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Forum Review

Role of Oxidants in Lung Injury During Sepsis

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ABSTRACT

The role of oxidative stress has been well appreciated in the development of sepsis-induced acute lung injury (ALI). Oxidative stress in sepsis-induced ALI is believed to be initiated by products of activated lung macrophages and infiltrated neutrophils, promptly propagating to lung epithelial and endothelial cells. This leads to tissue damage and organ dysfunction. On stimulation, neutrophils (PMNs) enable their migration machinery. The lung undergoes changes favoring adhesion and transmigration of PMNs, resulting in PMN accumulation in lung, which is a characteristic of sepsis-induced ALI. Oxidative stress turns on the redox-sensitive transcription factors (NF-κB, AP-1), resulting in a large output of proinflammatory cytokines and chemokines, which further aggravate inflammation and oxidative stress. During the process, transcription factor nuclear factor-erythroid 2-p45–related factor 2 (Nrf2) and heme oxygenase (HO) appear to play the counterbalancing roles to limit the propagation of oxidative stress and inflammatory responses in lung. Many antioxidants have been tested to treat sepsis-induced ALI in animal models and in patients with sepsis. However, the results are inconclusive. In this article, we focus on the current understanding of the pathogenesis of sepsis-induced ALI and novel antioxidant strategies for therapeutic purposes. *Antioxid. Redox Signal.* 9, 1991–2002.

INTRODUCTION

Despite technical developments in intensive care units (ICUs) and advanced supportive treatment, the death rate in septic patients remains high, with a range of lethality ranging from 30 to 50% (3). The acute respiratory distress syndrome (ARDS) has been defined as a severe form of acute lung injury (ALI), featuring pulmonary inflammation and increased capillary leak (98). ARDS may arise in a number of clinical situations, especially in patients with sepsis. As a common complication of sepsis, it has been considered a leading cause for death in sepsis. A well-described pathophysiologic model of ARDS is one form of acute lung inflammation mediated by inflammatory cells and mediators as well as oxidative stress (22).

Oxidative stress is caused by an imbalance between the production of reactive oxygen species (ROS) and the biologic scavenger system, which can readily detoxify the reactive oxygen intermediates under normal physiologic conditions. Oxidative-induced damage has been considered to be one of the underlying mechanisms that contribute to multiple organ failure in sepsis (7, 35, 102). Because of the natural oxidizing nature of the atmosphere and the direct exposure to many atmospheric pollutants, the lung is at high risk of oxidative injury. It has been recognized that oxidative stress can not only directly cause tissue damage, but it also can affect the molecular mechanisms that control lung inflammation. In the healthy lung, airway lining fluids and extracellular spaces are maintained in a highly reduced state (with millimolar levels of reduced glutathione, GSH) to preserve normal physiologic functions. Under normal conditions, the levels of antioxidants and oxidants in lung are balanced in favor of a reducing state. Decreases in antioxidants or increases in oxidants can disrupt this equilibrium and can cause oxidative stress. An imbalance in the oxidant–antioxidant system has been recognized as one of the first events that ultimately lead to inflammatory reactions in the lung (17).

Oxidative stress has been found to occur in many forms of lung disorders, such as pneumonia, ARDS, idiopathic pulmonary fibrosis, lung transplantation, chronic obstructive pul-

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monary disease, cystic fibrosis, bronchiectasis ischemia–reperfusion, and lung cancer (10, 18, 79). Based on composition, oxidants can be divided into two main categories, ROS and reactive nitrogen species (RNS). Superoxide, hydroxyl radicals, and hydrogen peroxide are generally classified as ROS in contrast to nitric oxide and peroxynitrite, which are nitrogen based. These molecules naturally function as neurotransmitters, second messengers, and as a part of the chemical host defense against infection. It is only when their concentrations become excessive, especially extracellularly, that the potential for adverse responses can occur. High ROS/RNS levels lead to alterations in normal cell function and eventually compromise local tissue and systemic homeostatic mechanisms. In this review, we focus on the understanding of the roles of oxidative stress in sepsis-induced ALI and potential therapeutic strategies by using antioxidants.

DETECTION OF OXIDATIVE STRESS IN LUNG

It is clear that a better understanding of the oxidative state in the lung is important for the diagnosis and treatment of lung diseases. Many methods have been developed for detection of free radicals from oxygen, ROS, RNS, and their byproducts to assess the presence of oxidative stress. The techniques include established standard protocols and advanced methods using high-performance liquid chromatography (HPLC), mass spectrometry, and electron paramagnetic resonance. Described later are the most frequently used methods for evaluation of lung oxidative status.

Monitoring oxidative stress in live cells

ROS in live cells can be detected by using a fluorogenic marker for ROS and observing under fluorescence microscopy. One frequently used marker is carboxy-H_2DCFDA, a cell-permeable fluorogenic marker, which is oxidized during oxidative stress in live cells and emits bright green fluorescence (97). $NO^{\cdot}$ production by lung cells can be measured by using the $NO^{\cdot}$ fluorescent indicator, DAF-2 (89). On reaction with an active intermediate (N_2O_3) formed during the oxidation of $NO^{\cdot}$ to nitrite, DAF-2 is converted to its fluorescent triazole form, which can be measured by fluorescent plate readers at excitation and emission wavelengths of 485 and 538 nm, respectively..

Hydrogen peroxide (H_2O_2) and superoxide products in bronchoalveolar fluids

H_2O_2 fluids in bronchoalveolar fluids (BALs) can be measured by the simple assay for detecting the presence of peroxides in both aqueous and lipid environments. The basis of these assays is the complexing of ferrous ion (Fe^{2+}) by H_2O_2 in the presence of xylenol orange. Peroxides will oxidize Fe^{2+} to Fe^{3+}, and Fe^{3+} will form a colored complex with xylenol orange that can be read at 560 nm (46, 47). Superoxide radical generation can be estimated by nitroblue-tetrazolium reduction assay (60).

