Postexposure aerosolized heparin reduces lung injury in chlorine-exposed mice

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Chlorine (Cl2) is a corrosive, highly irritant, and reactive gas that, when inhaled, may cause acute lung injury culminating in death from respiratory failure. In this study, we tested the hypothesis that exposure of mice to Cl2 causes intra-alveolar and systemic activation of the coagulation cascade that plays an important role in development of lung injury. C57Bl/6 mice were exposed to Cl2 (400 for 30 min or 600 ppm for 45 min) in environmental chambers and then returned to room air for 1 or 6 h. Native coagulation (NATEM) parameters such as blood clotting time and clot formation time were measured in whole blood by the viscoelastic technique. D-dimers and thrombin-anti-thrombin complexes were measured in both plasma and bronchoalveolar lavage fluid (BALF) by ELISA. Our results indicate that mice exposed to Cl2 gas had significantly increased clotting time, clot formation time, and D-dimers compared with controls. The thrombin-anti-thrombin complexes were also increased in the BALF of Cl2 exposed animals. To test whether increased coagulation contributed to the development of acute lung injury, mice exposed to Cl2 and returned to room air were treated with aerosolized heparin or vehicle for 20 min. Aerosolized heparin significantly reduced protein levels and the number of inflammatory cells in the BALF at 6 h postexposure. These findings highlight the importance of coagulation abnormalities in the development of Cl2-induced lung injury.

Research conducted within the past 5 years in animal models has increased our understanding regarding the lung pathophysiology resulting from Cl2 gas exposure and the mechanisms involved. Airway (12, 45) and distal lung (30, 46, 58) injury is caused by increased concentrations of oxygen-nitrogen and chlorine-reactive intermediates that persist even when animals are returned to room air. Influx of activated inflammatory cells (mainly neutrophils), which secrete myeloperoxidase and reactive intermediates, exacerbate lung injury. Moreover, we reported that in rats exposed to Cl2 gas there was systemic endothelial dysfunction due to the inhibition of endothelial nitric oxide synthase-dependent signaling, revealing the potential of Cl2 gas exposure to compromise systemic vascular function (21, 40).

Previous studies have documented that inflammatory mediators activate the coagulation cascade, which plays an important role in the pathogenesis of ALI/ARDS (17, 23, 24). This association has been investigated in animal models with a number of different causes of lung injury, such as endotoxin, hyperoxia, trauma, hemorrhage, or pneumonia (2, 7, 8, 14, 44, 54), but not in Cl2-induced lung injury. Moreover, pulmonary inflammation can cause local disturbances in fibrin turnover whereas increased intra-alveolar fibrin deposition with decreased breakdown may induce inflammation (44). Given this tight cross talk between coagulation and inflammation, the administration of an agent (such as heparin) that has both anti-coagulant as well as anti-inflammatory properties (53) may reduce the disturbance in pulmonary coagulopathy and inflammation, thereby ameliorating the clinical course of ALI. Aerosolized heparin has been shown to be effective in patients with ALI, making this agent a candidate for clinical use in patients with Cl2-induced lung injury (11, 19, 20, 49).

Herein we performed a rigorous study testing the hypothesis that exposure of mice to Cl2 causes both intra-alveolar and systemic coagulation abnormalities and that aerosolized heparin reduces Cl2-induced injury to the blood-gas barrier of mice.

MATERIALS AND METHODS

Animals. Pathogen-free C57Bl/6 male mice, weighing 20–25 g, were purchased from Charles River Laboratories (Wilmington, MA). They were housed for at least 4 days in groups of three in regular mouse cages (Maxi-Miser no. 9, floor area of 435.7 cm²; Thoren Caging), under a 12:12-h light-dark cycle, in a temperature-controlled environment, in the animal facilities of the University of Alabama at Birmingham. Access to food and water was ad libitum. All procedures involving animals were approved by the University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC).

