompany lung transplantation. We will discuss how the insights gained from studies of lung I/R can be employed for better management and storage of lungs prior to transplant and will identify potential interventions that can alter key events in the mechanosignaling cascade to prevent or reduce I/R-related injury. We will not consider the effects of I/R on other lung cell types, the various other mechanisms of lung transplant-related injury such as infections or rejection that are not directly associated with I/R, or the signaling pathways of other mechanical stresses such as those associated with lung ventilation. Since our laboratory has been primarily responsible for studies related to acute decrease in shear stress, the presentation will of necessity reflect in large part the contributions from the authors of this review. The overarching theme will be the revisiting of our earlier findings to derive potential strategies to mitigate shear stress-induced oxidant production and thus reduce lung transplant-related injury.

Definitions: Ischemia vs. Hypoxia

I/R has been studied in detail in most organs of the body, with a focus on the reduction of oxygen supply associated with the decrease in blood flow. The resultant hypoxia/anoxia leads to decreased cellular ATP production that has myriad ramifications for cellular function. Among other alterations, the Na+/K+-ATPase pump operates less effectively, resulting in the influx of sodium and water, cell swelling, and perturbation of organellum function. Perturbation of ionic homeostasis stimulates release of Ca2+ from intracellular stores and activation of store-operated Ca2+ channels; these combine to increase intracellular Ca2+ concentration, which in turn leads to activation of intracellular degradative enzymes (viz. aspartate proteases, matrix metalloproteases, and calpains), which disrupt membrane and cytoskeletal proteins (45, 84, 90, 101). With less prolonged hypoxia, the major injury appears to occur during the reperfusion period associated with the reintroduction of oxygen. A major effect has been attributed to the formation of superoxide anion (O2−), primarily via the xanthine-xanthine oxidase pathway but also by the mitochondrial respiratory chain and NADPH oxidases. These enzymes appear to be “primed” by varied mechanisms during hypoxia to then generate increased O2− upon reoxygenation. O2− and related oxidizing agents, termed reactive oxygen species (ROS), can peroxidize cellular membrane lipids, oxidize DNA, and denature enzyme proteins and can also disrupt cellular homeostasis through interaction with physiological signal transducers (88).

Clearly, hypoxia and subsequent reoxygenation can have profound effects on lungs as well as other organs. However, this discussion generally is moot in considering the lungs since ischemia per se does not lead to hypoxia in this organ. Unlike systemic organs, the lung parenchyma does not rely on its perfusion for its cellular oxygen requirements; rather, the flow of O2 physiologically is in the opposite direction, through the lung tissue to the blood. Thus alveolar ventilation maintains normal alveolar PO2 and even static inflation of the lungs can keep the lung cells oxygenated for a reasonable time. Reperfusion of the lung also is dissimilar from that in other organs since oxygen is present during ischemia and is not reintroduced during reperfusion. Below, we will consider circumstances when lung hypoxia may accompany ischemia.

Altered Shear Stress as a Manifestation of Ischemia

Since the lung does not become hypoxic with mere cessation of perfusion, it is reasonable to question whether lung ischemia does result in tissue injury and, if so, its mechanism. We initially addressed this question ~25 years ago (with considerable prodding from the National Heart, Lung, and Blood Institute via a Request for Applications). Indeed, we and others demonstrated that ischemia (±reperfusion) did result in lung injury that was in part the result of oxidative stress. Subsequent detailed study indicated that the activation of an ROS-generating pathway during ischemia was a function of the altered mechanical (shear) stress associated with the loss of blood flow. (As described above, the definition of ischemia is solely loss of blood flow; hypoxia per se is not part of the definition, although hypoxia clearly accompanies ischemia in systemic organs). These findings led us to conclude that it was not altered oxygen supply but rather altered shear that resulted in lung oxidative injury associated with ischemia (Fig. 1).

To study the response to pulmonary ischemia ± reperfusion, we have used in situ and in vitro models where constant alveolar PO2 can be maintained. For in situ experiments, lungs were ventilated throughout the ischemic episode (PO2 ~100 mmHg). For in vitro studies, cells were kept under flow (flow adapted) in artificial capillary chambers or parallel-plate chambers; cellular oxygenation with stop of flow was maintained in these cell culture studies by perfusion through abluminal side ports that did not expose the cells to shear (20, 25, 115) (Fig. 2). In this presentation, we will use the terms “stop of flow” and “ischemia” interchangeably.

