Carbon monoxide-mediated antiinflammatory effects in acute lung injury

-Doctoral thesis-

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Abstract

Acute lung injury (ALI) is a common and severe disease providing 5-15 % of all intensive care unit patients. Acute lung injury is often presented in its most advanced form, acute respiratory distress syndrome (ARDS) that has 40-50% mortality. Despite intensive investigation the mechanism of ALI is not well understood and its therapy is poor.

ALI was assessed in animal models where bacterium polysaccharide and mechanical ventilation was used to induce injury. Microarray profiling of mouse lung genes following injury lead to description of new target genes. The results suggest their important role in the mechanism ARDS. Major pathologic features of ARDS including alveolar edema formation, epithelial barrier disruption and inflammatory cell infiltration were described in an *in vivo* model. I showed the increased release of proinflammatory cytokine tumor necrosis factor-α (TNF-α) and the induction of oxidative stress-inducible, heme oxygenease-1 (HO-1) enzyme following mechanical ventilation. Neutrophil leukocyte infiltration and proinflammatory cytokine levels in the bronchoalveolar lavage fluid were attenuated with low dose inhaled carbon monoxide (CO) while antiinflammatory cytokine interleukin-10 (IL-10) levels increased. CO has antiinflammatory effects in other lung injury models as well but the intracellular mechanism is only partly understood. I showed that CO exerts its effects independent of two major inflammatory pathways nuclear factor-κB (NFκB) and activator protein-1 (AP-1) but involving p38 mitogen-activated protein kinase (MAPK) in the model.

My findings support the theory that inhaled carbon monoxide can reduce lung inflammation suggesting a new therapeutic approach to ALI/ARDS-related morbidity.

I would like to continue further investigation of the applicability of CO in ALI hoping that one day it can be used in medical intensive care.

Introduction and specific aims

Acute lung injury (ALI) became a highlighted topic of pulmonary medical care with the widespread availability of mechanical ventilation in the 1960`s. During the Vietnam War thousands of solders suffered multiple organ injuries and required mechanical ventilation. This sad event lead to the rapid development of the field of pulmonary intensive care medicine. Lung injured patients could be kept alive for longer periods of time and weaned from ventilators leading to new dimensions of patient care and research. This is when acute respiratory distress syndrome, the most severe form of ALI was described by Ashbaugh and colleagues. It was first referred to as adult respiratory distress syndrome to distinguish the disease from the respiratory distress syndrome that occur in neonates. However today the more preferred name is acute respiratory distress syndrome (ARDS). ALI is one of the most investigated and debated field of pulmonary-intensive care medicine. With new therapeutic modalities and mechanical ventilator setting patients who were previously prone to death are given a chance to return to life.

A milestone in the history of ALI/ARDS is the ARDS Network Trials. The original trial collected patients from 1996 to 1999 in 10 University Medical Centers in the USA. Patients with mechanical ventilation and ALI, determined according to the American-European Consensus Conference guidelines, were eligible to enroll the study. 861 patients received either 6ml/kg or 12ml/kg mechanical ventilation. The initial findings of the ARDS Network were reported in year 2000. The results have shown that ALI/ARDS-related death can be reduced with 22% in the lower tidal

volume ventilation. Another beneficial outcome was the number of ventilator-free days in the lower tidal volume group.

In a recent analysis of the ARDS Network trial Gross and colleagues found that the incidence of ALI in the United States is 22.4 cases/ 100,000 population. Other studies in developed countries showed similar results. Approximately 10 to 15 percent of patients admitted to an intensive care unit and up to 20 percent of mechanically ventilated patients meet criteria for ARDS. Ventilator-induced lung injury (VILI) is often observed in patients with mechanical ventilation and the pathological features of VILI are indistinguishable from ARDS. However it is debated whether mechanical ventilation alone can lead to ALI. Since the lower airways are one of the most harmed organs of the body a large variety of harmful noxa and trauma lead to acute lung injury. The major risk conditions for ARDS are sepsis (38-43%), documented aspiration of gastric content (30-37%), pneumonia (28-36 %) and chest contusion (11-17%). Brun-Buisson and colleagues found that (based on 6522 ICU admissions in 78 hospitals in 10 European countries) in their population that mortality is the highest among patients where the underlining disease is sepsis (43%), lower in patients with pneumonia (36%) and the lowest is in association with trauma (11%). The mortality rate of ARDS is appreciable and varies with cause, with most patients dying of multiorgan system failure rather than isolated respiratory insufficiency.

