Postexposure aerosolized heparin reduces lung injury in chlorine-exposed mice

Sotirios G. Zarogiannis,1,2* Brant M. Wagener,1* Susanna Basappa,1 Stephen Doran,1 Cilina A. Rodriguez,1 Asta Jurkuvenaite,1,2 Jean Francois Pittet,1,2* and Sadis Matalon1,2*

1Department of Anesthesiology, School of Medicine, University of Alabama at Birmingham, Birmingham, Alabama; and 2Center for Pulmonary Injury and Repair, School of Medicine, University of Alabama at Birmingham, Birmingham, Alabama

Submitted 9 June 2014; accepted in final form 15 July 2014

Zarogiannis SG, Wagener BM, Basappa S, Doran S, Rodriguez CA, Jurkuvenaite A, Pittet JF, Matalon S. Postexposure aerosolized heparin reduces lung injury in chlorine-exposed mice. Am J Physiol Lung Cell Mol Physiol 307: L347–L354, 2014. First published July 18, 2014; doi:10.1152/ajplung.00152.2014.—Chlorine (Cl2) is a highly reactive oxidant 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.

CHLORINE (Cl2) is a corrosive, highly irritant, and reactive gas produced in large quantities globally because of its multimodal industrial and domestic applications (40, 47, 57). The magnitude of the deleterious effects of Cl2 depends on the inhaled concentration, the duration of exposure, and the genetic background of exposed individuals (13, 16, 22, 31, 32, 36). The onset of respiratory symptoms ranges from minutes to hours, and exposures likely to be encountered near industrial accidents may result in acute lung injury (ALI) and occasionally death from acute respiratory distress syndrome (ARDS) (33, 51, 55, 58). Postexposure complications include increased susceptibility to fungal infections (16), reactive airway disease syndrome (12, 21), subepithelial fibrosis (48), and partial occlusion of main airways by thrombus formation (38).

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...
At the end of exposures, they returned to their cages where they breathed room air.

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).

Aerosol administration of heparin to Cl2-exposed mice. Within 5 min post-Cl2 exposure (400 ppm for 30 min), mice breathed aerosolized heparin or vehicle generated by an AirLife Brand Misty Max 10 disposable nebulizer for 20 min. Each mouse was placed inside a conical tube, open on both ends, that prevent it from turning around. At a flow rate of 5 l/min, this nebulizer delivers aerosols with mass median aerodynamic diameter of 2.2 μM with a geometric standard deviation of 2 μM at a flow rate of 5 l/min. Heparin (1,000 Unit/ml) or saline was placed in the nebulizer. The inhaled dose (ID) was calculated from the following equation (online supplement of Ref. 58): ID = Cl2 × Ve × T, where Cl2 = aerosol concentration (mg/l), Ve = minute ventilation, and T = aerosol delivery time (min). Mice that received either heparin or vehicle aerosols were euthanized at 6 h post-Cl2 exposure, and BAL was performed (Cl2 + heparin, n = 8; Cl2 + vehicle, n = 14) for measurements of number of inflammatory cell and protein in the BALF as discussed above. Another group of air-exposed mice receiving vehicle served as a control group (n = 8).

Statistical analysis. All values are presented as means ± SE for the indicated number of mice. Normality of the data was tested with the Kolmogorov-Smirnov normality test and statistical significance was performed by unpaired t-test or Mann-Whitney test for parametric and nonparametric data, respectively. In cases of parametric data with unequal variances, the unpaired t-test with Welch’s correction was used. Multiple comparisons of means were performed by one-way ANOVA with Newman-Keuls posttest. Differences were deemed significant with a P < 0.05.

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 regimes (400 ppm for 30 min) are shown (Fig. 2A 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 Cl₂ would affect systemic coagulopathy, we measured clotting and clot formation times immediately after 600 ppm of Cl₂ 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 Cl₂. However, clot formation time was significantly increased after both exposure and 1 h postexposure.

