Antifibrinolytic Mechanisms in Acute Airway Injury after Sulfur Mustard Analog Inhalation

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Abstract

Acute lung injury in response to mustard gas (sulfur mustard [SM]) inhalation results in formation of fibrin casts, which obstruct the airway. The objective of this study was to identify fibrinolytic pathways that could be contributing to the persistence of airway casts after SM exposure. Rats were exposed to the SM analog, 2-chloroethyl ethyl sulfide, via nose-only aerosol inhalation. At 4 and 18 hours after exposure, animals were killed and airway–capillary leak estimated by measuring bronchoalveolar lavage fluid (BALF) protein and IgM content. The fibrin clot–degrading and plasminogen-activating capabilities of BALF were also assessed by activity assays, whereas Western blotting was used to determine the presence and activities of plasminogen activator inhibitor-1, thrombin activatable fibrinolytic inhibitor and α 2-antiplasmin. Measurement of tissue-specific steady-state mRNA levels was also conducted for each fibrinolytic inhibitor to assess whether its synthesis occurs in lung or at extrapulmonary sites. The results of this study demonstrate that fibrin-degrading and plasminogen-activating capabilities of the airways become impaired during the onset of 2-chloroethyl ethyl

sulfide–induced vascular leak. Findings of functionally active reservoirs of plasminogen activator inhibitor-1, thrombin activatable fibrinolysis inhibitor, and α 2-antiplasmin in BALF indicate that airway fibrinolysis is inhibited at multiple levels in response to SM.

Keywords: sulfur mustard; lung injury; plasminogen activator inhibitor-1; α 2-antiplasmin; thrombin activatable fibrinolysis inhibitor

Clinical Relevance

Our study identifies several antifibrinolytic pathways that are contributing to the persistence of airway obstruction after sulfur mustard inhalation, and to the eventual outcome of bronchiolitis obliterans observed in survivors of such attacks. Similar mechanisms may be contributing to disease states involving airway fibrin casts, including congenital heart disease and palliative surgeries, such as the Fontan procedure.

Sulfur mustard (SM; bis(2-chloroethyl) sulfide) is a vesicant and chemical weapon that has been employed in several 20th century conflicts. It remains a threat because of its ease of synthesis, the existence of large stockpiles, and improper disposal (1). Acutely, SM inhalation results in an asymptomatic period of 4–12 hours, followed by pain and cough with progressive rhinosinusitis and tracheobronchitis that can be followed by hemorrhagic pulmonary edema and respiratory failure within 20–48 hours, depending on the dose inhaled. Studies

conducted in the aftermath of World War I and the Iran/Iraq conflicts have documented the presence of necrotic debris obstructing the large airways in SM inhalation casualties (2, 3). On a biochemical level, SM's vesicant properties are due to an intermolecular chemical reaction, resulting in formation of a highly reactive electrophile, which is capable of alkylating DNA, protein, and lipid membranes (4).

Excessive procoagulant activity in airways can exacerbate the pathophysiology

resulting from acute and chronic lung diseases (5–7). Previously we have found inhalation of SMs to cause formation of fibrin casts in conducting airways of rats, resulting in obstruction, impaired gas exchange, and death (8). Factors driving this process include in situ activation of tissue factor/FVII complex, and leakage of components of the coagulation cascade into the airway after injury to the bronchial circulation. Early treatment with anticoagulants, such as tissue factor pathway inhibitor, can limit cast formation and airway obstruction (9).

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These findings have demonstrated that procoagulant activities are enhanced in the conducting airways after SM inhalation.

More recently, our laboratory has demonstrated that fibrinolytic drugs can reverse the accumulation of obstructive casts, improve tissue oxygen delivery, and prevent death after SM inhalation, even when treatment is delayed 5–6 hours after the initial insult. The persistence of airway casts 24–48 hours after SM inhalation suggested that normal airway fibrinolytic pathways could also be compromised. Herein, we demonstrate that three major antifibrinolytic proteins are elevated in response to SM inhalation. Thus, SM-dependent airway obstruction may involve antifibrinolytic mechanisms in addition to activation of procoagulant clotting pathways.

Materials and Methods

Expanded methodology can also be found in the online supplement.

