Lung-derived soluble mediators are pathogenic in ventilator-induced lung injury

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LUNG INJURY IS COMMON in critically ill patients receiving mechanical ventilation and contributes to morbidity and mortality (27). Acute lung injury, and its more severe form, the acute respiratory distress syndrome (ARDS), is characterized by elevated levels of multiple classes of inflammatory mediators (43). It is widely supposed that circulating mediators, triggered by the underlying disease process or by injurious mechanical ventilation, can cause or worsen lung injury and contribute to distant organ failure: this notion was formally proposed as the “Biotrauma Hypothesis” (35).

Strong supportive evidence for this biotrauma hypothesis exists, and several classes of candidate mediators have been proposed, ranging from proteins (e.g., cytokines, hormones, procoagulants) to smaller lipid molecules (e.g., eicosanoids, platelet-activating factor, sphingolipids). For example, proinflammatory cytokine concentrations correlate with experimental (37, 41) and clinical (30, 36) lung injury as well as with clinical outcome (2, 25, 26). Also, anticytokine interventions, including antibodies (14), soluble receptors (45), receptor antagonists (23), and corticosteroids (13), confer some protection from experimental lung injury. Other candidate proteins include angiotensin II (16) and coagulation factors (33). More recently, lipid mediators such as eicosanoids (22), platelet-activating factor (10), and sphingolipids (38) have been proposed as mediators of lung injury.

However, in (experimental) ventilator-induced lung injury (VILI) or (clinical) ventilator-associated lung injury (VALI), formal proof is lacking for a pathogenic role of circulating molecules. Important inconsistencies include significant lung injury in the context of only minimally elevated mediator levels (32, 39, 40), lack of injury following administration of exogenous mediators (5), and the absence of clinical outcome benefit following inhibition of candidate cascades such as coagulation (1, 20).

With multiple classes of candidate mediators identified, it is likely that broad classes of candidate molecules, rather than an individual “responsible” molecule, account for any putative circulating mediation of the inflammatory effect in VILI.

Circulating molecules may be markers (9), i.e., nonpathogenic molecules that mirror disease activity such as C-reactive protein or procalcitonin, or mediators, i.e., pathogenic molecules that induce or worsen the disease in question. Where the pathogenic roles of classes of mediators are known (e.g., B lymphocytes, cytokines), mediator-directed therapies can be highly effective. For example, rituximab, which targets and depletes B lymphocytes (7), and etanercept, which binds to and inactivates tumor necrosis factor (TNF) (34), are highly effective in rheumatoid arthritis and inflammatory bowel disease. Such therapies are usually administered empirically, without ascertaining the levels of individual molecules that may be blocked because the pathogenic roles of the classes of molecules are understood.

Circulating mediators would make attractive therapeutic targets for evolving lung injury in the critically ill, but, before such therapy can be pursued, convincing evidence of pathogenicity, beyond association, is required. We have previously suggested (15) that such evidence might be obtained by applying the principles of Koch’s postulates to an established model.
of VILI, the isolated-perfused lung. In compliance with these postulates, we aimed at providing evidence of the pathogenic role of soluble mediators in VILI by hypothesizing that: 1) high tidal volume (VT), but not low VT, ventilation would cause accumulation of candidate mediators, 2) recirculation of perfusate would worsen lung injury induced by high VT, 3) perfusate from injured lungs could cause injury ex vivo (other isolated lungs) and in vitro (lung epithelial monolayer), and 4) candidate mediators could be biochemically characterized.

**METHODS**

Ethics approval was granted by the Animal Care Committee of the Hospital for Sick Children Research Institute in accordance with guidelines from the Canadian Council on Animal Care.

**Surgical Preparation**

A previously described isolated perfused mouse lung model (41) was used. Male C57Bl/6 mice (18–28 g; Charles River, Montreal, QC) were anesthetized with ketamine and xylazine, a tracheotomy was performed, and the mice were placed inside the isolated lung perfusion chamber (IL-1: Harvard Apparatus, Montreal, QC). Positive-pressure ventilation was commenced [VT 120 µl, positive end-expiratory pressure (PEEP) 3 cmH₂O, respiratory rate 90/min]. A midline incision was performed, the diaphragm was exposed, and the mice were then heparinized and exsanguinated. The diaphragm was removed, and the pulmonary artery and left atrium were cannulated with stainless steel cannulas while perfusate was flowing (0.02 ml/min). The chamber was closed, and lungs were ventilated with negative pressure under baseline ventilation parameters (transpulmonary pressure −8 cmH₂O, end-expiratory pressure −3 cmH₂O, VT ~200 µl), with a deep inflation (−20 cmH₂O) every 5 min to prevent atelectasis. Perfusion was increased to the experimental flow rate (1.0 ml/min). The lungs and perfusate were both exposed to 5% CO₂ and 30% O₂.