Antioxidant status in lung

The antioxidant status in the lungs can be evaluated by lung levels of superoxide dismutase (SOD) and catalase (CAT) and their activities. SOD activity can be assessed by the OxyScan SOD-525 assay, which measures the activity of all forms of SOD. The method is based on the SOD-mediated increase in the rate of autoxidation of 5,6,6a,11b-tetrahydro-3,9,10-trihydroxybenzôfluorene in aqueous alkaline solution to yield a chromophore with maximum absorbance at 525 nm. Catalase (CAT) activity can be determined by a two-step reaction scheme (catalase-520 assay). First, catalase reacts with a known quantity of H_2O_2 to generate H_2O and $O_2^{\cdot}$. In the presence of horseradish peroxidase (HRP), the remaining H_2O_2 reacts with 3,5-dichloro-2-hydroxybenzenesulfonic acid and 4-aminophenazone to form a chromophore with a color intensity. Lipid peroxidation levels in lung can be measured by thiobarbutiric acid–reactive substances assay (25). The GSH/GSSG ratio, a useful measure of oxidative stress, can be determined by a colorimetric method by using Bioxtech GSH/GSSG-412.

Protein oxidation and lipid peroxidation in lung

The levels of protein carbonyls and nitrotyrosine are widely used for the detection of oxidative modification of proteins. Protein carbonyls are considered to be one of the most reliable methods to evalute the protein damage mediated by oxidative stress. Nitrotyrosine levels in tissues and BAL fluids correlate with oxidant stress in animal and human studies (33). Protein carbonyls can be measured through the reaction with dinitrophenylhydrazine (DNPH) (57). The proteins are first precipitated by the addition of 20% trichloroacetic acid and then redissolved in DNPH. Nitrotyrosine is often determined by enzyme-linked immunosorbent assay (ELISA), Western blotting, and immunohistochemistry with specific antibodies. Lipid peroxidation, oxidized lipids, and lipid mediators are believed to play an important role in lung inflammatory diseases. The detection of products of lipid peroxidation has been widely used to estimate the overall status of oxidative stress in lung. Among them, thiobarbituric acid reactants (TBARs) and malondialdehyde (MDA) are commonly used indicators (16).

DNA damage

The most common type of damage caused by reactive oxygen species in the body is oxidative damage to DNA. Hydroxydeoxyguanosine (8-OHdG), a product of this type of DNA damage, is used as a biomarker for oxidative stress. It can be measured by the immunohistochemical procedure and an HPLC system equipped with an electrochemical detector (HPLC-ECD) (63). More recently, it has been reported that capillary electrophoresis-mass spectrometry (CE/MS) can be also used for the analysis 8-OHdG to study oxidative stress (101).

Analysis of expired air for oxidation products

Studies have shown that expired $NO^{\cdot}$ and CO can serve as biomarkers for oxidative stress, whereas ethane can serve as a marker of lipid peroxidation (73). CO can be detected electrochemically, and it can also be measured by a laser spectrophotometer and near-infrared CO analyzers. The levels of exhaled

NO˙ can be assessed by chemiluminescence. Ethane content can be detected by using gas chromatography. Oxidative stress in lung appears to be an important factor in predicting or assessing (or both) lung injury. Methods for detection of oxidative stress have a broad range from well-established protocols to newly developed technologies. It is very difficult to measure oxygen free radicals directly because of their short half-lives and reactivity with other molecules. Radical spin-trapping agents have been used to form stable radical adducts, which can be detected by electron paramagnetic resonance spectroscopy (EPR). Trapping agents are generally nitrone- or nitroso-containing molecules, such as 5,5-dimethyl-1-pyrroline-*n*-oxide (DMPO), that react with oxygen free radicals to form stable nitroxide free radicals (72). The sampling procedure to collect exhaled breath condensate can be used to measure H_2O_2, leukotrienes, isoprostanes, and 3-nitrotyrosine in lung inflammation (73). Given the nature of complexity and importance of lung biology, it is essential to develop sensitive and reliable tools to monitor the status of lung oxidative stress. A standardized protocol is also required for clinical application.

EVIDENCE OF OXIDATIVE STRESS IN SEPSIS-INDUCED ALI

Low antioxidant and high plasma levels of oxygen free radicals have been well documented in patients with sepsis and in animal models of sepsis (30, 35, 96). Many oxidative indicators have been reported in lungs from septic patients and animals. In patients with sepsis, protein carbonyls were significantly elevated in both blood and BAL fluids during the initial phase of sepsis, decreasing within a few days but remaining above control values (102). In the same study, myeloperoxidase (MPO) activity was also markedly increased in BAL fluids from septic patients. Strong correlations were found between carbonyl concentrations in BAL fluid and plasma, when compared with protein carbonyls, TBARS, and MPO in lung, suggesting that neutrophil oxidants might be chiefly responsible for oxidative stress in lungs during sepsis. All patients with ARDS had higher levels of hypoxanthine, a prooxidant substrate for xanthine oxidase (75). Hypoxanthine levels were 2 times higher in nonsurvivors than those in survivors. Nonsurvivor ARDS patients appeared to have higher levels of oxidative stress and damage when compared with survivors. Surprisingly, hypoxanthine levels were normal in patients in intensive care with sepsis but no lung injury. These data suggest that oxidative stress plays an essential role in the pathogenesis of ALI in sepsis, and directly contributes to the bad outcome.

Oxidative stress in animal models of sepsis has been also well described. In rodent sepsis models induced by cecal ligation and puncture (CLP), the activities of enzymatic antioxidants, including SOD, CAT, and glutathione peroxidase (GSH-PX), in lung were significantly decreased during the early- and late-sepsis phages (21), indicating that sepsis sets up an environment favorable for oxidative stress in lung. As expected, MDA concentrations and nitrate (NO_3^-)/nitrite (NO_2^-) levels were also elevated in the septic lung. In addition, MPO activity was found to be enhanced, and a large number of neutrophil infiltrates were observed by histology in lungs from septic animals. Many other oxidative indicators in lung, including 8-isoprostane, exhaled NO˙, superoxide anion, glutathione, protein carbonyls, and TBARS, were all changed in favor of an oxidative-stress status during experimental sepsis (2, 19, 51, 59, 85, 86, 96).