Exposure of mice to Cl2. Mice (2 at a time) were exposed to Cl2 (400 ppm for 30 min or 600 ppm for 45 min) in custom-made glass environmental chambers (Specialty Glass) as described previously.
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In vivo experimental protocols. C57Bl/6 mice that were exposed to 400 ppm for 30 min and were euthanized at 1 (n = 9), 3 (n = 6), or 6 h (n = 6) post-Cl2 exposure, and their lungs were lavaged with 2 ml of normal saline as described previously (13, 16, 45). Unexposed mice served as air controls (n = 6). In another set of experiments, mice that were exposed to 600 ppm for 45 min were euthanized immediately (0 h; n = 12) or 1 h after exposure (n = 6). Approximately 500 μl of blood were drawn from the inferior vena cava and placed in tubes containing citrate for coagulation studies (ratio 1:9).

BALF cell count and protein determination. The bronchoalveolar lavage (BAL) fluid (BALF) was centrifuged to pellet cells and debris and the cell-free BALF was collected. For assessment of inflammatory cells, live and dead cells collected from BALF were counted by Trypan blue staining. Cell differential counts were determined from 300 live cells per cytopsin slide, which were prepared using a cytopsin centrifuge (Shandon, Pittsburgh, PA) and stained with Wright protocol (Kalamazoo, MI). Protein concentrations in the cell-free BALF were measured by the BCA protein assay (Pierce Endogen) as previously described (42, 43). A standard curve was prepared by assaying known concentrations of BSA in 0.9% NaCl (10).

RESULTS

Cl2 gas exposure induces intra-alveolar hypercoagulation. Direct insults to the lung epithelium, such as pneumonia or inhalation injury, are known to increase intra-alveolar hypercoagulation (17) measured by TAT formation, resulting from increased thrombin production and release. As seen in Fig. 1A, TAT complexes in the BALF of mice exposed to Cl2 (400 ppm for 30 min) and returned to room air for 1 h were significantly higher than those of air breathing mice. TAT levels in the BAL remained elevated in four mice at 6 h postexposure (Fig. 1B). Exposure of mice to 600 ppm of Cl2 for 45 min increased TAT at 1 h postexposure to levels significantly higher than exposure to 400 ppm for 30 min (Fig. 1A). Interestingly, no TAT formation was observed immediately postexposure to either level of Cl2 (data not shown).

Cl2 gas exposure induces systemic coagulopathy. In addition to intra-alveolar coagulation, pulmonary insults are known to result in systemic coagulation abnormalities (8). To determine whether exposed to Cl2 also resulted in systemic coagulopathy, we measured coagulation parameters (clotting time and clot formation time) in whole blood using a viscoelastic technique. Clotting time is the latency time from adding the start reagent to blood until the clot starts to form. Clot formation time is the time from CT until a clot firmness of 20 mm has been reached and denotes the speed at which a solid clot forms. Representative records of clotting and clot formation times for control (air breathing) mice and mice exposed to sublethal Cl2 regimens (400 ppm for 30 min) are shown (Fig. 2, A and B). Mice exposed to 400 ppm of Cl2 and euthanized 1 h postexposure had significantly higher clotting times compared with controls (Fig. 2C). However, CT was not different from controls at 3 and 6 h postexposure. Blood clot formation times (Fig. 2D) in Cl2-exposed mice were not significantly different compared
with unexposed controls at any time postexposure. To determine whether the dose of Cl$_2$ would affect systemic coagulopathy, we measured clotting and clot formation times immediately after 600 ppm of Cl$_2$ exposure and 1 h postexposure (Fig. 2, E and F). The clotting time was significantly increased 1 h postexposure, but not immediately after exposure to Cl$_2$. However, clot formation time was significantly increased after both exposure and 1 h postexposure.