**Ex Vivo Model**

Briefly, the lungs were perfused at constant flow with RPMI (Roswell Park Memorial Institute) 1640 medium with 4% endotoxin-free bovine albumin (Sigma-Aldrich, St. Louis, MO). The perfusate osmolality was adjusted to 340 mmol/l. The perfusion circuit, reservoir, and mouse lungs were kept at 37°C. The perfusion circuit volume was 4 ml during single-pass perfusion and 10 ml (circuit + reservoir volume) during perfusate recirculation.

Preexperimental exclusion criteria were as follows: decrease of dynamic compliance during baseline ventilation (>10%, or <35 µl/cmH₂O); perfusate leakage; pulmonary artery pressure >10 cmH₂O; or detection of air in the circuit.

**Physiological Measurement**

Chamber pressure was measured with a Differential Pressure Transducer MPX, Type 399/2 (Hugo Sachs Elektronik, March-Hugstetten, Germany), and the VT and inspiratory and expiratory flow were measured with a Validyne DP45 Very Low Range Differential Pressure Transducer (Validyne Engineering, Northridge, CA) using a Fleisch-type pneumotachograph. Dynamic compliance and airway resistance were calculated by the monitoring software (Pulmodyn; Hugo Sachs Elektronik, March-Hugstetten, Germany) as previously described (41), and data were recorded at 200 Hz. Fluorescein isothiocyanate-Dextran (FITC-Dextran, mol wt 70 kDa, 20 µg/ml) was added to the perfusate of selected experiments, and the bronchoalveolar lavage (BAL) concentration was assayed. After each experiment, the right lung was removed, and the lung wet-to-dry (W/D) weight ratio was determined. A BAL was performed in the left lung by injecting 0.5 ml of normal saline three times and removing a maximum of fluid. Protein and FITC-Dextran concentration (fluorescence plate reader, SpectraMax; Molecular Devices, Sunnyvale, CA) were determined in separate samples of BAL.

**Study Protocol**

Ventilation was with negative pressure (41) with either high VT (transpulmonary pressure −15 cmH₂O, end-expiratory pressure −1.5 cmH₂O, VT 17–25 ml/kg) or low VT (identical to baseline parameters established during surgical preparation above; transpulmonary pressure −8 cmH₂O, end-expiratory pressure −3 cmH₂O, VT 8–11 ml/kg). Experiments with high VT and recirculating perfusate were terminated when the VT fell to the threshold VT of 200 µl.

**Effect of Perfusion Recirculation on High VT Injury**

High VT ventilation with recirculating perfusate were compared with the following three control experiments: control 1, high VT ventilation with nonrecirculating perfusate; control 2, low VT ventilation with recirculating perfusate; and control 3, low VT ventilation with nonrecirculating perfusate. Each control experiment was time-matched to a previous high VT with recirculating perfusate experimental lung preparation (i.e., stopped after the same duration).

**Impact of Previously Recirculated Perfusate on Low VT Ventilated Lungs**

Recirculated perfusate from lungs ventilated with high VT (i.e., donor) was used to perfuse a lung ventilated with low VT (i.e., recipient) in a recirculating manner. The experiment was stopped when the VT reached the threshold of 100 µl. The experimental group was compared with two time-matched groups to control for the type of perfusate used: control 1, low VT, recirculating perfusate donated from a previous low VT recirculated lung and control 2, low VT lung recirculating with fresh perfusate (Fig. 2A). In this and other experiments using donor perfusate, the donor perfusate was filtered (0.2 µm pore size) before perfusing the recipient to remove debris and cells.

**Impact of Previously Recirculated Perfusate on High VT Ventilated Lungs**

Recirculated perfusate from a high VT lung (i.e., donor) was used to perfuse a lung undergoing high VT ventilation (i.e., recipient) in a recirculating manner. Experiments were terminated when the VT fell to the threshold VT of 200 µl.