NEUTROPHIL ACCUMULATION IN LUNG DURING SEPSIS

The pathophysiology of the sepsis-induced ALI/ARDS is very complicated and not completely defined. However, increased production of ROS/RNS from PMNs has been proposed as one of the significant mechanisms underlying the development of lung inflammation. Additionally, the contribution by lung macrophages may be an important source for ROS/RNS. Sepsis-induced ALI is characterized by the activation of a variety of cells, including inflammatory cells such as PMNs and macrophages, and increased levels of inflammatory mediators. PMN infiltrates occur in lungs from both humans and animals with sepsis. The number of neutrophils in BAL fluids from patients with ARDS is significantly increased and associated with poor survival (1, 61, 100). Circulating PMNs infiltrate and accumulate in the lung *via* transmigration through the endothelium, interstitium, and alveolar epithelium to enter the alveolar compartment or be sequestered in lung capillaries. Upregulation of chemoattractant molecules (chemokines) occurs, establishing a concentration gradient that directs the neutrophils into the lung. Adhesion molecules, including integrins, selectins, and ICAMs, are also involved in the migration process (40). These events are described in Fig. 1.

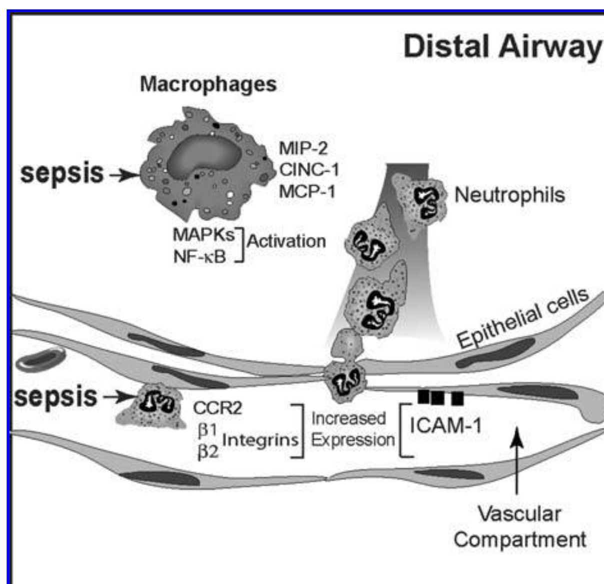

FIG. 1. The mechanism of PMN accumulation in the lung during sepsis. In rat and mouse models of sepsis, activated alveolar macrophages release MIP-2 (rat), CINC-1 (rat), and MCP-1 (mouse), which chemoattract PMNs into the lung. Increased levels of CCR2 as well as β_1 and β_2 integrins on PMNs, together with enhanced expression of ICAM-1 on endothelial cells, facilitate PMN adhesion and migration.

Local production of CXC chemokines is essential for the accumulation of PMNs in the lung under conditions of acute inflammation. For PMN chemoattractants, although sufficient local concentrations of CXC chemokines are necessary for PMN recruitment, the dictating factor is the ratio of lung-to-blood chemokine concentrations (11). In experimental sepsis, lung production of two important CXC chemokines, MIP-2 and CINC-1, increased but lagged behind in comparison with the levels of MIP-2 and CINC in blood (38). As a result, lung levels of CXC chemokines increased when blood levels of those decreased. The discrepancy might be important for CXC chemokines to create a gradient difference between the two compartments (vascular and alveolar), which is necessary for initiation of PMN migration. CXC chemokines have been implicated in all steps in the extravasation process of leukocytes, including rolling, adhesion, and transmigration *in vivo* (104). Thus, the role of blood CXC chemokines may primarily focus on the activation of PMNs and endothelial cells, setting the stage for PMN migration, whereas local CXC chemokines chiefly function chemotactically during sepsis. In addition, blood CXC chemokines produced in the early stage of experimental sepsis may provide vital signals for PMN survival, given the fact that CXC chemokines and C5a reduce PMN apoptosis (23, 39). Under such conditions, the greatly prolonged life span of PMNs may be related to the development of the symptoms of sepsis. The reason for higher BAL levels of CXC chemokines is likely the activation of lung macrophages. It is clear that a phase exists in which alveolar macrophages are prone to activation during the early stage of sepsis, and then may go into a refractory (deactivation) stage (38, 82). Conversely, PMNs quickly enter into this refractory phase, and their capability for producing CXC chemokines and other inflammatory mediators is greatly compromised. It was found that CXC chemokine macrophage-inflammatory protein-2 (MIP-2) production in both alveolar macrophages and PMNs was dependent on mitogen-activated protein (MAP) kinases (p38 and p42/p44) and NF-κB pathways (38). Interestingly, alveolar macrophages still maintain, and even increase, their capacity for activation of p38 and p42/p44 MAPKs during sepsis, whereas septic blood PMNs become nonresponsive to stimulations. Paralysis of signaling pathways in PMNs is likely caused, at least in part, by overproduction of C5a in sepsis. In addition, the CC chemokine, MCP-1, seems to also play an important role in attracting PMNs into lung (89). PMNs in sepsis used a novel migration pathway that is CC chemokine receptor CCR2 dependent. Ordinarily, PMNs do not express CCR2, but in sepsis, this receptor is clearly expressed (88).

Sepsis involves widespread upregulation of both PMN and endothelial adhesion molecules (74). In a rat model of sepsis, it was observed that the content of β_1 and β_2 integrins on circulating PMNs was elevated after CLP. The increased expression of β_1 integrin on blood PMNs followed β_2 integrin elevation, which was seen as early as 3 h after CLP. Rapid elevation of β_2 integrin may represent an important role in host defense by directing PMNs into inflamed organs. Several lines of evidence support such a role for β_2 integrin in the mobilization of PMNs into tissues. Patients with inherited deficiencies of β_2 integrin are much more susceptible to bacterial infection (41). In a canine model of lung inflammation (i.v. infusion of TNF-α), anti-CD11b treatment reduced PMN accumulation early (within the first 24 h), but not later (>24 h) (74), suggesting that leukocyte trafficking may differ between the early and late stages of inflammation. Expression of β_1 integrin in PMN migration is amplified in sepsis. Fully activated β_1 integrin at the later stage of sepsis may alter the balance of integrin cooperativity. It was previously shown that blood PMNs from septic, but not control, patients expressed $\alpha_4\beta_1$ integrin, which caused increased adhesiveness to immobilized VCAM-1 (43). Anti-β_1 integrin antibody interferes with cell motility of septic PMNs from CLP rats, indicating that sepsis alters the trafficking of PMNs into the lung by engaging a β_1 integrin–dependent pathway (37). The adhesion molecule, ICAM-1, is also involved in PMN migration during sepsis. Absence or blockade of this molecule impairs the ability of PMNs to migrate into organ tissues and reduces consequent secondary organ damage, resulting in improved clinical indicators and overall survival (42). We previously demonstrated that PMN migration into lung during sepsis is ICAM-1 dependent, but not VCAM-1 dependent, perhaps related to VCAM-1 shedding that occurs during the course of sepsis development (54).