Male Sprague-Dawley rats were exposed to 2-chloroethyl ethyl sulfide (CEES) for 15 minutes using a Jaeger nose-only inhalation system (CH Technologies, Westwood, NJ). Control rats were exposed identically to diluent (100% ethanol). After mice were killed, lungs were lavaged with PBS. Bronchoalveolar lavage fluid (BALF) cells were pelleted by centrifugation and the supernatant aliquoted and stored at -80°C .

IgM and Total Protein Measurements in BALF

IgM was measured in BALF using standard ELISA (Bethyl Laboratories, Inc., Montgomery, TX). Total protein was measured using bicinchoninic acid protein assay (Pierce, Rockville, IL).

Plasminogen Activator Activity Assay

BALF (8 μl) was incubated with reaction buffer containing rat plasminogen and D-Ala-Leu-Lys-7 amido-4-methylcoumarin. A total of 10 μl of this reaction was transferred to 90 μl of reaction buffer on a 96-well plate and fluorescence was measured. For inhibitory reactions, rat urokinase-type plasminogen activator (uPA), previously incubated for 15 minutes with leupetin or plasminogen activator inhibitor (PAI)-1, was substituted for BALF.

Fibrin Clot Assay

BALF (50 μl) was tested for fibrinolytic activity by addition into 96-well plates

containing fibrinogen (3 mg/ml), CaCl_2 (3 mM), and plasminogen (1 $\mu\text{g/ml}$) in 150 μl of PBS. Clotting was initiated by adding rat thrombin (2 ng/ml) and absorbance ($\lambda 405$) recorded for 4 hours. Fibrinolytic activity was determined by calculating mean absorbance decline between 120 and 180 minutes.

Western Blot Analysis

BALF was added to $5\times$ Laemmli buffer (1:4) and (20 μl) loaded onto SDS-polyacrylamide gels. After transfer, membranes were incubated overnight with antibodies recognizing $\alpha 2$ -antiplasmin ($\alpha 2\text{AP}$; ab62771; Abcam, Cambridge, MA), thrombin-activatable fibrinolytic inhibitor (TAFI; ab6133679; Abcam), PAI-1 (ab637205; Abcam), prothrombin (ab83981; Abcam), or fibrinogen (ab92572; Abcam). Blots were washed, followed by incubation with a goat anti-rabbit secondary antibody (1:7,000; Southern Biotechnology, Birmingham, AL). Experiments were performed under nonreducing conditions except for TAFI.

TAFI, PAI-1, and Thrombin–Antithrombin Complex Measurements in BALF

TAFI in BALF was measured by Pefakit TAFI assay (Pentapharm, Basel, Switzerland) according to the manufacturer's instructions, except that 30 μl of BALF was added in lieu of 10 μl of plasma, and volumes of activator and incubation reagents were reduced from 100 to 90 μl . To inhibit TAFI, 100 μl of BALF was preincubated with 1 μl of 100 μM

carboxypeptidase inhibitor from potato tuber. Carboxypeptidase B (Worthington Biochemical, Lakewood, NJ) was used as a standard curve preparation. For PAI-1 measurement, 99 μl of BALF from rats exposed to CEES (18 h) was preincubated with 1 μl of BSA (100 ng) or rat uPA (1–100 ng) before performing PAI-1 active assay (Molecular Innovations, Novi, MI). Thrombin–antithrombin (TAT) complex was measured in BALF using an Enygnost TAT microassay (Siemens, Tarrytown, NY).

Measurement of Steady-State mRNA Levels

Total RNA was isolated by RNeasy mini system (Qiagen; Valencia, CA) from tissue derived from the left lobe of perfused lung, liver, kidney, or the entire cell pellet isolated from BALF. Next, cDNA was synthesized using Taqman reverse transcriptase kit (Applied Biosystems; Foster City, CA) followed by real-time PCR.

Statistical Analysis

Prism 4 software (GraphPad Software, La Jolla, CA) was used for statistical analyses. Means were compared by one-way ANOVA followed by Tukey's test. *P* values less than 0.05 were considered significant.