**Inhibition of Cyclooxygenase**

To test whether prostaglandins contribute to the injury, their production was inhibited by adding ibuprofen (1,100 µg/ml) dissolved in distilled water (LifeBrand, Toronto, ON) or vehicle to the recirculating perfusate of n = 5 high VT lungs. Lung preparations perfused with ibuprofen-containing perfusate were time-matched to corresponding vehicle controls.

**Lung Epithelial Cell Monolayers**

To investigate the impact of perfusate from high VT ventilated lungs on the integrity of the alveolar epithelial barrier, we used cultured primary rat fetal distal lung epithelial (FDLE) cells as previously described (29). When the cell monolayer reached confluence, the cell culture medium was replaced by perfusate, recovered after recirculation from high VT ventilated lungs, and compared with control medium (i.e., fresh RPMI, 4% albumin that had not been previously used to perfuse lung). Transepithelial resistance (TER) was measured with an ohmmeter before and 24 h after this medium change.

**Characterization of Mediators**

Several approaches were undertaken to characterize the nature of the circulating mediators in the perfusate from isolated lungs.
Lipid Extraction

FDLE monolayers. TER was determined after 24 h of incubation. (80°C, 20 min) to inactivate heat-labile compounds and added to

Heat Denaturation

Recirculated perfusate from high VT ventilated lungs was heated (80°C, 20 min) to inactivate heat-labile compounds and added to FDLE monolayers, and TER was determined after 24 h of incubation.

Lipid Extraction

Recirculated perfusate from high VT ventilated lungs was subjected to a Folch-Pi and and Sakura (8) lipid extraction. The extracted lipids were dried and resuspended in fresh perfusate by sonication and added to FDLE monolayers. TER was determined after 24 h of incubation.

Measurement of Mediators

Proinflammatory cytokines [TNF-α, interleukin-6 (IL-6), and macrophage inflammation protein-1α (MIP-1α)] were measured in the perfusate (Luminex, Austin, TX). Eicosanoids and sphingolipids were measured by mass spectrometry (4).

Acid Sphingomyelinase Activity

The Amplex Red Sphingomyelinase Assay Kit (Invitrogen, Paisley, UK) was used to detect acid sphingomyelinase (ASM) activity in lung tissue and perfusate.

Statistics

Results were expressed as means ± SD. Groups were compared using one- or two-way ANOVA, with Newman-Keuls post hoc analysis. Student’s t-test was used for two-group comparisons. P < 0.05 was considered statistically significant.

RESULTS

Effect of Perfusate: Recirculation and High VT Injury

A total of 56 mice lungs were isolated, of which 12 did not pass inclusion criteria (n = 11/group). Preparations in the experimental group (high VT, recirculating perfusate) were stopped once the predefined threshold VT was reached. This occurred after 103 ± 22 min, corresponding to a mean reduction in compliance of 60%. The following groups were time-matched (i.e., experiments stopped after the same duration): high VT nonrecirculating perfusate, low VT recirculating perfusate, and low VT nonrecirculating perfusate. Dynamic compliance was impaired in both high VT groups, but to a greater extent in the lungs with recirculating perfusate. It did not change in either low VT group (Fig. 1A). Starting experimental compliance (after 30 min baseline ventilation) was similar in all four groups. A similar pattern of injury induced by high VT, and exacerbated by perfusate recirculation, was evident by assessing the microvascular permeability using BAL concentrations of protein (Fig. 1B) and FITC-Dextran (Fig. 1C). W/D ratio was performed on five lungs in each group. High VT ventilated lungs with recirculating perfusate had a significantly higher W/D ratio than lungs with high VT and nonrecirculating perfusate and lungs with low VT and recirculating or nonrecirculating perfusate (Fig. 1D). Additional control preparations (nonventilated, nonperfused) yielded BAL protein and W/D ratios similar to low VT groups (Fig. 1, B and D).

Transfer of Effect: Perfusate from High VT Lungs Induces Injury

We collected recirculated perfusate from lungs that had been injured by high VT and exposed uninjured lungs to that perfusate using low VT conditions. A total of 53 mice lungs were isolated; 21 did not pass inclusion criteria (n = 6/group, two groups with n = 6 perfusate donors each).

Low VT ventilated lungs perfused with recirculated perfusate from high VT ventilated lungs were ventilated until the predetermined threshold VT was reached (135 ± 39 min). The following low VT groups were time-matched (i.e., experiments stopped after the same duration) and perfused with perfusate from one of two sources (Fig. 2A): 1) recirculated perfusate from low VT ventilated lungs or 2) fresh perfusate (not previously exposed to any lung preparation).