Response of the Lung to Altered Shear Stress: ROS Generation

Our studies with isolated but otherwise intact lungs from rats and mice showed that stop of perfusate flow resulted in the production of ROS. Using various ROS-sensitive fluorophores (dihydroethidine for superoxide, dichlorofluorescein (DCF) for tissue H2O2, Amplex red for intravascular H2O2), we observed that ROS generation occurs within seconds following stop of flow (5). Using fluorescence microscopy imaging techniques, we observed that ROS with stop of flow (ischemia) were...
produced in the intact lung predominantly by the pulmonary microvascular endothelial cells (7, 25, 124, 125). This major role in the response to ischemia was confirmed by studies with pulmonary microvascular endothelial cells in culture; cells that had been flow adapted to mimic in vivo conditions demonstrated ROS production with stop of flow while similar experiments with cells cultured under static conditions showed no ROS response (25, 115). Reperfusion resulted in a further increase in the markers of oxidative stress (37, 43, 44). As both stop and restart of flow caused the production of ROS, we posit that endothelial mechanosensing responds to decrease or increase in shear by a similar pathway, the important stimulus being the change in shear from a flow adapted state.

**ROS Generation: Mechanism for Linkage to Altered Shear Stress**

We studied the linkage between the mechanical event of decreased shear stress and ROS production using the intact isolated perfused lung ex vivo and several models of flow adapted endothelial cells in culture. An early response to stop of flow is endothelial cell membrane depolarization that was detected initially by the use of membrane potential sensitive dyes. Subsequently, patch-clamping experiments confirmed that depolarization with stop flow occurred via closure of an inwardly rectifying K⁺ channel (25). Experiments with chemical agents (agonists and inhibitors) provided strong evidence that KATP channels are responsible for the response to altered shear (4, 6, 25); this was corroborated with use of mice that were null for the KIR 6.2 component of those channels (25, 124, 125). We concluded that KATP channels are responsible for determining the resting membrane potential in pulmonary microvascular endothelium. Presumably these channels are maintained in the open state by the normal shear stress associated with blood flow, whereas channel closure resulting in membrane depolarization occurs when shear is abolished because of the cessation of flow. The link between membrane depolarization and ROS production was confirmed by treating cells with high external K⁺ and observing a response similar to that seen with the loss of shear stress (4, 21). What is the biochemical source of the increased ROS production with altered shear? We ruled out xanthine oxidase since allopurinol had no effect on the response and turned our attention to NADPH oxidase, now considered the major source of ROS in response to physiological stimuli (14, 48). NADPH oxidase (NOX) has long been associated with polymorphonuclear neutrophils (PMN) and is known to be the enzyme responsible for the respiratory burst in these cells; this burst is the source of oxidants for the bactericidal activity of PMN (48, 89). Recent studies have demonstrated that the NOX family of enzymes consists of seven widely distributed members (86). The enzyme found in PdMN and now called NOX2 also is present in a variety of cell types including microvascular endothelium, where it plays an important role in cellular homeostasis (7, 21, 25, 73, 74, 115). In our models of altered shear stress, endothelial NADPH oxidase clearly is the source of ROS with ischemia since use of inhibitors of NOX2 activation as well as lungs and cells from NOX2-null mice abolished the effect. In these latter experiments, the upstream events (i.e., membrane depolarization and kinase activation) were similar in NOX2-null and wild-type lungs.

How is the signaling pathway for endothelial ROS production cell membrane depolarization coupled to NOX2 activation? We investigated cellular signaling pathways with special attention to the protein kinases. Depolarization resulted in activation of the PI3K-Akt complex in pulmonary endothelial cells and activation of NOX2. On the other hand, lungs and cells studied in the presence of a PI3K inhibitor (wortmannin) or isolated from Akt1-null mice showed depolarization but generated little ROS with stop of flow (21). Thus PI3K-Akt activation is downstream of membrane depolarization but upstream of ROS production (Fig. 3A). The pathways for phosphorylation events related to activation of PI3K-Akt are still under investigation; the sequence of events appears to be the phosphorylation of ERK or JNK resulting their activation (72, 114) followed by phosphorylation of peroxiredoxin 6 and the cytoplasmic components (p67phox, p47phox, p40phox) as required for activation of NOX2. This sequence of events following stop of flow is shown schematically in Fig. 3B.

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**Fig. 1.** Pathways for reactive oxygen species (ROS) production with ischemia-reperfusion (I/R). In systemic organs, I/R compromises oxygen delivery and results in anoxia-reoxygenation. ROS production occurs by the xanthine-xanthine oxidase pathway (left). Ox Phos, oxidative phosphorylation. We investigated lung I/R in which the mechanical component of blood flow is altered but the lung parenchyma does not depend upon the circulation for oxygenation and is remain oxygenated through the alveoli. ROS is produced by activation of NADPH oxidase (NOX2) pathway as shown at right.
The onset of flow or its removal or restart each alter membrane polarity. We observed that, similar to loss of shear with ischemia, suddenly increased shear with reperfusion triggers signaling that leads to NOX2 activation (1, 62). NOX2 activation in this case is driven via hyperpolarization that occurs with onset of flow signaling. In other words, endothelial mechanosensing responds to decrease or increase in shear by a similar pathway, the important stimulus being a change from set point.