Results

Effect of Half-Mustard (CEES) Inhalation on Lung Permeability

Measurements of protein and IgM levels in BALF were performed to assess the

Figure 1. Progression of airway–capillary leak in response to 2-chloroethyl ethyl sulfide (CEES)-induced airway injury. Permeability was assessed by measurement of total protein (A) and IgM (B) concentrations of rat bronchoalveolar lavage fluid (BALF) after vehicle (ethanol, 18 h) or 7.5% CEES (4 and 18 h) inhalation. Appearance of increasing levels of BALF protein and IgM are consistent with the time-dependent leak in response to CEES. Data are mean ($\pm$ SD). Group comparisons ($n = 6$ for ethanol and 18-h CEES; $n = 3$ for 4-h CEES) were analyzed by one-way ANOVA with Dunnett's *post hoc* test and revealed significant differences (*) between ethanol and 18-hour CEES-exposed groups. EtOH, ethanol.

progression of airway–capillary leak in response to CEES injury (Figures 1A and 1B). The total protein concentration of BALF obtained at 18 hours after exposure of rats to ethanol control, the diluent for CEES, was $0.066 (\pm 0.012)$ mg/ml. In contrast, mean BALF protein concentrations from CEES-exposed rats demonstrated a time-dependent increase in protein content after exposure at 4 hours (0.227 ± 0.071) and 18 hours (1.065 ± 0.67). To determine if airway permeability extended to very large proteins, BALF was also analyzed for IgM (900,000 mol wt) concentration (see Figure 1B). BALF from ethanol-exposed rats had a mean IgM concentration of $19.51 (\pm 2.80)$ ng/ml. BALF IgM concentrations were elevated at 4 hours (49.38 ± 20.28 ng/ml) and

18 hours (331 ± 188.3 ng/ml) after CEES exposure. These findings were consistent with the onset of vascular–epithelial leak due to increased permeability in response to mustard inhalation.

Effect of CEES on Airway Patency and Associated Fibrinogen, Prothrombin, and TAT Complex Levels in BALF

Microscopic inspection of large airway lumens in 18-hour CEES-exposed rats revealed the formation of airway-occlusive casts, which were not evident in those receiving only inhaled ethanol diluent (Figure 2A). Consistent with the appearance of luminal airway cast material, Western blot analysis using rat BALF after CEES exposure demonstrated the time-dependent appearance of fibrinogen in

airways (Figure 2B). Elevations in the levels of prothrombin and TAT complexes (Figures 2B and 2C) provide further evidence that the key proteolytic steps necessary for fibrin-dependent clot formation are activated in response to CEES inhalation.

Effect of CEES Exposure on Airways Plasminogen Activator Activity

Conversion of plasminogen to the active fibrin-degrading protease, plasmin, is the central step in the fibrinolytic process. D-Ala-Leu-Lys 7-amido-4-trifluoromethylcoumarin (D-ALL) has previously been shown to be an effective substrate for plasmin *in vitro* (10). Hydrolysis of D-ALL by plasmin releases the 7-amido-4-trifluoromethylcoumarin fluorophore, which can then be quantified via spectrophotometric methods at excitation/emission wavelengths of 380 nm/500 nm. To investigate whether D-ALL could be used to quantify plasminogen activator activity in BALF, we first validated substrate cleavage specificity. As shown in Figure 3A, test reactions containing only D-ALL substrate did not generate significant fluorescence. Similarly, reactions containing D-ALL substrate with plasminogen only, or uPA only, generated minimal fluorescence (see first three columns on left in Figure 3A). A large increase in fluorescence was observed, however, when all three (D-ALL + plasminogen + uPA) were incubated together. Fluorescence due to cleavage of the substrate could be inhibited in these *in vitro* test reactions by addition of the serine protease inhibitor, leupeptin, or by incubation with PAI-1, the latter being the endogenous inhibitor of uPA activity. As demonstrated in Figure 3B, there was a concentration-dependent increase in fluorescence intensity after incubation of uPA with fixed concentrations of D-ALL substrate and plasminogen. Total plasminogen activator activity (uPA + tissue plasminogen activator [tPA], indiscriminate) was also quantified in BALF by use of D-ALL and plasminogen. A significant decline in activity was detected between BALF from ethanol-exposed rats (206 ± 40.4 units) and that obtained from rats at 4 hours (163.7 ± 40.1 units) or 18 hours (56.8 ± 9.8 units) after CEES exposure. Thus, native plasminogen activating activities were diminished after SM inhalation.