Starting experimental compliance (at the beginning of the experiment, after 30 min baseline ventilation) was similar in all three groups. Dynamic compliance was impaired in low VT recipients of high VT perfusate but not in the two control groups (Fig. 2B). These effects were paralleled by BAL fluid protein concentration reflecting microvascular leak (Fig. 2C).

Filtration (0.2-μm pore size) of perfusate between the donor and the recipient experiment, to remove cellular elements or debris, did not impact on the development of compliance impairment [Supplemental Fig. S1 (Supplemental data for this article can be found on the American Journal of Physiology: Lung Cellular and Molecular Phys- iology website.)].

Transfer of Effect: Perfusate from High VT Lungs Worsens Injury

We compared the injury in high VT preparations receiving fresh vs. donated perfusate. The predefined threshold VT of 200 μl was reached earlier in the perfusate recipients vs. the corresponding perfusate donors (91 ± 13 vs. 100 ± 10 min, P = 0.015). A total of 17 mouse lungs were isolated; 7 did not pass inclusion criteria.

Transfer of Effect: High VT Perfusate Impairs In Vitro Epithelial Barrier Function

Because of the pivotal role of epithelial barrier function in acute lung injury (18) and the increased permeability induced in recipient lungs by the recirculated perfusate, we investigated its effects on in vitro TER. Recirculated ("donor") perfusate from high VT ventilated lungs was applied to high resistance (TER = 2.71 ± 0.29 kΩ-cm²; n = 121) FDLE monolayers in vitro, and the change in TER was assessed at 24 h. We chose 24 h for our measurement of TER since our preliminary experiments demonstrated a significant drop in resistance in FDLE exposed to high VT perfusate relative to control fluids at this time (data not shown).

Donor perfusate impaired the TER in a dilution-dependent manner; the maximal effect occurred with undiluted perfusate, and the effect disappeared with a 1:3 dilution (Fig. 3). A 3:1 dilution with albumin-supplemented Dulbecco’s modified Ea-
gle’s medium resulted in a significant drop in TER (i.e., 60–80% of baseline) while maintaining a TER routinely $\leq 500 \Omega \cdot \text{cm}^2$, indicating a viable monolayer; this dilution was therefore used for subsequent experiments.

**Characterization: Determining the Physicochemical Nature of the Active Elements**

Subsequent experiments were designed to provide physicochemical characterization (i.e., size, heat lability, lipid solubility) of compounds in the perfusate that are responsible for the transmitted injury.

**Ultrafiltration**

Ultrafiltration of the recirculated perfusate from high VT ventilated lungs produced an ultrafiltrate (molecules $< 3$ kDa, i.e., passing through a 3-kDa pore size filter) and a retentate (molecules $> 3$ kDa). Both ultrafiltrate and retentate significantly impaired the TER of FDLE monolayers compared with identically prepared fractions from fresh perfusate (Fig. 4A). This indicates activity of low- and high-molecular-weight macromolecules with a predominance of the former.

**Heat Denaturation**

The perfusate was heated (80°C, 20 min) to inactivate heat-labile compounds. Such heat treatment of recirculated perfusate from high VT ventilated lungs resulted in a significant decrease in its impact on TER of FDLE monolayers, suggesting that heat-labile molecules were necessary for the biological effects (Fig. 4B).

**Lipid Extraction**

The lipid fraction from high VT perfusate [extracted using the Folch-Pi and Sakura (8) extraction method and resuspended in fresh perfusate] decreased the TER as effectively as untreated high VT perfusate (Fig. 4C). In contrast, the
lipid fraction from fresh perfusate did not drop the TER more than control medium. This suggests that lipid-soluble compounds are necessary for the drop in TER.

Do Commonly Cited Protein Mediators Account for the Injury?

Three candidate proinflammatory protein mediators (TNF-α, IL-6, MIP-1α) were assayed in both the recirculated and nonrecirculated perfusates of high and low VT ventilated lungs. At the end of the experiments, recirculation of the perfusate, but not the choice of VT, led to an accumulation of mediators (Fig. 5, A–F). The cytokine levels in lungs with recirculating perfusate were similar after high vs. low VT ventilation, despite significant differences in lung injury.