Despite intensive investigation ALI/ARDS-related mortality remains high and its therapy is poor.

Accumulating data exist in the literature supporting the paradigm that the stress inducible enzyme HO-1 or its catalytic by-product, CO, can confer potent cytoprotective effects in various models of tissue and cellular injury. One mechanism by which HO-1 or CO mediate a cytoprotective effect is via its potent

antiinflammatory properties. Our laboratory has recently demonstrated that exogenous administration of low concentration of inhaled CO can markedly decrease lung inflammation and confer potent cytoprotection in various tissue injury models.

In my dissertation work my goal is dual. 1. I describe the main pathologic and immunologic features of ALI with gene expression analysis and with molecular biologic techniques in animal models. 2. I show the antiinflammatory effects of CO in an in vivo model of lung injury.

Specific aims

Aim #1: Gene expression analysis of mouse lung genes following acute lung injury.

1. To determine which genes show significant expression changes in acute lung injury. Functional grouping of these genes.
2. To describe groups of genes modified by bacterium lipopolysaccharide (LPS)-induced lung injury and/or high-volume mechanical ventilation. Description of a distinct group of genes modulated only by mechanical ventilation injury.

Aim #2. To describe novel target genes in ventilator-induced lung injury.

1. To confirm the gene expression changes of target genes on the RNA and protein level.
2. The role of amphiregulin, a new candidate gene in acute lung injury

Aim #3: To determine the effects mechanical ventilation in acute lung injury

1. To demonstrate the effects of mechanical ventilation on alveolar edema and inflammation in LPS-induced lung injury.
2. To describe heme oxygenase activation and induced cytokine production in ventilator-induced lung injury (TNF-α, IL-10).

Aim #4: To describe and confirm the aniinflammatory effects of low dose, inhaled CO in ventilator-induced lung injury

1. To present the effects of CO on the direct and indirect indices of acute lung injury (altered inflammatory cell infiltration, reduced proinflammatory cytokine levels).
2. To describe the effect of CO cardio-respiratory function (blood pressure, blood gases)
3. To determine the mechanism through witch intracellular pathway CO provides antiinflammatory effects in ventilator-induced lung injury. CO exerts antiinflamatory effects independent of NF-κB and AP-1 involving p38 mitogen-activated protein kinase in this model.

Methods

1.To assess the gene expression changes during mechanical ventilation I used an isolated, perfused and ventilated mouse lung model. This model is often called *ex vivo* to represent its transitional position between *in vitro and in vivo* experimental settings. The *ex vivo* model has the advantage of keeping the chest almost intact without loosing the chest wall component of breathing and at the same time applied high pressure mechanical ventilation does not compromise cardiac function as often seen in models of VILI. In brief, 9 Balb/c mice (22-30g) were ventilated with negative pressure for 1 hour with an end-expiratory pressure of -3 cm H_2O and an end-inspiratory pressure (EIP) of -10 cm H_2O. Subsequently, they were randomized to 3 groups and ventilated for the next 3 hours (n=3/group). Control: ventilation with -10 cm H_2O EIP; LPS: ventilation with -10 cm H_2O EIP in the presence of 3 mg/kg Escherichia coli bacterium lipopolysaccharide; Serotype O127: BO (Sigma, St. Louis,

MO) in the perfusion buffer; OV: overventilation with -25cm H_2O EIP. I analyzed the gene expression of the 3 groups of animals with microarray. Genes with significant expression changes were grouped functionally. Following I identified genes that were modulated only by mechanical ventilation but not by LPS treatment. 5 genes` expression was confirmed with quantiative real-time polymearase chain reaction (QRT-PCR) and amphiregulin`s expression was analyzed on the protein level.