Figure 1 illustrates a simplified version of pathways for PMN migration in the lung during sepsis. CCR2 and β_1 and β_2 adhesion molecules on PMNs are upregulated during the course of sepsis, whereas PMN capability to produce CXC chemokines is reduced. In lung, activated macrophages release a large amount of PMN chemoattractants and other inflammatory cytokines, whereas ICAM-1 is activated on endothelial cells, setting the stage for PMN migration. These processes are tightly regulated by MAPKs and NF-κB pathways.

ROLE OF OXIDANTS IN SEPSIS-INDUCED ALI

Inflammation occurs after activation of PMNs and macrophages, resulting in ROS/RNS generation and the release of lysosomal enzymes and cationic proteins. Oxidative stress is initiated by ROS such as superoxide ($O_2^{\cdot}$) and hydrogen peroxide (H_2O_2). These oxidants are not very harmful *per se*, but can be converted into more dangerous oxidants, causing harmful reactions in tissues (16). Generation of superoxide-derived products continues to be the main pathway responsible for the production of ROS. $O_2^{\cdot}$ can be generated through various enzymatic systems, including the mitochondrial respiratory chain, xanthine oxidase, cyclooxygenase, and NADPH oxidase (16). The NADPH oxidase pathway is well defined in phagocytic cells. Activation of the oxidase can be initiated by a variety of inflammatory mediators and is likely to be a major source of oxidant generation in sepsis-induced lung injury. Translocation of cytosolic subunits of NADPH oxidase components from the cytoplasm to the cell membrane occurs in PMNs, macrophages, and monocytes on cell stimulation, thus representing a potential therapeutic target in the treatment of ROS-mediated lung injury during sepsis. NADPH oxidase catalyzes the transfer of an additional electron to molecular oxygen (O_2) to form the $O_2^{\cdot}$ anion. Because NADPH oxidase adds single electrons to O_2, oxygen-derived intermediates are also produced. Reduction of Fe^{3+} to Fe^{2+} or Cu^{2+} to Cu^{+} by superoxide in the Fenton re-

action facilitates the generation of hydroxyl radicals (OH^-). In the presence of Fe^{2+}, superoxide is reduced to H_2O_2, which can then be metabolized in the presence of transition metals and chloride to form hypochlorous acid (HOCl). This reaction is catalyzed by MPO and provides a practical marker for PMN accumulation in tissue. Although it is not a free radical, H_2O_2 can permeate cellular membranes, thus extending the damage beyond the originating cell. These products injure cells of the lung and airway and interfere with gas exchange.

$NO^{\cdot}$ is an abundant signaling molecule. Like H_2O_2, it is able to cross cell membranes to alter various physiologic processes through binding and activation of guanylate cyclase. *Via* this intermediate, $NO^{\cdot}$ functions as a secondary messenger in the maintenance of systemic vascular tone. $NO^{\cdot}$ also affects platelet aggregation and can stimulate immune responses, activate genes, and cause apoptosis (32, 50). It is fairly unreactive with bioorganic molecules; however, it does react with aromatic amino acids to form stable nitrotyrosine adducts in proteins and peptides, which may impair cell function. $NO^{\cdot}$ is synthesized by three nitric oxide synthase (NOS) isozymes, so named by the origin of the cell in which they were originally discovered. Two of the three forms (nNOS and eNOS) are constitutively expressed and generate small amounts of $NO^{\cdot}$, which generally are not sufficient to cause cellular damage. Binding to calcium and calmodulin is required for NOS activation, serving as factors that regulate NOS activity. In contrast, the third isoform (iNOS) is an inducible calcium-independent enzyme that produces copious amounts of $NO^{\cdot}$ for several hours to even days after induction. It is during circumstances that initiate induction of this third NOS form that the physiologic roles of $NO^{\cdot}$ are superseded by its implication as the culprit behind various injurious inflammatory responses and potentially cytotoxic events.

In the setting of lung, all three NOS isozymes are present. Various forms of $NO^{\cdot}$ can be observed as *S*-nitrosothiol, nitrate, and nitrite in exhaled air and bronchoalveolar lavage from human lungs (24, 95). Upregulation of iNOS amplifies the conversion of L-arginine to L-citrulline and $NO^{\cdot}$ formation. In the presence of superoxide, $NO^{\cdot}$ is converted to peroxynitrite ($ONOO^-$), which is then protonated to form an unstable species, peroxynitrous acid (ONOOH). The presence of iNOS has been reported in alveolar macrophages, PMNs, and endothelial and lung epithelial cells (99). Accordingly, these cells are all potential sources of peroxynitrite. $ONOO^-$ reacts with protein thiols and is thought to be the predominant mechanism by which $NO^{\cdot}$ production leads to cytotoxicity. Peroxynitrous acid is degraded to form the hydroxy radical, NO_3^- and NO_2^- by hemolytic cleavage, or it can react with CO_2 to form peroxycarboxylate ($ONOOCO_2^-$). Detection of NO_3^- and nitrite NO_2^- serves as a convenient experimental marker for $NO^{\cdot}$ production. Activation of infiltrating and resident phagocytes can cause an increase in both $NO^{\cdot}$ and superoxide, resulting in apoptosis. On a molecular level, the resulting peryoxynitrite interacts with DNA to cause DNA fragmentation, and with membrane lipids to cause peroxidation of the endothelial or alveolar epithelial cell plasma membranes. Low concentrations of $NO^{\cdot}$ suppress peroxidation, but as superoxide levels increase, cell membrane injury becomes intensified. Reduced amounts of $NO^{\cdot}$ serve to decrease endothelial cell membrane permeability, thus limiting PMN transmigration from the vascular compartment into the lung tissue. $NO^{\cdot}$ is an endogenous inhibitor of leukocyte adhesion to endothelial cells, but this phenomenon is reversed by increasing amounts of superoxide anion. The potential pathways that generate oxidants in sepsis-induced ALI are illustrated in Fig. 2.