Because recirculation, and not VT, was associated with the elevated levels of perfusate cytokines (Fig. 5, A, C, and E), the cytokine levels in recirculated perfusate were plotted against injury (expressed as BAL protein leak; Fig. 5, B, D, and F). Here, the high (vs. low) VT determined the extent of lung injury, but the perfusate cytokine levels are similar in high and low VT groups. There was no correlation between injury and cytokine levels for TNF-α (Fig. 5B), IL-6 (Fig. 5D), and MIP-1α (Fig. 5F).
Therefore, cytokine levels in the perfusate did not mirror the presence or extent of the lung injury.

Do Commonly Cited Lipid Mediators Account for the Injury?

Cyclooxygenase-dependant lipid mediators (prostanoid series). The perfusate concentrations of three central cyclooxygenase (COX)-dependant eicosanoids (prostaglandin E2, prostaglandin D2, and thromboxane B2, the stable metabolite of thromboxane A2) were measured. The baseline levels were low in all cases. Although the perfusate concentrations accumulated during recirculation with both low and high VT, the highest levels were seen with high VT (Fig. 6, A–C). However, while the COX inhibitor ibuprofen effectively attenuated the VT-induced increase in all three mediators (Fig. 6, A–C), it had no impact on the development of lung injury (Fig. 6D).

Lipoxygenase- or cytochrome P-450-dependent lipid mediators. There was no consistent relationship between the level of VT during recirculating perfusion and the pattern of perfusate lipoxygenase metabolites [hydroxyeicosatetraenoic acid (HETE) series, HETES: 5-HETE, 8-HETE, 9-HETE, 11-HETE, 12-HETE, and 15-HETE; lipoxin-A4, and leukotriene-B4; Fig. S2] or cytochrome P-450-dependent mediators [epoxyeicosatrienoic acids (EET) series, EETs: 5,6-EET; 8,9-EET; 11,12-EET; 14,15-EET; Fig. S3].

Ceramides and sphingolipids. Because of the recent reports of a potential role for novel sphingolipid-derived mediators in the pathogenesis of VILI (38, 42), we assayed the concentrations of ceramides (ceramides C-14, C-16, C-18, C-20, and C-24) in the lung tissue and in the perfusate. The ceramide concentrations in the perfusate (Fig. S4), or lung tissue (Fig. S5), were not greater with high vs. low VT ventilation.

Similarly, the sphingomyelin levels in the perfusate and lung tissue were similar between the two groups (data not shown). Finally, because we were unable to confirm consistent differences in the perfusate ceramide levels, we examined the possibility that ASM (which catalyzes the metabolism of sphingomyelin to ceramide) might be the injurious factor. ASM protein concentration in the perfusate was not altered by high VT or recirculation (Western blot, data not shown). The ASM enzyme activity was detectable in both the lung tissue and the perfusate but was not increased by high VT or by recirculation.

DISCUSSION

We provide evidence that circulating mediators are pathogenic contributors, and not merely markers, of high VT-induced lung injury using an isolated perfused lung model of VILI. This evidence is based on Koch’s postulates where the presence and absence of a substance can be associated with the presence and absence of disease, and selective exposure can be shown to be pathogenic (17, 21). As hypothesized, no single class of previously reported mediators accounted for the development of lung injury, but our characterization indicates a spectrum of features, including lipid solubility, heat lability, and active molecules, both greater than and less than 3 kDa in molecular weight.

Conformity with Koch’s Postulates

The major insights from the current study parallel the lines of evidence described for the satisfaction of Koch’s postulates (15). First, we demonstrated the presence of a “positive association.” High VT ventilation causes lung injury, but to a greater extent when the concentration of putative mediators is elevated by recirculation. Indeed, the elevated concentration of several lung-derived inflammatory molecules was confirmed (e.g., cytokines, lipid mediators). Second, we verified the presence of a “negative association.” Although low VT ventilation produces some inflammatory responses (i.e., elevated cytokines), this was not sufficient to cause injury as assessed by compliance impairment or microvascular leak (in this model and over this time course), irrespective of the type of perfusion (recirculating or nonrecirculating). Third, we confirmed a “transferable effect.” Here, the perfusate from lungs ventilated with an injurious ventilation strategy resulted in several important effects when used to perfuse other lungs: it induced injury (compliance impairment, increased BAL protein) in lungs ventilated with low VT. In addition, such perfusate perturbed the TER of distal lung epithelial cells grown in primary culture. Fourth, we characterized the nature of the soluble factors that appeared to be responsible for mediating the injury: the effect required the presence of both smaller (ultrafiltrable, <3 kDa) and larger (>3 kDa) molecules, heat labile and partially lipid-extractable factors. We also establish that the following molecules are not sufficient for the effect: certain proinflammatory cytokines (proteins, TNF-α, IL-6, or MIP-1α), COX-dependent prostanoids, isolatable ceramides or their precursors, or sphingomyelins. Taken together, the overall characterization suggests that the responsible factors are likely protein-associated lipids.
Evidence for candidacy of cytokines. Proinflammatory cytokine proteins, for example, TNF-α and IL-6, were thought to contribute to VILI because they were associated with experimental (44, 48) and clinical (2, 30, 36) lung injury; as well, cytokine inhibition using antibodies (14, 28), soluble receptors (46), recombinant receptor blockade (19, 23), and lowering cytokine concentrations using corticosteroids (13) have all provided protection in experimental models.