2. In a second, in vivo model of lung injury I accessed the cellular changes, pulmonary edema formation, cytokine production and intracellular signaling pathway activation following high volume (26ml/kg) mechanical ventilation without positive end expiratory pressure (PEEP). Furthermore I tested the antiinflammatory effect of low-dose inhaled CO in the model. Adult 275-375 g male Sprague-Dawley rats (n=88) were purchased from Harlan (Indianapolis, IN). Animals received either 3mg/kg Escherichia coli bacterium lipopolysaccharide (LPS), Serotype O127: BO (Sigma) in 0.25ml Phosphate Buffered Saline (PBS) or PBS alone, injected into the tail vein under ketamine (75mg/kg) and acepromazine (2.5mg/kg) intraperitoneal anesthesia (Sigma). After one hour of spontaneous breathing, tracheotomy was performed and a canula was inserted into the trachea. I designed seven experimental conditions: control, LPS, ventilation, ventilation/CO, LPS/ventilation, LPS/ventilation/CO and SB203580/LPS/ventilation/CO (n=6/condition). Pairs of CO treated and non-treated animals were formed and treated one after another. Animals treated according to condition ventilation, ventilation/CO, LPS/ventilation, LPS/ventilation/CO and SB203580/LPS/ventilation/CO received 26ml/kg tidal volume mechanical ventilation with room air or with 250 parts per million (ppm) CO mixed with room air for 15minutes to 4 hours without PEEP. Condition SB203580/LPS/ventilation/CO were injected intraperitoneally with SB203580 p38-

kinase inhibitor (20mg/kg) 30 minutes prior to the experiment. Arterial blood pressure and arterial blood gases were measured in condition LPS/ventilation and LPS/ventilation/CO (pressure transducer UFI, Morro Bay, CA; blood gas analyzer Radiometer ABL5, Copenhagen, Denmark). In CO dose-response experiment LPS/ventilation/CO treated animals received 10, 100 or 250 ppm CO (n=3-6/dose). In these experiments I measured cell numbers and cytokine levels in the bronchoalveolar lavage fluid (BALF) as markers of lung injury. ALI was also assessed with histology, electrophoretic mobility shift assay (EMSA), western and northern blotting.

Results

Specific Aim #1.Gene expression profiling in a mouse model of isolated perfused and ventilated lung

I compared the gene expression patterns of -10 cm H_2O EIP ventilated (controls), -25 cm H_2O EIP ventilated (overventilation) and LPS-treated animals by microarray analysis. The lungs of the animals in the three treatment protocols exhibited distinct gene expression profiles. I identified 3 clusters of genes that behaved different in the 3 treatment groups. The expression of cluster A genes was decreased in the overventilated animals (OV) and unchanged in the LPS-treatment group (LPS). These genes show a generally lower expression. Cluster B genes displayed decreased expression in the OV group but their expression was increased in the LPS group. Cluster C genes were increased by OV treatment. Many of them were also increased in the LPS group but to a lesser extent. Using two different statistical programs: Significance Analysis of Microarrays (SAM, Stanford, CA) program and Scoregene software package (Scoregene Package, available at http://compbio.cs.huji.ac.il/scoregenes/) (Scoregene TNoM=0, SAM p-value<0.05) I

identified 567 genes that had significantly different expression levels following LPS and/or OV treatment when compared to controls. A total of 182 genes were changed by OV and not by LPS. The expression of 36 genes was induced, and that of 146 genes decreased. Included with increased expression were genes of general metabolism, growth factor related genes, intracellular transport, one cytokine ligand and one antioxidant. The genes with decreased expression featured growth factor-related genes, cell cycle regulators, antioxidants, oncogenes, complement and coagulation related genes. Both LPS and OV regulated 385 genes. Among these the expression of 246 was increased and that of 139 decreased. Among those many are cytokines, transcription factors and apoptosis-related genes. Certain growth factor genes also appear, such as granulocyte-macrophage colony stimulating factor (GM-CSF) as well as Nur77. LPS and OV reduced the expression of certain well-known protein kinases, cell cycle regulators, transcription factors and growth factors.

Functional grouping of genes following lung injury

I assessed cellular functions that were modified by LPS treatment and/or overventilation. First I identified genes with increased or decreased expression by creating $\log_2$ base ratios of the gene expression results (lps/c, ov/c and ov/lps respectively). Following I used GeneXpress (http//: www.genexpress.stanford.edu) to determine whether a specific cellular function annotations were enriched in one or more of the pairwise combinations. Statistical significance was determined using hypergeometric model and corrected for multiple testing using false discovery rate (FDR) methods. A 95 % confidence interval was set for the FDR correction to correct for multiple testing. Findings were visualized on a color map where orange represents the up-regulation of gene expression related to the cellular function and blue stands

for reduced expression in groups of genes related to the same function. Genes induced by OV but not by LPS were enriched for genes involved in cytoskeleton and cell communication. Genes induced only by LPS were enriched for genes involved in cytokine activity and in nucleic acid binding. Genes induced by both OV and LPS were involved in the immune response, carrier activity, and protein binding physiological processes.