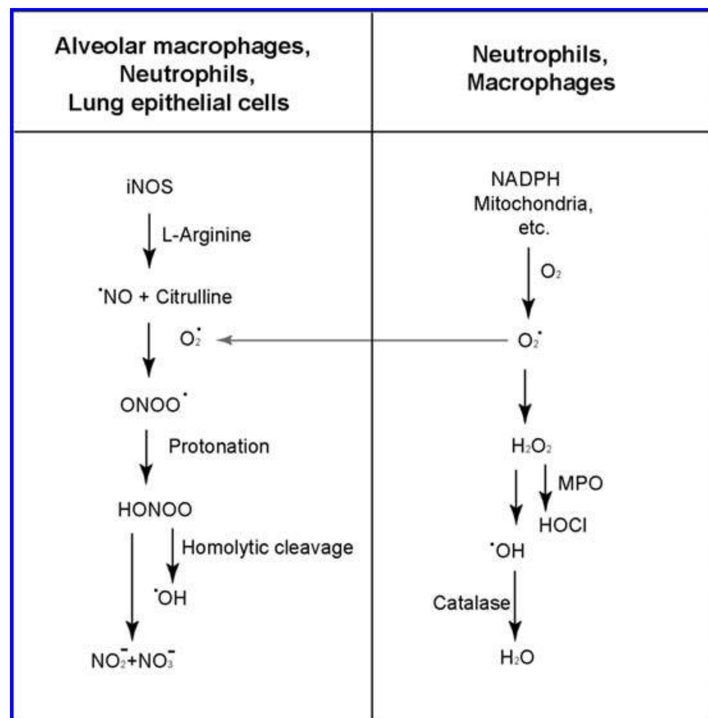

FIG. 2. Mechanisms for production of reactive oxygen species in sepsis-induced ALI.

Role of $NO^·$ in sepsis-induced ALI

Sepsis and ALI are associated with a high level of $NO^·$ production after activation of iNOS. $NO^·$ has been implicated in the pathophysiology of ALI in humans and animal models of ALI. However, NOS inhibition with nonselective inhibitors, such as N^G-monomethyl-L-arginine (L-NMMA), in animals with sepsis and ALI has resulted in worsened outcomes (65, 71), suggesting complex roles of $NO^·$ in the pathogenesis of sepsis. The results obtained from iNOS-deficient mice have been controversial. In the CLP-induced sepsis model, no pulmonary protein leak developed in iNOS-deficient mice, despite increased pulmonary MPO activity (27). By using the same model, it has been demonstrated that pulmonary oxidant stress is completely iNOS dependent and is associated with tyrosine nitration, and that pulmonary oxidant stress and nitrosative stress are dependent on the presence of iNOS in inflammatory cells (macrophages and PMNs), with no apparent contribution of iNOS in pulmonary parenchymal cells (80, 81). Therefore, the authors proposal that iNOS inhibition targeted specifically to inflammatory cells may be an effective therapeutic approach in sepsis and acute lung injury. In the bacterial lipopolysaccharide (LPS) model of ALI, lung-injury parameters such as MPO levels and albumin leak into lung were not affected by eNOS deficiency, but substantially were intensified in mice with iNOS deficiency. In LPS-indued lung injury in iNOS-deficient mice, BAL levels of CXC chemokines (MIP-2, KC) did not show any difference when compared with wild-type (WT) mice, but CC chemokines (MCP-1, MCP-3) were enhanced. Blockade of MCP-1 in iNOS-deficient mice reduced lung MPO to the levels present in WT mice. Thus, iNOS appears to play a protective role in this ALI model by limiting PMN accumulation in lung (89). Reasons for the discrepancy for iNOS-deficient effects from two ALI models are not clear. Tissue-specific KO conditions and reliable specific inhibitors for iNOS may help clarify the role of $NO^·$ in sepsis-induced ALI, thereby assisting in the design of future clinical trials. Nevertheless, the role of $NO^·$ in oxidative stress in lung is indisputable.

Role of $O_2^·$ in sepsis-induced ALI

Generation of $O_2^·$ by the NADPH oxidase complex of PMNs is crucial for host defense responses, and it is essential for killing invading microorganisms. However, $O_2^·$ may also exert harmful effect in tissues. NADPH oxidase is a multisubunit complex in which gp91^{phox} and p47^{phox} are essential for $O_2^·$ generation. $O_2^·$ can work as a "double-edged sword," so it is not surprising that the results from p47$^{phox-/-}$ and gp91$^{phox-/-}$ mice are perplexing in the setting of sepsis (31). More PMN infiltrates and higher bacterial loads were seen in lungs from p47$^{phox-/-}$ and gp91$^{phox-/-}$ mice compared with WT mice after live *Escherichia coli* challenge, whereas lung microvascular injury was prevented in these mice. Thus, PMN infiltration in lung tissue did not result in overt lung microvascular injury, when the $O_2^·$ generation was impaired. In the same study, it was found that increased bacterial load in NADPH-deficient mice was a critical factor for activating the release of chemokines, which subsequently enhanced PMN sequestration and migration into lung tissue. A selective SOD mimetic, M40401, was found to be protective in a live *E. coli* model of sepsis, but it improved survival only in severe sepsis and was less effective and even harmful with less-severe sepsis (19). Another SOD mimetic, "Tempol," has been reported to improve survival in CLP-induced septic rats, reduce the plasma levels of $NO^·$ and IL-1β, and decrease the levels of organ $O_2^·$ and tissue injury (59). It seems that $O_2^·$ is indispensable for killing bacteria, but also leads to tissue damage during sepsis.