Figure 2. (A) Representative images of airway microdissection used to assess airway obstruction after exposure to vehicle (ethanol, 18 h) or 7.5% CEES inhalation. (B) Western analysis of fibrinogen (top panel) and prothrombin (bottom panel) levels in rat BALF after vehicle (ethanol, 18 h) or 7.5% CEES (4 and 18 h) inhalation. (C) Thrombin–antithrombin (TAT) complex levels in BALF after ethanol or CEES inhalation. BLD, below the limit of detection (of the ELISA). Data represent the mean values ($\pm$ SD) from five rat lungs.

Figure 3. Plasminogen activation activity in BALF. (A) Use of an *in vitro* diagnostic test to verify the specificity of D-Ala-Leu-Lys 7-amido-4-trifluoromethylcoumarin (D-ALL) as a substrate for urokinase-type plasminogen activator (uPA)-dependent plasminogen activation. uPA (60 nM) was incubated with or without plasminogen (120 nM) before addition of D-ALL. Substrate cleavage was dependent on the presence of both plasminogen and uPA, and was inhibitable by use of inhibitors of uPA (plasminogen activator inhibitor [PAI]-1 and leupeptin). (B) Effect of varying concentration of uPA on cleavage of D-ALL substrate. (C) BALF obtained from rats exposed to ethanol (control) or CEES was incubated with plasminogen for 30 minutes, followed by determination of plasminogen activator activity by use of the D-ALL assay. Data represent means ($\pm$ SD) of two determinations for each individual BALF sample ($n = 6$ rats per group for ethanol- or 18-h CEES-exposed groups; $n = 3$ per group for 4-h CEES group). * $P < 0.05$ versus corresponding ethanol-exposed control.

Figure 4. The effect of BALF from ethanol- or CEES-exposed rats on clot formation and lysis. BALF procedure was performed on rats 18 hours after exposure to ethanol (A), or 4 and 18 hours after exposure to CEES (B and C). After centrifugation, 25 μ l of each sample was added to reactions containing rat fibrinogen, plasminogen, thrombin, and CaCl_2 . Clot formation and dissolution of these clots were then determined by measurement of turbidity at 2-minute intervals for 4 hours. Each panel shows three representative histograms depicting the impact of BALF type on clotting. Each BALF sample was run in duplicate reactions, and rates of fibrinolysis were determined by calculating the absorbance decline per minute occurring over the interval of 120–180 minutes. (D) Columns represent the average absorbance decline per minute ($\pm$ SD) for each BALF group and for PBS negative control in which PBS was substituted for BALF. * $P < 0.05$ versus BALF from ethanol-exposed rats.

Figure 5. Effect of CEES on the level of fibrinolytic inhibitors in rat airways. Western blot analysis of naive plasma and BALF obtained from rats at 4 or 18 hours after inhalation exposure to vehicle (EtOH) or 7.5% CEES. (A) Increased expression of latent and active forms of thrombin-activatable fibrinolysis inhibitor (TAFI) in response to CEES. (B) Time-dependent appearance of α 2-antiplasmin (α 2AP) and the high-molecular weight α 2AP-plasmin complex in BALF from rats exposed to CEES or its EtOH diluent. (C) Increased expression of PAI-1 protein in 18-hour CEES BALF. Western blots (A, B, and C) were performed using the same set of three experimental animals for each BALF type.

Effect of SM Exposure on Airway Fibrinolytic Activity

To determine whether SM exposure also had an impact on fibrinolytic activity, we performed fibrin lysis time experiments by introducing BALF into reactions containing fibrinogen, plasminogen, and CaCl_2 . The initial clotting phase of the reaction commenced after addition of thrombin, and clot formation was monitored by measurement of increased absorbance at 405 nm. As shown by the representative histograms in Figure 4A, reactions containing BALF from ethanol-exposed rats undergo a rapid clotting phase (0–15 min), followed by a period of absorbance decline back to baseline, representing the phase of clot lysis activity. Reactions incubated in the presence of BALF from CEES-exposed rats (Figures 4B and 4C) also demonstrated a rapid clotting phase, but appeared to have diminished rates of clot lysis. Differences in clot lysis rates between sample groups were quantified by determining absorbance change during the time intervals of 120–180 minutes (Figure 4D). Reactions performed in the absence of BALF (PBS only) exhibited a mean decline of $0.00014 (\pm 0.0001)$ absorbance units per minute. Addition of BALF from ethanol-exposed rats

accelerated the rate of absorbance decline (0.0012 ± 0.0005 absorbance units). In contrast, absorbance declined at slower rates in reactions incubated with BALF from CEES-exposed rats (4 h, -0.0007 ± 0.0002 absorbance units; 18 h, -0.0001 ± 0.00017 absorbance units). In these experiments, analysis of clot lysis was limited to the 120- to 180-minute time interval, because samples exhibiting initial rapid clot breakdown had decreased rates of absorbance decline at later time points. Thus, SM analog exposure caused an impairment of native airway fibrinolytic activity.