However, several lines of evidence suggest that these cytokines are not causative factors: severe lung injury has been described in the setting of only minimal cytokine elevation (12, 28, 32, 40), administration of exogenous cytokines does not...
necessarily cause injury (5), the levels of circulating cytokines appear similar in patients ventilated with injurious vs. protective parameters (47), and elevated levels of circulating cytokines can occur during low VT ventilation in the absence of demonstrable lung injury (39).

In the current study, the presence of lung injury depended on the VT used but did not reflect the cytokine levels. In fact, there was no correlation between any of the cytokine levels and the extent of lung injury (Fig. 5). Instead, the levels of cytokines appear to be determined by the presence of recirculation alone; indeed, in the context of recirculation, the choice of VT had little impact on the cytokine levels. It is possible that the choice of cytokines assayed, or the timing of the sampling, resulted in missing an important link between circulating cytokines and injury.

Fig. 5. Cytokine levels in recirculated vs. nonrecirculated perfusate. The perfusate cytokine levels were increased by recirculation but not by high VT [tumor necrosis factor-α (TNF-α, A), interleukin (IL)-6 (C), and macrophage inflammatory protein (MIP)-1α (E)]. Cytokine levels in recirculated perfusate were plotted against lung injury (expressed as BAL protein leak), and there was no correlation between injury cytokine levels for TNF-α (B), IL-6 (D), and MIP-1α (F) (n = 6/group). Data are expressed as individual data points and as means ± SD. Analysis was with 2-way ANOVA (VT, recirculation), and post hoc tests were performed with the Holm Sidak method. All groups with recirculating perfusate were significantly different from groups with nonrecirculated perfusate regardless of VT but were not significantly different from each other (*P < 0.05).
lung injury. We believe this to be unlikely because the cytokines selected are proximal in the pathogenesis of many inflammatory processes, and the patterns of elevation are consistent among them. In addition, transiently expressed molecules should still be detected in recirculated perfusate. Thus the current data suggest that the cytokine proinflammatory mediators we measured (TNF-α, IL-6, or MIP-1α) are not sufficient for the mediation of lung injury in this context. However, we did not perform inhibitory experiments in the current study, and potential roles as cofactors remain possible.

**Evidence for candidacy of lipid mediators.** Eicosanoid levels correlate with lung injury in several models of VILI (11, 22). Eicosanoids control pulmonary vascular tone and microvascular permeability and have multiple inflammatory actions. Inhibition of the rate-limiting synthetic enzyme (cytosolic phospholipase A2) reduces microvascular leak caused by injurious ventilation (22), but, for comprehensive protection, simultaneous inhibition of all downstream enzymes (i.e., COX, lipoxygenase, and cytochrome P-450) is necessary (22), suggesting functional overlap among them.

In the current study, high V T was associated in general with higher levels of prostanooids, in the setting of recirculation. However, effective blockade of COX, while resulting in comprehensive suppression of COX-dependent prostanooids in the perfusate, had no impact on the development of lung injury (Fig. 6). In addition, there was an overall increase in the final vs. baseline levels of other eicosanoids (HEETs, EETS, and leukotrienes) following ventilation with either high or low VT, but the magnitude of the V T had no impact on the lipid concentration. Thus eicosanoids (COX-dependent or COX-independent) are not sufficient for the induction of this type of lung injury.