**Cl$_2$ gas exposure increases fibrinolysis.** To this point, our results are consistent with the simultaneous presence of intra-alveolar hypercoagulation and systemic hypocoagulation. To determine whether systemic fibrinolysis was present as a component of systemic coagulopathy, we measured plasma levels of D-dimers in mice after exposure to Cl$_2$ (Fig. 3A). Mice exposed to 400 ppm of Cl$_2$ and euthanized 1 h postexposure had significantly higher levels of D-dimer compared with...
controls. In contrast, mice exposed to 600 ppm of Cl₂ did not have increased levels of D-dimers. To determine whether coagulation had been activated via thrombin, TAT complexes were measured in the plasma after Cl₂ exposure (Fig. 3B). Although no increase in TAT complexes could be measured immediately after exposure to 400 ppm of Cl₂, a significant increase was seen at 6 h postexposure. Furthermore, exposure to 600 ppm of Cl₂ led to significantly increased TAT formation.

Aerosolized heparin mitigates alveolar-capillary barrier perturbation and lung inflammation after Cl₂ exposure. Aerosolized heparin has been shown to be a safe and effective regimen for the mitigation of several types of ALI (11, 19, 20, 49). On the basis of our previous findings demonstrating intra-alveolar hypercoagulation, we administered aerosolized heparin within 20 min post-Cl₂ exposure and measured the number of inflammatory cells and concentration of plasma protein in BALF at 6 h postexposure. On the basis of the mean geometric diameter of our aerosolized particles (2.2 μm), and existing information in the literature (41), we calculated that 40 and 5% (25 units of heparin) of the inhaled dose were deposited in the upper airways and distal lung spaces, respectively. The alveolar-capillary barrier perturbation and the lung inflammation in the 400 ppm Cl₂ gas model has been shown to start as early as 30 min postexposure and be sustained for at least 6 days (13, 16, 45, 46, 58). Therefore, we opted to assess the levels of BALF protein and leukocyte cell content and differentials at 1 and 6 h postexposure. Whereas aerosolized heparin did not decrease markers of alveolar injury or lung inflammation at 1 h postexposure (data not shown), the results were dramatically different at 6 h postexposure. Aerosolized heparin significantly decreased BALF total protein and total leukocyte counts after exposure to Cl₂ (Fig. 4, A and B) compared with those receiving aerosolized saline. In contrast, aerosolized heparin had no effect on BALF protein of air breathing mice [air + vehicle = 31 ± 2 (n = 8 mice); air + heparin = 34.5 ± 2.5 (n = 8 mice); means ± SE]. Furthermore, the percentage of neutrophils was significantly decreased (Fig. 4C) while the percentage of monocytes was significantly increased (Fig. 4D) after aerosolized heparin administration. No changes occurred in the percentage of lymphocyte populations (data not shown). These findings indicate that administration of aerosolized heparin 6 h post-Cl₂ exposure mitigates lung inflammation and neutrophil recruitment and restores the integrity of the alveolar-capillary barrier.

Aerosolized heparin administration does not worsen systemic hypocoagulation of Cl₂ gas-exposed mice. Our previous data demonstrate that administration of aerosolized heparin after exposure to Cl₂ mitigates acute lung injury. To determine whether heparin had an effect on systemic coagulation, we measured coagulation parameters using a viscoelastic technique. Neither clotting time nor clot formation time was measured coagulation parameters using a viscoelastic technique. Neither clotting time nor clot formation time was measured coagulation parameters using a viscoelastic technique. Neither clotting time nor clot formation time was compared with saline vehicle. These findings indicate that administration of aerosolized heparin in mice does not worsen systemic hypocoagulation induced by Cl₂ gas exposure.

DISCUSSION

Cl₂ gas exposure is a documented public health threat mainly because of accidents involved in its trafficking and deliberate release involved in warfare; therefore increased preparedness is required to avoid mass casualty situations (26, 51, 55, 56). Apart from the fact that significant morbidity and mortality are entailed in cases of acute exposure, there are also long-term residual clinical symptoms, such as reactive airway disease syndrome, reduced residual volume, and reduced forced vital capacity (4, 12, 16, 45).