Also, it is not clear how both depolarization and hyperpolarization lead to similar signaling pathways that eventually cause oxidant production. With depolarization, we observed PI3K-Akt-PKC activation that caused NADPH oxidation and ROS production (21). In the case of hyperpolarization, we and others have observed ROS production that seems to be via NOX/NOX2 activation, but the intermediate links between altered membrane potential and NOX activation in this case have not been adequately delineated (1, 2, 62). Onset of shear is reported to activate tyrosine kinases (including ERK, JNK, Akt, and PKC). These could be responsible for NOX2 activation since activation of PKC and MAPK cause NOX assembly and ROS production in other nonmechanosensitive pathways (35, 39, 51). Thus the intermediate link between hyperpolarization and NOX activation and ROS production with start and stop of flow may occur via various kinases and/or kinase receptors. This aspect of the mechanosensing cascade remains to be elucidated in greater detail.

**Cellular Ca\(^{2+}\) Flux and Activation of NO Synthase**

With the use of fluorescent dyes, we investigated possible changes in intracellular [Ca\(^{2+}\)] in association with cell membrane depolarization with ischemia. There was a rapid rise in intracellular Ca\(^{2+}\) that occurred within the first minute after onset of ischemia. The rise in cellular Ca\(^{2+}\) was significantly inhibited by pretreatment with thapsigargin, an agent that depletes Ca\(^{2+}\) from intracellular stores. This indicated that the increase in intracellular Ca\(^{2+}\) occurs chiefly through Ca\(^{2+}\)

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**Fig. 2.** In vitro and in situ models to study signaling with lung ischemia. **A**: in situ (ex vivo) model of lung ischemia. Rat or mouse lungs isolated and cleared of blood are ventilated and perfused on the stage of a microscope. Lungs are preperfused with fluorescent dyes, placed on the stage of a microscope, and imaged for ROS, Ca\(^{2+}\), or NO, etc. **B**: apparatus for flow adapting endothelial cells in vitro. Left: parallel-plate chamber (Warner Instruments, Hamden, CT) and perfusion circuit. Cells are seeded on coverslips and inserted into the chamber. After 24 h of flow adaptation, these cells can be labeled with fluorescent dyes (that monitor membrane polarity or ROS production) and imaged with stop and restart of flow in real time. Right: artificial capillary chamber that mimics blood vessels. Cells are seeded into the narrow polypropylene capillaries and allowed to attach for 24 h. During this period cells are not exposed to shear and are fed via perfusion through abluminal ports. For flow adaptation, medium is perfused through luminal ports and are then subjected to flow (shear from 1–10 dyn/cm\(^2\)) for 24–72 h. Ischemia is achieved by stop of luminal flow; medium is routed through side ports to prevent hypoxia. Cells are removed from the capillaries by trypsin and assessed by immunocytochemistry.
entry from the extracellular space (9, 94) and raises the question of the pathway for entry of calcium ions into endothelial cells. We observed that an inhibitor with some specificity for T-type Ca\(^{2+}\) channels (mibefradil), but not an L-type Ca\(^{2+}\) channel blocker (nifedipine), blocked Ca\(^{2+}\) influx into endothelium in the stop of flow experiments (116). Others have identified T-type Ca\(^{2+}\) channels in pulmonary endothelial cells (120). We calculated that the change in membrane potential with ischemia was around \(-20\) mV; since the resting potential of endothelial cells is approximately \(-70\) to \(-80\) mV, depolarization is compatible with the known “voltage window” for T-type channels (99, 120). We concluded that the change in membrane potential with ischemia was around \(-20\) mV; since the resting potential of endothelial cells is approximately \(-70\) to \(-80\) mV, depolarization is compatible with the known “voltage window” for T-type channels (99, 120). We conclude that membrane depolarization induced by K\(_{\text{ATP}}\) channel closure with ischemia activates T-type Ca\(^{2+}\) channels that are responsible for Ca\(^{2+}\) influx into endothelial cells. Membrane depolarization was unaffected by pretreatment with mibefradil, indicating that depolarization is upstream of T-type Ca\(^{2+}\) channel opening. (116).

Besides ROS generated by the pulmonary endothelium, we observed the production of NO with ischemia (9, 94). Using the fluorescent dye diaminofluorescein diacetate (DAF-2DA), we were able to detect NO release within 1 min after stop of flow. NO generation was preceded by an increase in intracellular Ca\(^{2+}\). Conversely, perfusion with a Ca\(^{2+}\)-free medium inhibited NO generation with ischemia. These results implicate the activation of a Ca\(^{2+}\)-dependent enzyme such as endothelial NO synthase (eNOS) in NO generation as a component of the ischemic response (69, 94, 107). The generation of a vasodilator (NO) can be considered as an appropriate physiological response to the loss of blood flow.