Other lipid mediators such as ceramides and other sphingolipids (e.g., sphingosine 1-phosphate or sphingomyelin) may be important in lung injury (38). These molecules have important roles in the regulation of apoptosis and maintenance of intercellular junctions. Increased ASM activity, which elevates local ceramide levels by converting sphingomyelin to ceramide, has been reported to be elevated in the lungs of patients with acute lung injury (31). Indeed, in patients with sepsis,
higher levels are associated with a greater risk of death (3, 6), and, in experimental studies, inhibition of ASM stabilizes surfactant (42) and attenuates pulmonary inflammation in experimental in vivo lung injury (38). In the current study, there was no association in preparations with recirculating perfusate between high vs. low VT ventilation and the concentrations of ceramides or sphingomyelins in either the perfusate or the lung tissue.

Characterization of the classes of mediators. We took two approaches to preliminary delineation of the nature of the responsible mediators. First, we assayed specific mediators (proteins, lipids) previously known to be closely associated with the development of ARDS or VALI and demonstrated that none of the levels reflected the development of injury. Second, we examined the physicochemical features of the “injurious” perfusate and confirmed that activity required molecules that are heat-labile, lipid extractable (8), and of various sizes (i.e., less than and greater than 3 kDa). These features point to a multifactorial requirement for pathogenicity of lung-derived circulating factors consistent with protein-associated lipid mediators. Of course, estimates of molecular size are complicated by posttranslational modification, including dimerization, conjugation, or simple binding, for example, to large transport proteins (e.g., albumin).

Limitations of the model. This (41) or similar (24, 37) models have been used to make seminal observations relating to the pathogenesis of lung injury and, in the current series, is strengthened by the concomitant use of in vitro lung cell monolayers. These models have several important strengths, including strong specificity for the interface between the circulation and the lung, thereby avoiding other (e.g., neurohumoral or cellular) influences.

However, there is limited direct application to the human situation, with important differences including species, use of a blood-free perfusate, absence of the integrative physiological impact of other organ systems due to closed circulation, the necessarily brief time course, and the artificial nature of the delivered VT. Because it is not possible to concomitantly determine perfusion pressure and flow, perfusion pressure was recorded but not regulated to allow control of flow. Although the experiments were not conducted under strictly sterile conditions, the entire circuit was disinfected between uses, and the time course of the experiments is insufficient for the development of de novo sepsis. Although ventilating pressures and stopping thresholds were artificially preselected, these were based on extensive pilot experiments and review of previously published ex vivo lung studies thereby designed to provide a robust model with a consistent separation between unjured low VT lungs and injured high VT lungs and low within-group physiological variability. In the isolated lung setup, the perfusate comes in contact with a large amount of foreign material (e.g., circuit tubing). Therefore, it is possible that the observed increase in mediators (and also the observed injury) was due to bio(in)compatibility. Nevertheless, the use of a blood-free perfusate, lacking cellular and humoral components of the immune system, has probably reduced this issue to a minimum as demonstrated by the fact that recirculation by itself did not result in lung injury in the low VT preparations (low BAL protein and FITC-Dextran concentration, absence of compliance impairment) during our observation period.

In terms of characterizing the responsible mediators, several limitations must be considered. The cut off at 3 kDa during ultrafiltration does not take cognizance of molecular shape or adsorption on cell membranes or macromolecules. In addition, the lipid extraction process is invariably associated with quantitative or qualitative molecular alterations.

In conclusion, we have provided a novel proof of concept of the bioratrauma hypothesis (circulating mediators contribute to VILI) through application of Koch’s postulates, showing that: 1) recirculation of perfusate worsens high VT-induced lung injury; 2) perfusate from injured lungs can transfer injury, impairing compliance and increasing permeability, to additional lungs ventilated under protective (i.e., low VT, high PEEP) conditions; and 3) such perfusate impairs TER in a tissue culture model of lung epithelia. Although protein-dependent lipid mediators appear to be the most active class of compounds, further characterization is needed. Classically, sphingolipids, platelet-activating factor, and eicosanoids were reported contributors to VILI, but we could not show their implication in the present model. Other liposoluble compounds like lipoxins or peroxidized membrane lipids can be investigated, and modern lipidomics can help to identify novel substances. Subsequent verification experiments (e.g., pathway inhibition, synthetic analogs, administration of high VT perfusate to intact animals) will be needed to confirm their pathogenic role before such mediators can be targeted by pharmacological interventions in vivo or in the clinical setting.

DISCLOSURES

The authors have no conflicting financial interests.

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