Specific aim #2.Genes with significant changes in expression

Based on gene clustering and gene scoring results my colleagues and I decided to validate the RNA expression of 5 target genes: Areg (Amphiregulin), Akap12 (A kinase anchoring protein), Cyr61 (Cystein-rich protein 61), Nur77 (Nuclear receptor subfamily 4, group A, member 1) and Il11 (Interleukin-11). These genes are all found in cluster C with significantly increased expression (Scoregene TNoM=0, SAM p-value<0.05) following overventilation and LPS-treatment. The increased mRNA expression of the 5 genes was validated by QRT-PCR. The findings were further substantiated by immunoblotting for amphiregulin. Amphiregulin protein expression was increased in the OV group when compared to the control and LPS treatment groups. Finally, I used the same antibodies to immunostain the lungs of the 3 experimental groups. Lungs subjected to overventilation showed increased amphiregulin staining compared to controls and LPS-treated lungs with primarily epithelial localization.

Specific aim #3.Ventilation enhances LPS-induced lung injury in rats

To determine the magnitudes of lung injury caused by LPS, ventilation, and LPS/ventilation I performed two-hour experiments. LPS animals were sacrificed 2

hours after injection of LPS. In the LPS/ventilation condition, animals received LPS injection, and allowed to spontaneously breath for 1 hour, and then treated with 1hour mechanical ventilation. Ventilation only animals received 2 hours of mechanical ventilation, and then sacrificed. For all animals, I measured total cell number and total protein from the BALF. LPS or ventilation alone significantly augmented the total cell number measured in the lavage fluid, as did LPS followed by ventilation. LPS treatment followed by ventilation also significantly enhanced the total protein levels in BALF whereas ventilation or LPS alone did not increase BALF protein levels. The histology further supports the finding that ventilation further enhances LPS-induced lung injury. When compared to PBS treatment, LPS or ventilation alone caused inflammatory cells infiltration into the alveolar septa and thickening of the alveolar wall. The combined effect of LPS and ventilation was the most injurious that resulted in the destruction of the alveolar structure. Pro-inflammatory cytokine TNF-α levels dramatically increased in BALF after LPS/ventilation treatment compared with the control or ventilation alone treatment conditions. Kinetic experiments show significantly elevated TNF-α levels in LPS/ventilation treated animals after 30 minutes ventilation. The maximal TNF-α level was measured after one-hour ventilation on the 3-hour ventilation time course.

VILI induces HO-1 expression

In order to examine whether VILI can induce expression of the stress inducible HO-1, I performed Northern and western blot analyses to determine HO-1 expression levels in the lung tissues after ventilation. LPS or mechanical ventilation alone increased HO-1 gene and protein expression. The use of LPS, as a primer of lung injury, followed by ventilation increased HO-1 mRNA and protein expression

the most when compared to controls. These data suggest that HO-1, an important cellular stress response gene product, may play a role in defense against VILI.

Specific aim #4. Inhaled CO inhalation attenuates ventilator-induced lung injury

Inhaled CO significantly reduced the total cell number increased by LPS/ventilation in the BALF. Ventilation alone with CO (ventilation/CO condition) markedly reduced the total cell number, but it did not reach significance. CO did not affect cell count in control and LPS treatment conditions. CO treatment did not affect the elevated total protein levels.

I observed a dose-dependent decrease of the pro-inflammatory cytokine TNF-α in BALF when the animals inhaled CO during mechanical ventilation. I also measured the anti-inflammatory cytokine IL-10 in the BALF. LPS, ventilation or their combination did not affect IL-10 levels. CO treatment in LPS/ventilation/CO condition significantly increased IL-10 in the BALF. The effect was not observed in ventilation/CO condition. CO did not have an effect on cytokine levels in control and LPS treatment conditions. Modest decrease in hypercellularity and inflammation was observed in tissue histology after CO treatment.

Differential cell count showed significantly reduced number of macrophages in the BALF after 2 hours treatment with 250ppm CO mixed with room air. The number of neutrophil leukocytes in the BALF at this timepoint was negligible. To examine whether CO inhalation can effect neutrophil leukocyte infiltration to the alveoli, I performed LPS/ventilation and LPS/ventilation/CO condition experiments where animals received LPS injection, and allowed to spontaneously breath for 1hour, and then they were mechanically ventilated for 4 hours. Treatment with 250 ppm CO resulted in significantly reduced total cell count and neutrophil cell count in the

BALF. This finding suggests that CO may also reduce lung injury via inhibiting neutrophil leukocyte infiltration into the alveolar space.