In this study we used an established animal model of Cl₂ gas exposure involving whole body exposure of mice, thus mimicking real-life scenarios (6). Exposures during the accidental release of Cl₂ have been modeled based on the Graniteville accident and have shown that Cl₂ levels during a 30-min exposure period were 6,868, 837, and 89 ppm at 0.2, 0.5, and 1 km downwind from the epicenter of the accident (5). Previous studies from our group and those of others have shown that mice exposed to 400 ppm Cl₂ for 30 min and returned to room air develop acute hypoxia, lung inflammation accompanied by perturbation of the integrity of the alveolar epithelial barrier, and significant lung oxidative stress (30, 33, 48, 57, 58). Moreover, there were also alterations in respiratory mechanics (34, 45), compromised immune response to fungi (16), and impaired locomotion (13). Additionally, using this model we were the first to demonstrate that Cl₂ gas exposure exerts extrapulmonary manifestations in rats involving inflammation and endothelial dysfunction due in part to the inactivation of endothelial nitric oxide synthase an event linked to atherosclerosis and hypertension (21).

Herein we expand this knowledge by reporting that mice exposed to 400 ppm of Cl₂ gas for 30 min and returned to room air were euthanized at 1 and 6 h post-Cl₂ exposure. Aerosolized heparin significantly decreased BALF protein of air breathing mice [air + vehicle = 31 ± 2 (n = 8 mice); air + heparin = 34.5 ± 2.5 (n = 8 mice); means ± SE]. Furthermore, the percentage of neutrophils was significantly decreased (Fig. 4C) while the percentage of monocytes was significantly increased (Fig. 4D) after aerosolized heparin administration. No changes occurred in the percentage of lymphocyte populations (data not shown). These findings indicate that administration of aerosolized heparin 6 h post-Cl₂ exposure mitigates lung inflammation and neutrophil recruitment and restores the integrity of the alveolar-capillary barrier.
Specifically, we show that mice exposed to Cl2 and returned to room air also develop acute systemic hypocoagulation and pulmonary hypercoagulation at the same time, similar to that reported in patients with severe trauma (8) and smoke inhalation (17). Specifically, we show that mice exposed to Cl2 and returned to room air also develop acute systemic hypocoagulation and pulmonary hypercoagulation at the same time, similar to that reported in patients with severe trauma (8) and smoke inhalation (17).

We observed significantly longer clotting and clot formation times at 1 h postexposure compared with those exposed to 400 ppm for 30 min (Fig. 2). It is unclear why D-dimer formation was not observed in the plasma of mice following exposure to 600 ppm for 45 min. It is possible that D-dimers will have been formed and disappeared rapidly prior to sampling. Alternatively, sufficient levels of D-dimers may not have been formed until later times. A large number of additional experiments are needed to establish the time course of D-dimer formation and disappearance.

The mechanisms by which Cl2 gas activates the coagulation system are not clear. When inhaled, Cl2 hydrolyzes to form hypochlorous (HOCl) acid and its conjugate base (OCl-) as well as hydrochloric acid. HOCl and OCl- react with proteins, plasmalogen lipids, as well as components of the lung extracellular matrix (46, 47). Products of these reactions, such as chloramines, chlorinated lipids, low molecular weight hyaluronan, and other danger signals, have considerable pulmonary and systemic toxicity on their own even after the cessation of Cl2 inhalation. Injured lung epithelial, endothelial, and inflammatory cells may release tissue factor and procoagulant microparticles, which have been shown to activate the coagulation cascade (2, 52). Furthermore, there is a large body of experimental evidence indicating that thrombin increases lung endothelial permeability via a protease-activated receptor-dependent mechanism (28) and alveolar epithelial permeability via an αvβ6 integrin- and TGF-β-dependent mechanism (25) and compromises vectorial sodium transport via activation of PKCζ (50). In addition, it may act synergistically with reactive intermediates that are known to be generated both during and
after exposure to Cl2 both in vitro and in vivo (30, 57), to activate the small GTPase RhoA and suppress Rac1, which also contribute to increased alveolar permeability and pulmonary edema (3, 15). Heparin may abate these processes by preventing the continued formation of thrombin or by acting as an anti-inflammatory, which prevents epithelial damage and exposure of pulmonary thrombin to the systemic coagulation system. Recent studies reported enhanced activation of the coagulation in the lungs of mice that developed ALI due to sulfur mustard inhalation (39) and smoke inhalation (17), which may account for airway obstruction by fibrin clots. However, these authors did not report the occurrence of systemic hypocoagulation in mice exposed to nitrogen mustard.