$NO^·$ and $O_2^·$ appear to play dual roles in the pathogenesis of sepsis-induced ALI. They may function by totally different mechanisms at different stages of sepsis. Thus, in clinical trials that involve antioxidant inhibitors, a compelling need exists to monitor closely the bacterial load and oxidative status. For example, it might be reasonable to scavenge $O_2^·$ at a stage when the systemic bacterial load is partially contained during sepsis.

OXIDANT-RELATED MOLECULAR EVENTS IN SEPSIS-INDUCED ALI

NF-κB and activator protein-1 (AP-1)

NF-κB and AP-1 are two well-defined redox-sensitive transcription factors. Oxidative stress activates multiple stress kinase pathways and transcription factors (NF-κB, AP-1) by modifying cysteine residues subsequently regulating gene expression for proinflammatory cytokines as well as the protective antioxidant molecules (76). Activation of NF-κB occurs in lung macrophages and in lung tissue during sepsis (28, 45, 62). NF-κB is a heteromeric dimer composed of a complex of proteins from the RelA family. NF-κB is constitutively relegated to the cytosolic compartment. The dimer most commonly comprises p50 (NF-κB1) and p65 (RelA) subunits bound to members of the inhibitor κB (IκB) family. Activation of NF-κB occurs in response to an appropriate stimulus. After phosphorylation and ubiquitination of an IκB subunit, it is subsequently degraded by a 28S proteasome. This allows the heterodimer complex to translocate to the nucleus and bind to specific DNA promotor sequences. Access to DNA is dependent on the degree of histone coiling, which is in turn regulated by the degree of acetylation of histone core residues. As the DNA becomes more acetylated, it unwinds, thus allowing binding of transcription factors, initiating gene transcription. H_2O_2 and TNF-α have both been shown to increase histone acetylation, providing a potential mechanism for oxidant-mediated inflammation (56). This effect may be antagonized by $NO^·$, which has been shown to be capable of maintaining levels of IκB, thus hindering NF-κB activation (95). Whether this is achieved by decreasing IκB degradation or increasing its synthesis has yet to be determined. Depletion of glutathione leads to ubiquitination of NF-κB and subsequent activation (77, 78). As glutathione levels increase, IκB degradation is inhibited so that NF-κB does not become activated. NF-κB also regulates iNOS and the inducible form of cyclooxygenase (COX-2). Thus, regulation of the inflammatory response may proceed through a negative-feedback loop *via* NF-κB and nitric oxide. An endogenous protease inhibitor, secretory leukocyte protease inhibitor (SLPI), inhibits IκB degradation, suppressing NF-κB activation. PMN accumulation, ROS activity, and levels of IL-1 and IL-8 were shown to decrease in LPS-treated rabbits that in-

haled NO$^{\cdot}$ (48). These decreases correlated with a concomitant decrease in NF-κB activation. Depletion of lung macrophages in rats by airway instillation of liposome-encapsulated dichloromethylene diphosphonate suppressed activation of NF-κB and resultant BAL levels of TNF-α and MIP-2 (55). PMN accumulation and vascular permeability were also decreased, suggesting that activation of alveolar macrophages served as the source of an initial inflammatory stimulus. Activated alveolar macrophages and infiltrated PMNs generate ROS and cytokines, propagating the cycle of oxidant stress and inflammation. This scenario is likely to be the key event that drives pulmonary oxidant stress and inflammation during sepsis-induced ALI.

The promoter regions of various cytokines and chemokines contain binding sites for AP-1, suggesting that AP-1 plays a critical role in coordinating the gene expression of various inflammatory mediators. Like NF-κB, AP-1 activation occurs in lung during sepsis and likely modulates inflammation (4). AP-1 is a complex multisubunit protein composed of members of the Jun and Fos families. Much is known regarding its role in events regulating cell proliferation, transformation, differentiation, and apoptosis (49). AP-1 activation was found to be detectable in alveolar macrophages as well as in whole-lung lysates. Macrophage depletion or anti–TNF-α treatment significantly decreased the level of AP-1 activation in lung after IgG immune complex deposition, but complement depletion had no effect (36). AP-1 also responds to oxidative and cellular stress, DNA damage resulting from UV irradiation, and exposure to proinflammatory cytokines (TNF-α, IFN-γ, and TGF-β). TNF-α has been shown to activate AP-1 upstream of MAPK kinase pathways. Activation of AP-1 appears to occur concurrent with activation of other transcription factors, including Elk-1, ATF-2, and CEBP (58, 78). The AP-1 family of transcription factors has been found to play a critical role in regulating the stress-inducible protein heme oxygenase-1 (HO-1) gene after LPS treatment in rat lung (12), suggesting that AP-1 may also participate in the negative regulatory loop of the inflammatory chain.

Nuclear factor-erythroid 2-p45–related factor 2 (Nrf2)

Nrf2 is a transcription factor that is expressed in many organs, including lung. Nrf2 is directly involved in transcriptional activation of ARE-driven redox-related genes including GST, NADPH/quinine reductase, UDP-glucosyltransferases, epoxide hydrolase, heme oxygenase-1, glutathione peroxidase-2, peroxiredoxins, and glutathione reductase (GSSG-R). Therefore, it appears to be an important modulator in regulation of redox status in cells. Nrf2 has been reported as a critical intracellular molecule for regulation of the innate immune response and survival in mouse models of sepsis. Disruption of Nrf2 dramatically worsened the survival of mice in response to endotoxin and CLP-induced septic shock. Inflammation in these Nrf2-deficient mice was greatly intensified after LPS challenge. In response to LPS, Nrf2-deficient cells showed greater activation of NF-κB, which appeared to be regulated *via* the modulation of the oxidant–antioxidant system (93). In addition, these mice have been shown to be more susceptible to hyperoxic lung injury and lung inflammation induced by the oxidant butylated hydroxytoluene (BHT) (14, 15). Activation of Nrf2 by a chemical agonist, CDDO-Im, attenuated LPS-induced inflammatory responses and oxidative stress in lung, and decreased mortality in Nrf2-sufficient mice (94). Nrf2 appears to be an important transcription factor that limits progression of oxidative stress during sepsis-induced ALI. Therefore, activation or overexpression of this molecule in lung appears to be an attractive strategy for antioxidative defense.