Shear Sensing: Initiation of the Signaling of Cascade

Cell signaling associated with mechanotransduction comprises a cascade from cell membrane depolarization to altered transcription factor expression. Although cell membrane depolarization represents an early event in the cell signaling cascade, it is unlikely to be the actual sensor for detecting the change in shear. This is illustrated by our finding that statically cultured endothelial cells demonstrate low K\(_{\text{ATP}}\) channel expression whereas channel expression is increased with flow adaptation (20). Thus there is a shear sensor that “senses” flow leading to induction of K\(_{\text{ATP}}\) channel expression. It has been postulated that a mechanosensory complex comprising the

Fig. 3. A: summary of the mechanosensing cascade with stop of shear based on our research from the past 2 decades. Shear sensing occurs by mechanosomes (consisting of caveolae, PECAM-1, VEGFR2, and VECadherin) (24, 109) followed by transduction of the mechanical signal via cellular components that in turn lead to activation of bioactive transmitters that modify transcription factors to alter gene expression and elicit physiological responses. B: temporal events in signaling with loss of shear. Instantaneous or immediate events (<1 min) such as ion channel activation-deactivation lead to intermediate signaling (1 min-24 h) that finally leads to late or adaptive (>24 h) responses.
platelet endothelial cell adhesion molecule-1 (PECAM-1), the vascular endothelial growth factor receptor (VEGFR2), and vascular endothelial cadherin (VE-cadherin) can transduce mechanical forces including shear stress (109). Our investigations based on genetically engineered mice revealed that the shear-sensing molecular complex also includes PECAM-1 and caveolae as important component of the mechanosensing machinery in the pulmonary endothelium. We observed that a significant fraction of cellular PECAM-1 is located in caveolae (79). Thus we posit a mechanical stress-responsive “mechanosome” comprising caveolae, PECAM-1, VEGFR2, VE-cadherin, and possibly other elements (24). Although the exact mechanism by which the mechanosome works or indeed its linkages to the subsequent signaling moieties in the mechanosignaling cascade are unclear, we and others have provided evidence that the elements that comprise the mechanosome (caveolae, PECAM-1, VEGFR2, and VE-cadherin) are closely associated with one another and the lack of any of these elements disrupts the mechanosignaling cascade (79, 109).

Because the stimulus of flow keeps the mechanosome activated and the K<sub>ATP</sub> channel open, we thus reasoned that for the mechanosome to sense both onset and stop of shear, it should exist in Off-On states. How that Off-On mode may translate into opening or closing of K<sup>+</sup> channels is not clear at this time. Possibly removal of this stimulus (stop of flow) is sensed by the mechanosome; signals emanating from the mechanosome perturb the membrane and activate intrinsic proteins that trigger stop of flow signaling.

**The Physiological Role of the Mechanosignaling Cascade**

It is now appreciated that ROS can function as a signaling molecule and can influence cellular growth, differentiation, motility, and other homeostatic functions (14, 54). Thus ROS generated in endothelial cells exert their physiological effects by interaction with transcription factors, protein tyrosine phosphatases, protein tyrosine kinases, mitogen-activated protein kinases, and other cell signaling-related components. Many of these modified proteins transmit their “signal” through phosphorylation and dephosphorylation of specific amino acid residues (serine, threonine, tyrosine, and histidine). ROS modify these signaling-related proteins through the oxidation of specific residues that result in reversible activation or inactivation of enzymatic activity.

ROS generation in endothelial cells with stop of flow was linked to cell proliferation as indicated by both increased DNA synthesis and FACS analysis for cell number. Proliferation was blocked by inhibitors of shear stress-induced ROS generation including cromakalim or diphenyleneiodonium chloride (DPI) (72–74, 114). Although it would be desirable to confirm these effects in vivo, evaluation of endothelial cell proliferation in the intact lung does not seem feasible based in large part on the extensive vascularity of this organ. To address this issue, we utilized expression of VEGF as a surrogate index for endothelial cells subjected to a stimulus for proliferation. There is utilized expression of VEGF as a surrogate index for endothelial cell proliferation. There is limited VEGF expression in the normal lung. However, ischemia in vivo subsequent to the introduction of microbeads in the lung caused a significant increase in VEGF expression (79). This appeared to be linked to mechanotransduction since ROS production/VEGF expression was not increased with microbeads in NOX2-null or PECAM-null lungs (i.e., lungs that generate relatively little ROS with stop of flow) (79). Thus the increased VEGF expression with ischemia in the intact lung could correspond to the increased cell division observed in vitro. We propose that increased cell division with ischemia represents an attempt to generate new capillaries to restore impeded blood flow.