Inhaled low dose CO exerts negligible effects on hemodynamics and oxygenation

In order to confirm that mechanical ventilation and low dose inhaled CO used for our studies did not exert untoward effects on hemodynamics and gas exchange; I measured blood pressure and blood gases in LPS/ventilation and LPS/ventilation/CO conditions. Following tracheostomy a canula was inserted in the right carotid artery and blood pressure was continuously measured during the ventilation. Blood was sampled from the canula for blood gases in the beginning and the end of mechanical ventilation. I did not observe statistically significant differences in blood pressure, pH, PCO_2, and PO_2 in LPS/ventilation and LPS/ventilation/CO conditions during the course of the experiment. Inter-group differences were not significant either. LPS treatment or ventilation alone had no effect on the hemodynamic of the animal model. The carboxy-hemoglobin level was significantly elevated in CO treated animals, as expected.

Mechanisms of CO-induced anti-inflammatory effects

Transcription factors are involved in cellular stress-induced gene expression and regulate biological processes including inflammation, cell proliferation and cell survival, all of which are important in conferring protection against cell and tissue injury. I examined whether transcription factors such as AP-1 and NF-κB are involved in CO-induced anti-inflammatory effects in VILI. LPS and ventilation both induced activation of AP-1 and NFκB; however, CO treatment did not modulate the

activation of AP-1 or NFκB binding activity compared with ventilation or LPS/ventilation treatment.

Another intracellular pathway that is activated in lung injury is p38 MAPK. In our model LPS and ventilation increased activation of p38 MAPK; CO significantly increased p38 MAPK activation when compared to ventilation and LPS. In order to examine whether CO-induced p38 activation exerted biological effects, I measured the levels of the anti-inflammatory molecule IL-10. I demonstrate that inhibition of p38 with the chemical inhibitor SB203580 compound significantly attenuates CO induced IL-10 levels and histology shows hemorrhagic, inflamed lung tissue.

Discussion

According to an international study, an average of 39% of intensive care unit patients require mechanical ventilation worldwide. Many of these patients develop ventilator-induced lung injury. Eventually VILI contributes to ARDS. While clinical trials showed that ARDS/VILI-related mortality could be attenuated with lower tidal volume ventilation, PEEP ventilation and more recently, with recruitment maneuver combined with protective ventilation strategy, the syndrome remains a major problem in intensive care units.

1. Gene expression profiling of target genes in ventilator-induced lung injury (Specific aim #1)

Cumulating experimental and clinical evidence suggests that VILI is triggered by mechanical overstretch of the alveoli, frequently facilitated by other causes of acute lung injury such as microbial agents. Lipopolysaccharide (LPS), acid aspiration and cecal ligation/perforation induced sepsis are commonly used models for approximating previous lung injury in ventilator-induced lung injury model.

However, the extent to which overstretch and microbial agents activate similar or separate intracellular mechanisms it is not yet established. I approached this problem by comparing the gene expression profiles of overventilated and LPS-treated mouse lungs. Gene expression changes in ventilator-induced lung injury can be key to understand the detailed mechanism of ALI/ARDS. However the application of this approach to ventilator-induced gene expression *in vivo* is complicated by at least two factors: (i) in intact, non-injured animals alveolar overdistension is limited by both the chest wall and adjacent alveoli, (ii) infiltrating inflammatory cells may alter the gene expression profile. These two problems are circumvented in isolated blood-free perfused lungs. Therefore, in the present study I have used microarray analysis to study the effect of overventilation on gene expression and compared this gene expression pattern to that induced by LPS. I identified 567 genes whose expression was modified by overventilation and/or LPS. Many genes, in particular those that code for cytokines, growth factors and apoptosis-related proteins were altered both by overventilation and by LPS suggesting the presence of common intracellular pathways. However, the expression of some genes including growth factors, metabolizing enzymes, mediators and cytoskeletal proteins were modified by alveolar distention only. It is tempting to speculate that these genes activate mechanisms different from the well-known lung inflammatory pathways that have been established for microbial infections.