Heme oxygenase-1 (HO-1)

Cumulative evidence has demonstrated that the stress-inducible protein, HO-1, is an auxiliary antioxidant molecule, closely involved in the regulation of lung oxidative status and inflammatory responses. LPS induces high mRNA levels of HO-1 expression in the rat lung, which correlates with increased HO-1 protein levels and enzyme activity (12, 13). Redox-sensitive transcription factor AP-1 plays a critical role in regulating HO-1 gene activation after LPS exposure. In a murine model of sepsis, mice treated with a lethal dose of LPS and subsequently exposed to the HO-1 enzymatic product, CO, had significantly improved survival and lower serum IL-6 and IL-1β levels than controls. The same effect was obtained when endogenous CO was induced through overexpression of HO-1 (68). Interestingly, AP-1 binding was decreased by CO exposure. CO, ferrous iron, and biliverdin are main enzymatic products of HO activation. With the rat model of CLP-induced sepsis, biliverdin treatment offered a potent defense against lethal endotoxemia, as well as in the lungs, and effectively abrogated the lung inflammatory response. Biliverdin administration before a lethal dose of LPS led to a significant improvement in long-term survival, reduced lung permeability and lung alveolitis, and decreased proinflammatory cytokine IL-6. In the same case, augmentation of IL-10, a potent antiinflammatory cytokine in lung injury, occurred (84). Iron (Fe) released as a result of HO-1 activation returns to a transient chelatable pool, where it may potentially promote oxidative stress and inflammation. However, this pathway can be effectively inhibited by ferritin generated through HO-1. Apparently, HO-1 works as a strong negative regulator in the development of oxidative stress and lung inflammatory responses during sepsis-induced ALI. Endogenous CO indeed increased in patients with severe sepsis (103), suggesting that the HO-1 pathway has been activated, and provides a protective role in patients with sepsis.

Other important molecular events in sepsis-induced ALI

Cellular oxidative stress regulates cell function from many perspectives, including receptor function, enzymatic activity, transcription factor activation, and gene expression. As described earlier, it has been documented that protein modifications frequently occur during sepsis. Oxidation-involved molecular events such as tyrosine phosphorylation, activation of MAP kinases, protein kinase C, phospholipase A_2, are found in the lung during sepsis and are likely contribute to the pathogenesis of sepsis (45, 53, 69, 90). Other oxidation-involved events such as protein carbonylation, tyrosine chlorination, and tyrosine nitration are also reported in sepsis (20, 66). How these events regulate the development of sepsis-induced ALI has yet to be defined.

As depicted in Fig. 3, oxidative stress in the lung activates NF-κB and AP-1 pathways during sepsis-induced ALI, which in turn leads to amplified inflammatory responses. AP-1 and Nrf2 activation result in HO-1 expression, leading to CO production in the lung during sepsis. CO together with enzymatic antioxidants generated from Nrf2 activation may play an anti-inflammatory role in sepsis-induced ALI.

ANTIOXIDANT TREATMENT IN SEPSIS-INDUCED ALI

In an earlier study in patients with sepsis-induced ALI, it was found that oral intake of an antioxidant mixture reduced lung microvascular permeability, improved oxygenation and cardiopulmonary function, and reduced proinflammatory eicosanoid synthesis and lung inflammation (29). These findings provide hope for antioxidant strategies in the treatment of sepsis.

Both enzymatic antioxidants and nonenzymatic antioxidants have been widely tested in humans and animals with sepsis. *N*-acetylcysteine (NAC), a nonenzymatic antioxidant, is one of the most extensively tested antioxidants. NAC possesses powerful antioxidative roles by directly scavenging oxygen radicals (H_2O_2, OH , HOCl) and indirectly replenishing the cellular glutathione system. In the models of sepsis-induced ALI, NAC has been shown to be highly protective in lung inflammatory responses by reducing the levels of inflammatory mediators, inhibiting PMN activation and sequestration, suppressing the prothrombotic state, and preventing hypoxic pulmonary vasoconstriction (HPV) (6, 26). However, clinical trails have resulted in controversial findings. Although NAC improves the degree of organ-failure indexes in patients with septic shock, it reduces cardiac performance and tissue oxygenation (5). It was even harmful when initiation of NAC treatment occurred $>$24 h after hospital admission (67). A later clinical trial suggested that NAC treatment aggravated sepsis-induced organ failure, in particular cardiovascular failure (87). In a recent preclinical trial study using a porcine model of endotoxemia, NAC failed to improve any of the variables of the systemic, pulmonary, or hepatosplanchnic hemodynamics, gas exchange, and metabolism, although it significantly elevated glutathione levels (96). More experimental and clinical studies with new management approaches are required to take advantage of the potential therapeutic utility of NAC.

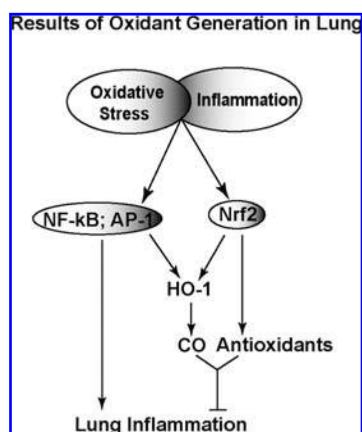

FIG. 3. Molecular events in sepsis-induced ALI. Oxidative stress and inflammation in lung activate the transcription factors, NF-κB and AP-1, leading to amplified inflammatory responses. AP-1 and Nrf2 activation result in HO-1 expression, resulting in CO production. CO, together with enzymatic antioxidants generated from Nrf2 activation, results in an antiinflammatory outcome in sepsis-induced ALI.