**Activation of Transcription Factors**

We envision endothelial ROS generation as the pivot point in the mechanotransduction signaling cascade. The sensing of altered shear and the responses upstream of ROS generation occur on an individual cell basis. However, ROS production as it occurs through NOX2 activation on the extracellular side of the plasma membrane allows the signal to spread over adjacent cells, albeit the importance of this observation is not yet clear. The subsequent downstream events also can be considered as part of the signal transduction cascade (Fig. 3, A and B). The major downstream effects of ROS-mediated signaling are the activation of various transcription factors. Experimentally, endothelial cells that were flow adapted in vitro and subjected to ischemia showed activation of NF-κB by electrophoretic mobility shift assay as reflected by increased nuclear content of p65 and p50 subunits; concurrent activation of activator protein 1 (AP-1) was detected by Western blot analysis showing an increase in both c-Jun and c-Fos subunits (115). Activation of these transcription factors was abolished by pretreatment of cells with inhibitors of ROS generation, indicating that their activation indeed is linked to ROS (42, 115). The specific responses to these changes in transcription factor expression may determine the longer term physiological response to altered shear stress.

There are many additional transcription factors such as Nrf2, ATF/CREB, and HIF-1α that are redox sensitive and might be modulated be ROS that are generated during ischemia. The activation of HIF-1α by ROS may be mediated by NF-κB (13). A possible role for NADPH oxidase-derived ROS in the regulation of HIF-1α was suggested by an in vitro study in mice in which overexpression of p22<sup>phox</sup> increased ROS generation and HIF-1α protein levels in smooth muscle cells (58). However, the involvement of these additional transcription factors in the mechanotransduction cascade has not yet been determined (66).

**Role of ROS in the Response to an Inflammation-Inducing Agent**

In addition to the homeostatic response to the signaling cascade described above, ROS produced by endothelial cells may have an important role in the lung response to inflammation, which may have an important bearing on lung function after transplantation. Blood cells associated with inflammation (PMN chiefly and monocytes) can attach to endothelial cells and subsequently migrate into lung interstitial spaces and even reach the alveoli. Endothelial ROS-dependent signals facilitate binding and adhesion of neutrophils to the vascular wall, although the mechanism is unclear. The major role of endothelial ROS in this process appears to be regulation of expression of those cell adhesion molecules that control PMN binding to endothelial cell membranes (47, 71). There was relatively little lung inflammation following LPS instillation in NOX2-null mice, whereas transgenic mice that expressed NOX2 only...
in endothelium did show an inflammatory response (82). This demonstrates the importance of ROS produced by endothelium to initiation of inflammation (55, 61, 85). Although PMN that are recruited into lungs can release ROS and can be a major cause tissue damage, the initial signal for their recruitment is ROS produced by the endothelium. By extrapolation, blocking ROS production resulting from the endothelial mechanosignaling cascade with I/R could reduce lung inflammation and subsequent tissue damage (Fig. 4).

The Response of Lungs and Cells to Reperfusion

Above, we have delineated the pathways that are activated with stop of flow. However, in the clinical setting, ischemia of the donor lung is followed by reperfusion after transplantation. In situ studies of lung I/R have utilized a variety of models, including those in which ventilation is continued, whereas others have used a hilar clamp, thus impeding pulmonary and bronchial blood flow as well as ventilation. In the latter case, the lung is often rendered hypoxic and also could be accompanied by trauma-induced inflammation. Clearly, it is important to recognize the role of the specific model for understanding the lung manifestations supposedly associated with I/R.

We have studied reperfusion with isolated rat lungs that were subjected to restart of flow after ischemia and found that reperfusion led to further oxidant production as monitored by lung tissue lipid peroxidation; the damage with reperfusion was severalfold greater than with ischemia (37, 43, 44). The degree of lipid peroxidation and lung damage in these studies showed dependence on alveolar oxygen tension in both ischemia and reperfusion. Studies using isolated mouse lungs showed that increased ROS production with reperfusion was NOX2 dependent (62, 63).

Signaling associated with the onset of flow also has been studied extensively in statically cultured cells (10, 81, 112). Although this may not be a physiological model, these studies have relevance for the response to the increased shear associated with reperfusion. Onset of flow causes the opening of an inwardly rectified K+ (KIR) channel, leading to membrane hyperpolarization and eventual ROS and NO production (28, 53, 56, 81, 123). Presumably, similar to loss of shear with ischemia, suddenly increased shear with reperfusion triggers signaling that also leads to NOX2 activation (63). NOX2 activation in this case may be driven via hyperpolarization that occurs with onset of flow signaling, although this has not yet been evaluated experimentally. It is interesting that the response to increased shear in cells is culture is similar to the response to decreased shear. This has been interpreted as the response to a change from a set point of cell adaptation (17, 24, 119).

Lung Transplantation: a Clinical Case of Ischemia and Reperfusion

The earliest mention of transplant of any organ comes from Chinese medicine, when Chinese physician Hua-Tuo is said to have replaced diseased organs with healthy ones (108). The first documented reference to modern organ transplantation occurred in 1869 when Swiss surgeon Jacques Louis Reverdin transplanted skin from one individual to another (40). The first successful lung transplant occurred almost a century later, in 1963 (50). Subsequent attempts resulted mainly in failure. It was only with an understanding of the immunological processes underlying tissue injury and rejection that a framework was established in this field with focus on lung storage techniques, immunosuppression, and control of inflammation.