The largest group of genes affected by overventilation was related to apoptosis. Epithelial apoptosis has been observed in both *in vitro* and *in vivo* models of VILI. The genes identified in this study include genes involved in the FAS ligand and the caspase pathways. Among the affected genes, many are expressed in nervous system pointing to a possible neuro-inflammatory axis. For example, expression of

the neuropeptide preprotachykinin-1 was related to acute lung in injury in a mouse VILI model. Also, Hayashi and colleagues have reported that in patients with spinal muscular atrophy, mechanical ventilation appears to contribute to neurodegeneration.

Differences between overventilation and LPS

Thus a number of important target genes were recognized by comparing arrays from different studies. Another approach is the use statistical methods to identify likely candidate genes within a given study. Herein I have used the combined statistical power of Scoregene and SAM. Applied to our data 90% of the significant genes identified by these procedures were identical, summing up to 567 genes regulated by overventilation and/or LPS.

Among the genes upregulated by both overventilation and LPS figured well-known pro-inflammatory (IL-6, GM-CSF, MIF) and anti-inflammatory (IL-10, IL-1ra) cytokines. Noteworthy cytokines upregulated by overventilation, which are new to the field of VILI, are IL-11 (confirmed by QRT-PCR), macrophage migration inhibitory factor (MIF) and IL-22 (IL10-related T cell-derived inducible factor). Other extracellular factors upregulated by overventilation are amphiregulin (see below in The potential role of Amphiregulin) and cysteine-rich protein 61 (Cyr61, confirmed by QRT-PCR). Cyr61 binds to cell surface integrins and thereby induces intracellular signaling events, some of which relate to cell proliferation and angiogenesis. Moreover, my colleagues and I identified several factors involved in intracellular signaling pathways such as gravin (Akap12, A-kinase anchoring protein 12) and Nur77 (Nr4a1) to become activated by overventilation. Since these genes may point to relevant mechanotransduction pathways, I confirmed their expression by QRT-PCR. AKAPs maintain multivalent signaling complexes by binding additional enzymes, including kinases and phosphatases to the cytoskeleton and may thus

communicate cell distortion inside cells. Nur77, also known as NGFIB or TIS1, represents an orphan nuclear receptor that is involved in cell proliferation and death and may act as an anti-apoptotic factor.

A selective response was observed for another set of genes that according to the microarray analysis was upregulated by overventilation but not by LPS. This list includes Tnfrsf12a (Fibroblast growth factor regulated protein 2, Fn14), which modulates NFκB activation and stimulates human bronchial epithelial cells to produce IL-8 and GM-CSF, the largely unexplored chemokine CXCL16, and the chemokine receptor CCR5. The majority of these factors has not yet been implicated in VILI and indicates the existence of unknown specific mechanotransduction pathways in VILI.

Also of note is the fact that we found that a number of genes were down regulated by overventilation. So far, gene regulation has been largely focused on upregulation of genes and it is only with the beginning of microarray experiments that downregulation gets some focus. Prominent among the genes downregulated by overventilation figure platelet-derived growth factor and hypoxia-inducible factor 1α (HIF1α).

2. The potential role of amphiregulin (a new candidate gene in ventilator-induced lung injury) (Specific aim #2)

Stretch can induce growth factors in epithelial cells. Tschumperlin and colleagues demonstrated that epithelial stretch increases epidermal growth factor receptor (EGFR) ligand shedding into the lateral intracellular space leading to intracellular signaling. The ligands bind to the EGFR that subsequently induces extracellular signal-regulated kinase (ERK) MAPK activation. This mechanism could explain why several growth factor-related genes exhibited modified expression during overventilation.

One of them is amphiregulin. It is a polypeptide growth regulator and part of the epidermal growth factor family. Together with other EGFR ligands amphiregulin binds to the extracellular domain of EGFR. EGFR binding leads to the activation of the ERK and the transcription factor NFκB. The EGFR induces cell proliferation and the release of IL-8 and/or MIP-2 cytokines. Amphiregulin is expressed in lung tissue and has an essential role in lung branching morphogenesis. In human pulmonary epithelial cells amphiregulin secretion is induced upon exposure to tobacco smoke and fine particulate matter. Furthermore, increased amphiregulin secretion also contributes to GM-CSF release. In a mouse model of chronic asthma, ovalbumin-challenged animals displayed increased amphiregulin immunostaining of epithelial cells. My immunostaining shows stretch-induced expression of amphiregulin in epithelial cells, which are exactly those cells in which ERK is activated during overventilation. The possibility that amphiregulin mediates some of the signaling responses during overventilation merits further investigation.