Tocopherol (vitamin E) is another nonenzymatic antioxidant that has potential for treatment of sepsis-induced ALI (83, 92). Tocopherol can directly scavenge ROS and upregulate the activities of antioxidant enzymes. It can terminate the chain reaction of lipid peroxidation by scavenging lipid peroxyl radicals. In the mouse model of endotoxin-induced ALI, liposomal tocopherol administration significantly decreased the number of PMNs in airspaces and reduced lung injury, as evidenced by decreased lactate dehydrogenase activity in airways and reduced lung edema. Tocopherol failed to inhibit NF-κB and AP-1 activation, as well as the endotoxin-induced expression of proinflammatory cytokines in lung tissue. In patients with ARDS, the antioxidative system is severely compromised, as evidenced by decreased plasma levels of α-tocopherol, ascorbate, β-carotene, and selenium (64). The early administration of α-tocopherol and ascorbic acid (vitamin C) in humans reduced the incidence of organ failure and shortened the ICU length of stay (70). Vitamin C is known to scavenge $O_2^{\cdot}$ by forming the semi-dehydroascorbate free radical that is subsequently reduced by GSH. Thus, oral intake of combined antioxidants appears to be beneficial in the setting of sepsis-induced ALI.

The antioxidant enzyme SOD, with a biologic function of dismutating $O_2^{\cdot}$ to H_2O_2, holds promise for the treatment of sepsis-induced ALI. Extracellular (EC)-SOD-deficient mice showed increased evidence of ALI that occurs after hemorrhagic shock, accompanied by increased lung PMN accumulation and MPO activity (8). Overexpression of pulmonary EC-SOD in the mouse lung significantly attenuated lung injury that occurs after hemorrhagic shock (9). Overexpression of EC-SOD in lung also attenuated influenza-induced lung injury by both ameliorating inflammation and attenuating oxidative stress (91). Unlike nonenzymatic antioxidants, antioxidant enzymes such SOD and CAT have high affinities and rates of reaction, which can effectively detoxify ROS in numerous cycles, representing a highly efficient mechanism in detoxification. Similar to SOD, CAT provided impressive protection against acute lung injury induced in experimental animals after administration of LPS. EUK-8, a low-molecular-weight salen-manganese complex that exhibits both SOD-like and CAT-like activities *in vitro*, significantly attenuated several features of lung dysfunction caused by endotoxin, including arterial hypoxemia, pulmonary hypertension, decreased dynamic pulmonary compliance, and pulmonary edema (34). Because of the obvious gap between human diseases and animals models, further clinical data are needed to assess the role of the SOD/CAT approach in sepsis-induced ALI.

Other antioxidants that have shown therapeutic merits in the animal models of sepsis-induced ALI include methylene blue (21), resveratrol (52), tempol (59), β-glucan (85), M4041 (19), melatonin (86), and AT1-receptor inhibitor (44). However,

these compounds have been tested only in animals. More experimental and clinical data are expected in the coming years to validate these compounds by using various animal models and eventually in humans with sepsis.

SUMMARY

Sepsis and sepsis-induced ALI represent unsolved clinical problems due to the extremely complicated pathogenesis, which involves an imbalance of the pro- and antiinflammatory networks, complement activation, endothelial cell activation, PMN and macrophage activation, oxidative stress, and transcription factor activation. Blockade of only one of the inflammatory mediators has not resulted in a satisfactory outcome in human clinical trials, perhaps because of the complexity of the inflammatory network and the redundancy of inflammatory mediators. As oxidative stress works through the initiation and progression phases in the development of sepsis, the importance of oxidant stress in sepsis-induced ALI in humans has been appreciated. The disappointing results of NAC clinical trials may reflect the inability to reestablish a redox balance in the setting of sepsis in patients. It has been realized that severity of sepsis alters the effects of $O_2^{\cdot}$ inhibition in sepsis (19). Further improvement of antioxidant interventions requires better understanding of the mechanisms and characteristics of oxidant stress in the specific setting of a disease condition and the development of more-effective delivery strategies. In addition, the redox-sensitive transcription factor, NF-κB, seems to be a logical target for therapy in ARDS patients. However, blockade of NF-κB inevitably turns off some gene expression, which may lead to immunosuppression. To this end, a local inhibition of lung NF-κB activation may be a less detrimental therapeutic strategy. Therapeutic strategies should be directed at the improvement of net proinflammatory, prooxidant, and cytotoxic imbalances that develop in ARDS.

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ABBREVIATIONS

ALI, acute lung injury; AP-1, activator protein-1; BAL, bronchoalveolar fluid; BHT, butylated hydroxytoluene; CAT, catalase; CE/MS, capillary electrophoresis–mass spectrometry; CLP, cecal ligation and puncture; COX, cyclooxygenase; DMPO, 5,5-dimethyl-1-pyrroline-*n*-oxide; DNPH, dinitrophenylhydrazine; EPR, electron paramagnetic resonance spectroscopy; Fe^{2+}, ferrous ion; GSH-PX, glutathione peroxidase; GSSG-R, glutathione reductase; HO-1, heme oxygenase-1;HPLC, high-performance liquid chromatography; HPV, hypoxic pulmonary vasoconstriction; HRP, horseradish peroxidase; H_2O_2, hydrogen peroxide; HOCl, hypochlorous acid; L-NMMA, NO_3^- nitrate; LPS, lipopolysaccharide; MIP-2, macrophage-inflammatory protein-2; MAP, mitogen-activated protein; MPO, myeloperoxidase; MDA, malondialdehyde; NAC, *N*-acetylcysteine; NO_2^-, nitrite; N^G-monomethyl-L-arginine; NOS, nitric oxide synthase; Nrf2, nuclear factor-erythroid 2-p45–related factor 2; $O_2^{\cdot}$, superoxide; OH^-, hydroxyl radical; 8-OHdG, hydroxydeoxyguanosine; $ONOO^-$, peroxynitrite; ONOOH, peroxynitrous acid; $ONOOCO_2^-$, peroxycarboxylate; RNS, reactive nitrogen species; ROS, reactive oxygen species; SOD, superoxide dismutase; SLPI, secretory leukocyte protease inhibitor; TBARS, thiobarbituric acid-reactive substances; vitamin C, ascorbic acid; vitamin E, tocopherol.

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