Cl2 exposure can result from accidental exposure or terrorism acts that are associated with severe trauma in addition to the lung injury caused by Cl2 exposure. It has been previously shown that around 25% of severe trauma and hemorrhagic shock patients suffer from systemic hypocoagulation upon hospital admission (9). This early posttraumatic hypocoagulation is associated with poor outcome and is the first cause of mortality for trauma patients during the first 48 h after admission to the hospital (27). Thus the association of Cl2 exposure and other traumatic injuries may cause the development of a severe systemic hypocoagulation in a much greater percentage of trauma patients and thus be associated with a significant increase in mortality.

A very important finding of our study is that aerosolized heparin administered immediately after Cl2 inhalation injury can lead to decreased BALF protein content and lung leucocyte infiltration and thus restoration of the alveolar-capillary barrier function as well as reduced lung inflammation and reduction of neutrophils in the distal air spaces in a rapid fashion (within hours postinstillation). These findings clearly show that heparin in aerosolized form is a potential drug candidate for Cl2-induced ALI. An important fact is that the systemic coagulopathy is not aggravated, rendering safety to this regimen. The anti-inflammatory effects of aerosolized heparin have already been shown in a mouse model of Legionella pneumonia (1); also, aerosolized heparin did not alter systemic coagulation in a sheep model of ALI burn and smoke inhalation (49).

Our data show that aerosolized heparin did not alter TAT formation in the BAL. Thus our findings are consistent with the notion that, at the very small doses used in this study, heparin was acting mainly as an anti-inflammatory agent. A systematic review using aerosolized heparin for the treatment of smoke inhalation has demonstrated decreased mortality without systemic coagulopathy (35); another trial demonstrated fewer days on the mechanical ventilator (11). In addition to its anticoagulant properties, heparin prevents neutrophil adhesion to endothelial cells, because of its similarity to ligands of P-selectins (37). In addition, binding of chemokines to heparins decreases their ability to activate their receptors, thus decreasing their proinflammatory effects (29). Finally, heparin downregulates IL-1b and inhibits syndecan-1 shedding in the intestinal mucosa in an experimental model of colitis in mice (53). Interestingly, early heparin treatment or late urokinase treatment prevents lung fibrosis in response to bleomycin (18). Airway fibrosis as well as bronchiolitis obliterans have been reported in mice exposed to Cl2 and returned to room air for 7–10 days (36, 38).

In conclusion, early effects of Cl2 gas exposure on the activation of the coagulation cascade in both intrapulmonary and extrapulmonary sites contribute to the formation of ALI. C57Bl/6 mice, postexposure to high concentrations of Cl2, exhibited decreased blood clotting time and thus systemic hypocoagulation with concomitant increased fibrinolysis. At the same time, in the distal air spaces the mice developed hypercoagulation activity that contributes to inflammation. Aerosolized heparin does not affect systemic coagulation while reducing lung inflammation and neutrophil infiltration, rendering it a potential therapeutic agent for of Cl2-induced ALI.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s). Dr. Matalon is the principal investigator of a grant from GlaxoSmithKline entitled “PostExposure Mitigation and Repair of Lung and Systemic Cl2 Induced Injury by Novel TRPV4 Inhibitors.” There is no overlap among experiments conducted in this study and those conducted for the above-mentioned grant. Dr. Matalon has not received honoraria or travel funds from this application.

Dr. Matalon is the Editor-in-Chief of the American Journal of Physiology for which he receives a stipend.

AUTHOR CONTRIBUTIONS


REFERENCES

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