Taken together, these findings suggest that overventilation activates specific cellular programs that clearly distinguish it from LPS. In the future it will be important to further characterize these pathways, which may finally allow to specifically interfere with the side effects of mechanical ventilation.

4.Carbon monoxide confers antiinflammatory effects in ventilator-induced lung injury (Specific aim #3 and #4)

The primary goal of the *in vivo* mechanical ventilation study was to test the hypothesis that inhaled CO can confer protective effects in an animal model of VILI. I used intravenous LPS injection and/or a relatively injurious ventilator setting to induce lung inflammation, in ventilated animals in the presence or absence of inhaled

CO. A protective effect of CO was observed. Then I described the potential mechanism by which CO confers anti-inflammatory effects against VILI.

I used a relatively injurious VILI model in rats, which resulted in lung injury that features inflammation, and edema as assessed by cell count, TNF-α production, and protein in the BALF. I performed TUNEL assay in our lung tissues and did not observe evidence of cell death after VILI. To better mimic a human disease course and maximize VILI, I used a sub lethal dose of LPS to prime and supplement ventilator induced lung injury, as often used by investigators. I measured blood gas and arterial blood pressure parameters and did not observe significant changes in blood pH, pCO_2, pO_2 and arterial blood pressure.

I initially observed a robust induction of HO-1 mRNA and protein in this model of VILI . Our laboratory and others have shown that HO-1 induction in response to cellular and tissue stress, *in vitro* or *in vivo*, is not only a reliable marker of cellular injury but also a physiologic response to defend against the inciting stress or cellular insult. Thus, in view of our observation that HO-1 was markedly increased in VILI, we sought to assess whether CO could be responsible in mitigating VILI.

Using the same concentration of CO (250 ppm) I have used previously for *in vitro* and *in vivo* studies, we observed that CO could markedly attenuate the inflammatory responses of VILI. Inhaled CO significantly reduced the BALF cell count and TNF-α levels. Interestingly, I also observed that CO increased levels of the anti-inflammatory IL-10 in the BALF. These results correlate with the previous observations of Otterbein and colleagues in mice and murine macrophages.

Macrophages are the principal cell type found in the BALF after LPS/ventilation treatment. Macrophages are known to be sensitive to mechanical stress; thus, the changes in BALF total cell count might reflect an important role of

macrophages in cytokine release in VILI. Belperio and colleagues described neutrophil leukocyte-predominant inflammatory response in mice following six hours high-volume (12ml/kg) mechanical ventilation. In my model neutrophil leukocyte infiltration to the alveolar space was observed after 4 hours of mechanical ventilation. Inhaled CO significantly reduced neutrophil recruitment to the alveoli. Additionally, inhaled CO also reduced BALF macrophage number at 2 hours. TNF-α is a well-known and well-investigated cytokine that has a proinflammatory effect in *in vivo* and *in vitro* models and IL-10 has antiinflammatory activity in LPS-induced inflammation. Of note, it is interesting to note that CO did not affect the BALF protein levels suggesting that CO exerts negligible effects on pulmonary permeability. This observation provide us additional clue as to the differential and specific anti-inflammatory effect of CO which at this time appears to act as a regulator of inflammation by attenuating proinflammatory cytokine and augmenting antiinflammatory cytokine.

The signaling pathway, by which CO acts as an anti-inflammatory agent, is not fully understood. Although it is well established that CO activation of soluble guanyl-cyclase (sGC) and cyclic GMP (cGMP) mediates much of the vasodilatory effects, we did not observe a cGMP dependent effect in our VILI model. I showed that NF-κB and AP-1 activation, two major pathways in ventilator-induced lung injury, are not modulated by CO inhalation in VILI. I observed p38 MAPK activation following CO treatment in VILI. The p38 MAPK is known to regulate TNF-α and IL-10 production. Although the molecular mechanism by which CO affects p38 MAPK to produce less TNF-α and more IL-10 needs to be further investigated, CO may have a posttranscriptional effect on TNF-α production.

I believe that my animal models can lead to a better understanding of the complex intracellular regulatory function of CO in lung injury. Based on the observations of this study, it is tempting to speculate that inhaled CO could represent a potential new therapeutic modality for counteracting VILI.

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