Oxidative injury with I/R is associated with influx of inflammatory cells (mainly PMN) from the recipient into the newly transplanted donor lungs; macrophages that (59) are resident within the donor lung also can contribute (76). Once activated, both resident macrophages and infiltrating PMN release inflammatory mediators. TNF-α released by resident donor macrophages has been shown to be an essential component in the cascade of inflammatory events causing lung I/R injury in animal and human studies (34, 87). In addition to inflammatory mediators, induction of matrix metalloproteases is associated with lung allograft rejection (34, 121, 122).

Examination of samples of lung allograft rejection shows lesions associated with lymphocytic infiltration, disruption of
the epithelium, and ingrowth of fibromyxoid granulation tissue into the airway lumen resulting in obliteration of the airway lumen (bronchiolitis obliterans syndrome). This is a predominant feature of chronic lung transplant rejection. Although the relationship between I/R and the presence of bronchiolitis obliterans is unclear, there is a significant correlation between the latter and excessive oxidative stress markers in lungs of patients after transplantation (12, 68, 85). Thus oxidative injury and immunological rejection appear to be the two major causes for lung graft failure.

The link between the episode of I/R that attends the transplant process and recruitment of inflammatory cells has been studied but is still not clear. Our research on pulmonary endothelial mechanosensing related to stop (and restart) of flow and the attendant signaling that leads to ROS production would appear to be relevant to understanding of the process of transplanted lung rejection. Examination of the mechanisms for endothelial ROS production could enable interventional strategies aimed at blocking elements of the mechanosignaling cascade that participate in amplifying the oxidant injury associated with lung transplantation (Fig. 4).

**Lung Transplantation: Ventilation and Oxygenation**

Donor lungs procured for transplant are usually stored without ventilation so they may become hypoxic during the storage period; in these circumstances, reoxygenation occurs when physiological conditions are restored. In an effort to improve lung allograft survival, attention has been devoted to various strategies for ventilation of the donor lung. The purpose of ventilation is to prevent alveolar collapse and also to accomplish alveolar oxygenation, thereby avoiding tissue hypoxia and reoxygenation. Oxygen consumption of the human lung has been estimated at 11 ml/min (at 36°C) (67). Assuming that lungs are not ventilated during storage, a residual lung volume (RV) of 1.5 liters (in a lung with total lung capacity of 6 liters) would provide sufficient O₂ for about 30 min; storage in the cold would significantly increase the time to hypoxia, approximately doubling the time if stored at 26°C. Maintaining the lung at functional residual capacity (FRC) rather than RV could also double the time to hypoxia. Finally, inflating the lung with an elevated concentration of O₂ rather than air also could be beneficial. So, inflating the lung with 40% O₂ to FRC during storage at 26°C could extend the time to hypoxia to ~4 h. As a potential side effect we have noted that the generation of ROS during I/R is increased as a function of the O₂ content in the gas used for lung ventilation (37, 44). In our experimental studies with isolated lungs, 5–10% CO₂ always is added to the alveolar gas to maintain normal lung tissue pH; in situ, CO₂ is supplied normally by the pulmonary perfusion. In the absence of ventilation longer term storage of lungs may result in manifestations of hypoxia, in addition to those related to ischemia.

Recognizing a possible role for hypoxiareoxygenation in oxidant production, several clinical groups have developed techniques to provide ex vivo lung perfusion (EVLP) and ventilation during storage (31, 32, 38, 110) or prior to transplantation. A recent practice [pioneered by the Keshavjee group (30, 31)] has been to use ventilation and perfusion in a reconditioning process prior to transplant. Called the EVLP technique, this involves reconditioning of donor lungs that either have been stored without ventilation and under hypothermic conditions or have been freshly procured. In EVLP, lungs are ventilated (tidal volume of 7 ml/kg donor body weight and a rate of 7 breaths/min) and perfused under normothermic conditions for a period of 3–4 h prior to the transplant. After 4 h of EVLP, the lungs are cooled to 10°C over a period of 10 min. Perfusion and ventilation are stopped and the trachea is clamped at full inspiration to maintain the lungs in a state of inflation. The lungs are then stored at 4°C in storage buffer (Perfadex) until transplantation.

Preclinical studies have shown that normal and injured donor lungs that were maintained for up to 12 h in the EVLP system had excellent, sustained lung function after transplantation. Indeed, the incidence of primary graft dysfunction 72 h after lung transplantation tended to be lower in the recipients of EVLP lungs than in the controls (15 vs. 30.1%) (31).

On the basis of these findings and our past studies, we believe that misdirected cell signaling with altered blood flow (i.e., loss of the mechanical component of pulmonary blood flow) plays an important role in the tissue injury associated with lung transplantation (21, 22, 25). These results support the concept of ex vivo perfusion in stabilizing the vascular side of the donor lung prior to implantation.