Cl₂ 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 Cl₂ (Fig. 3). Mice exposed to 400 ppm of Cl₂ and euthanized 1 h postexposure had significantly higher levels of D-dimer compared with

Fig. 2. Cl₂ gas exposure increases systemic coagulopathy. Mice were exposed to Cl₂ (400 ppm for 30 min or 600 ppm for 45 min) and returned to room air. A group of mice exposed to air served as control. Mice were euthanized at 0, 1, 3, or 6 h post-Cl₂ exposure. A and B: representative images of the ROTEM output for air (A) and Cl₂ (400 ppm for 30 min; 1 h postexposure) breathing mice, respectively. Clotting time (CT), which is the time from the start of the measurement until initiation of clotting, and clot formation time (CFT), the time from clotting initiation until a clot firmness of 20 mm is detected, are shown. C and D: clotting time and clot formation time in whole blood were measured with rotation thromboelastometry in mice exposed to 400 ppm of Cl₂. E and F: clotting time and clot formation time in the whole blood were measured with rotation thromboelastometry in mice exposed to 600 ppm of Cl₂ and returned to room air. Values are means ± SE; *P < 0.05 compared with controls, **P < 0.05 between treatments. In all cases n = 4 mice.
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 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 affected by heparin administration after Cl2 exposure (Fig. 4, E and F) 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...
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). 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).

The mechanisms by which Cl₂ gas activates the coagulation system are not clear. When inhaled, Cl₂ hydrolyzes to form hypochlorous (HOCI) acid and its conjugate base (OCl⁻) as well as hydrochloric acid. HOCI 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 α₅β₆ 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 immediately after exposure, they received a 20-min aerosol of either heparin or saline. Total protein (A), total leucocytes (B), percentage of neutrophils (C), and percentage of monocytes (D) in BAL fluid are shown. Clotting time (E) and clot formation time (F) in whole blood was measured with rotation thromboelastometry. Values are means ± SE; *P < 0.05 compared with controls, **P < 0.05 between treatments. N values as follow: A–D, air: n = 8; 400 ppm Cl₂ + vehicle: n = 14; 400 ppm Cl₂ + heparin: n = 8. E and F: 400 ppm Cl₂ + vehicle. n = 6; 400 ppm Cl₂ + heparin: n = 5.

Aerosolized heparin administration mitigates lung injury. Mice were exposed to 400 ppm of Cl₂ for 30 min and returned to room air. Immediately after exposure, they received a 20-min aerosol of either heparin or saline. Total protein (A), total leucocytes (B), percentage of neutrophils (C), and percentage of monocytes (D) in BAL fluid are shown. Clotting time (E) and clot formation time (F) in whole blood was measured with rotation thromboelastometry. Values are means ± SE; *P < 0.05 compared with controls, **P < 0.05 between treatments. N values as follow: A–D, air: n = 8; 400 ppm Cl₂ + vehicle: n = 14; 400 ppm Cl₂ + heparin: n = 8. E and F: 400 ppm Cl₂ + vehicle. n = 6; 400 ppm Cl₂ + heparin: n = 5.

Aerosolized heparin administration mitigates lung injury. Mice were exposed to 400 ppm of Cl₂ for 30 min and returned to room air. Immediately after exposure, they received a 20-min aerosol of either heparin or saline. Total protein (A), total leucocytes (B), percentage of neutrophils (C), and percentage of monocytes (D) in BAL fluid are shown. Clotting time (E) and clot formation time (F) in whole blood was measured with rotation thromboelastometry. Values are means ± SE; *P < 0.05 compared with controls, **P < 0.05 between treatments. N values as follow: A–D, air: n = 8; 400 ppm Cl₂ + vehicle: n = 14; 400 ppm Cl₂ + heparin: n = 8. E and F: 400 ppm Cl₂ + vehicle. n = 6; 400 ppm Cl₂ + heparin: n = 5.
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 did not alter systemic coagulation in a sheep model of ALI burn and smoke inhalation (49).

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.

REFERENCES
AEROSOLIZED HEPARIN IN CHLORINE GAS INHALATION INJURY

L353