**Lung Transplant Primary Graft Dysfunction: Role of Endothelial Mechanotransduction**

One of the aims related to study of endothelial mechanosignaling with I/R is to apply this understanding to the field of lung transplantation. This may allow the design of strategies that 1) minimize or abrogate oxidant production during the ischemic period associated with procurement and storage of lungs or ischemia, 2) allow for reconditioning (i.e., “repair”) of potential donor lungs to expand the pool of lungs appropriate for transplantation, and 3) block pathways that lead to lung injury associated with recruitment of inflammatory cells upon reperfusion following transplantation. Application of the research related to lung transplantation reflects our assumption that lungs “know” their physiological state and respond to change with a signaling cascade that can, in excess, disrupt organ and cellular function. As injury associated with I/R is a frequent complication after lung transplantation (27, 36, 64, 65, 102) various strategies derived from understanding aspects of the mechanotransduction cascade may be employed to reduce oxidant production during I/R, particularly in the setting of ex vivo perfusion.

**Storage of Lungs for Transplantation: Lessons from Studies of Lung I/R**

It is well recognized that the condition of the donor lung and the degree of injury related to I/R is critical in transplant outcome. However, the role of storage time in determining ischemic injury is not clear. Some studies found that ischemic time has no impact on early graft function (26, 41, 60), whereas others suggested that ischemic time in combination with donor characteristics has a negative impact on lung transplant outcome (92, 105). Smits et al. (91) analyzed data from 21 Eurocenters and reported that cold ischemic time did not have an impact on 1-year lung transplant survival. However, it needs to be noted that long ischemia times have been avoided for these studies; i.e., the ischemic time was longer than 6 h for
only 6% of the lung allografts. Likewise, Thabut et al. (104) found that total ischemic times above 330 min were a risk for primary graft dysfunction (PGD).

If longer cold ischemic time adversely affects graft outcome, function of the graft could be improved by blocking mechanosignaling with ischemia, thereby reducing the risk of oxidative injury during long-term storage. The following are potential strategies to maintain lung viability under these conditions informed by understanding the mechanotransduction cascade associated with lung ischemia.

**Lung perfusion.** Keeping the lungs perfused during EVLP may prevent ischemia during storage. Thus perfusion may be a maneuver to prevent ischemia-reperfusion signaling (31). In rat lungs, signaling was not activated until the perfusion rate decreased below a threshold level, i.e., a drop in shear by >90% of physiological flow was required for onset of ischemia signaling (8). However, the shear threshold for ischemic signaling in human lungs has not been definitively established. Such information will be valuable in maintaining lungs close to physiological conditions during EVLP while minimizing ischemia-related signaling and resultant injury.

The technique of EVLP involves perfusing explanted lungs and ventilating them in a closed ex vivo circuit (29–31). The lungs are perfused at a constant pressure (<20 mmHG) with a buffered extracellular normothermic solution at an optimal colloid osmotic pressure (Steen solution, Vitrolife, Sweden). Steen solution may block NOX2 activation in platelets and white blood cells and reduces oxidative stress as determined by a significantly lowered formation of ROS and isoprostanes by both platelets and white blood cells (18). The EVLP approach may have several important applications: 1) prevention graft of damage after procurement and before implantation, 2) repair of potential grafts that may otherwise not have been considered for transplantation (95–97).

**Treatment with a K⁺ channel agonist.** On the basis of the link between membrane depolarization and activation of NOX2, an agent that prevents KATP channel inactivation with loss of shear will prevent ROS generation. An appropriate choice for this purpose would be cromakalim (or the L-isomer lemakalim), a relatively nontoxic KATP channel agonist (9, 11, 94). We posit that use of the KATP channel agonist cromakalim during storage of lungs prior to reperfusion would reduce ROS production. Cromakalim has been studied for the treatment of ischemic heart disease but has not yet been utilized in clinical lung studies (49, 106). As an alternative, MJ33 (a nontoxic inhibitor of the PL2a activity that is required for NOX2 activation (62, 63)) could be a useful agent to prevent endothelial NOX2 activation even in the presence of endothelial cell membrane depolarization.

**Avoid depolarizing agents.** On the basis of well-known physiological principles (i.e., the Goldman equation), perfusion of lungs with high K⁺ leads to endothelial cell membrane depolarization and ROS generation analogous to that occurring with ischemia (7, 25). Interestingly, it formerly was common practice to store lungs in a solution containing high K⁺ concentration (52, 83). Increasingly, this practice has been reversed in favor of a solution containing physiological K⁺ concentrations (80, 93).

**Inhibition of ROS generation.** Inflammation with secondary tissue damage of lungs following transplantation can result in part from ROS generated by PMN and macrophages that have been recruited from the acceptor blood supply posttransplant. The use of antioxidants to inhibit tissue damage with inflammation has been disappointing (98, 100, 117). This may reflect the difficulty of exogenous agents in competing with tissue targets for scavenging of ROS. A more efficient approach may be to inhibit the activation of NOX2 (or other enzymes) and thereby prevent ROS generation by inflammatory cells. This could be achieved by the use of MJ33, a nontoxic inhibitor of the PL2a activity that is required for NOX2 activation (62, 63). This strategy is still experimental since it has been demonstrated in an animal model with inflammatory lung injury and I/R (62, 63) but not yet in human tissue. Alternatively, activation of NOX2 can be inhibited by blocking the various kinases that are active in the NOX2 activation pathway (23). However, inhibition of these kinases is not specific and their use might result in nonspecific clinical manifestations.

**Inhibition of NO generation.** NO generation occurs as part of the mechanotransduction signaling cascade. Although this can reverse vasconstriction, it would appear to be an inappropriate response related to the loss of blood flow such as associated with lung transplantation. In the case of transplantation, the loss of perfusion is not related to vasconstriction, and the vasodilatory function of NO would play no significant role. NO potentially can be damaging to tissue in the presence of O₂⁻ since the two compounds combine to generate the relatively strong oxidant, ONOO⁻. In these circumstances, inhibition of NO synthesis to prevent excessive NO generation might be beneficial. On the other hand, the verdict on the role of NO in animal models of lung transplantation is mixed. Supplementation of the preservation solution with NO donor nitroglycerin in rodents improved recipient survival after lung transplantation (75, 77), although NO inhalation in human randomized trials had mixed to no effects (70). Thus the potential benefits or detrimental effect of increased NO generation is not clear.

**Lung ventilation and oxygenation.** Another important parameter that may effect change during lung storage is the oxygenation of lung tissue. Lung hypoxia can occur if lungs are permitted to collapse or remain unventilated for prolonged periods. Injury under these conditions may arise from complex signaling cascades involving both hypoxia (and reoxygenation) and ischemia (and reoxygenation) (16). An earlier clinical practice had been to ventilate lungs during harvest and storage. Although this may abrogate the injury associated with hypoxia /reoxygenation, it will not alter the effects of ischemia unless combined with perfusion in the EVLP technique. The role of lung ventilation per se during storage and after transplantation is complex and it is not considered in this review but requires further investigation and documentation.

Some of the strategies mentioned above for lung preservation are in current use. On the basis of work from our and other groups (6, 57, 118), low K⁺ (i.e., physiological levels of K⁺) is now the accepted storage or preservation solution for lungs and other donor organs (46, 113). Next, continued ventilation and perfusion prior to lung transplantation (EVLP) is being tested as a maneuver to reduce lung damage. However, the use of a KATP agonist (cromakalim) or a blocker of NO synthesis (or the use of a NO donor) has not been well studied.
Summary and Conclusions

Our studies have shown that lung ischemia (stop of flow) during continued ventilation activates a mechanosignaling cascade that leads to production of ROS. This cascade is initiated by a mechanosensing complex (the mechanosome), is transmitted by endothelial cell membrane depolarization (due to closure of KATP channels), and results in activation of NADPH oxidase (NOX2 with generation of ROS), increased intracellular Ca\(^{2+}\), and activation of eNOS. These chemicals signals promote vasodilation and neovascularization. This signaling cascade plays an important physiological role but may be deleterious in the clinical setting of lung transplantation where procurement of donor lungs by current practices involves a period of stop of flow. An approach to prevent lung injury may be to maintain close to physiological conditions during the procurement and storage periods to minimize ROS and NO production. Studies with animal lungs indicate that a relatively low perfusate flow rate can prevent activation of the mechanotransduction signaling cascade, although the perfusion level required for human lungs remains to be firmly established. An alternate approach to prevent the mechanosignaling cascade would be the use of a KATP channel agonist to prevent endothelial cell membrane depolarization. Relatively nontoxic agents are available for the purpose but have not yet been trialed clinically. Similarly, agents are available to prevent activation of NOX2 and eNOS, thereby preventing generation of ROS and NO, the primarily signaling molecules of the mechanotransduction cascade. A full understanding of the response of lungs to altered blood flow could result in strategies to preserve lung viability during storage in preparation for transplantation and in the posttransplantation period.

ACKNOWLEDGMENTS

We thank Dawn Williams for secretarial support. We are also thankful to the numerous collaborators who have contributed to this research, over the past few decades.

GRANTS

Original research for this work was supported by National Institutes of Health Grants (HL079063, HL60290, HL41939, HL075587, HL087115, HL081619, HL096845, HL115354, and HL105509).

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

S.C. prepared figures; S.C. and A.B.F. drafted manuscript; S.C., G.F.N., J.D.C., and A.B.F. edited and revised manuscript; S.C., G.F.N., J.D.C., and A.B.F. approved final version of manuscript.

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