Effect of CEES Inhalation on Inhibitors of Fibrinolysis

TAFI, α 2AP, and PAI-1 proteins each negatively regulate fibrinolysis by suppressing serine protease activity. Western blotting was performed to determine the presence of each protein in biological fluids. TAFI is synthesized by liver and megakaryocytes, and is secreted into the circulation as a 401-amino acid proenzyme (56 kD). As shown in Figure 5A, this inactive form of TAFI was readily detectable in plasma obtained from naive rats. In BALF from ethanol-exposed control rats, TAFI content was low. In contrast, BALF obtained from rats after CEES inhalation demonstrated a time-dependent

increase in TAFI protein levels. At 4 hours, TAFI was detected in airway fluids in the latent proenzyme form. By 18 hours after CEES inhalation, a significant increase in TAFI proenzyme detectable in BALF was observed, along with the appearance of the activated 35-kD subunit (TAFIa). Identical experimental conditions were employed to determine the presence of α 2AP (Figure 5B). In plasma, α 2AP was detected at high levels, but it was not detected in BALF from ethanol-exposed control rats. After CEES injury, α 2AP levels increased in a time-dependent manner. In BALF obtained 18 hours after CEES inhalation, significant levels of α 2AP/plasmin complex also were observed. PAI-1 is the physiologic inhibitor of plasminogen activators (uPA, tPA). Using Western blot, PAI-1 protein was not detectable in plasma from naive rats, nor in BALF from ethanol-exposed control rats (Figure 5C). In response to CEES injury, however, significant levels of PAI-1 were detectable in BALF at 18 hours after exposure. Thus, SM analog exposure caused the appearance of three different native antifibrinolytic proteins in the airways, and, as best we could detect here and in further studies, each was active.

Effect of CEES Exposure on TAFI Activation

The antifibrinolytic action of BALF-derived TAFI was evaluated in a kinetic assay of carboxypeptidase activity. This method employed a synthetic substrate having an L-lysine/L-arginine residue that was selectively cleaved by TAFIa, producing a thiol derivative capable of interaction with Ellman's reagent. Initial experiments incubating BALF with the substrate did not detect any intrinsic TAFI activity in any BALF samples tested (data not shown). Activity could only be detected in BALF after an initial treatment step using thrombin-thrombomodulin, and such activity was only detected in BALF from rats 18 hours after CEES exposure (Figure 6B). Preincubation of BALF from this group of rats with the carboxypeptidase inhibitor completely inhibited this activity. Thus, TAFI appeared in airways after SM inhalation and retained its potential for activation.

Effect of CEES Exposure on Plasmin- and uPA-Neutralizing Capacity of BALF

To examine the plasmin-neutralizing capacity of BALF in rats 18 hours after CEES

Figure 6. Carboxypeptidase B activity measurements on purified enzyme and BALF samples. Spectrophotometric measurement of carboxypeptidase B activity was performed using a synthetic peptide substrate, the degradation of which releases a product capable of interaction with Ellman's reagent (5,5'-dithio-bis-[2-nitrobenzoic acid]; DTNB). Conversion of Ellman's reagent increases absorbance at 405 nm, the degree to which is proportional to the concentration of carboxypeptidase. (A) Time-dependent substrate cleavage after reaction with purified carboxypeptidase B enzyme. Data in the box display kinetic profiles after the addition of enzyme at known concentrations (0.21–3.45 U/ml). (B) Measurement of TAFI (carboxypeptidase B₂) activity in EtOH-exposed, CEES-exposed (4 or 18 h), or 18-hour CEES-exposed plus carboxypeptidase inhibitor (CPI) BALF samples after addition of thrombin (5 nM), thrombomodulin (20 nM), and CaCl₂ (5 mM). Each panel displays absorbance plots from three representative samples for each BALF type. (C) TAFI activity levels in BALF were quantitated by linear regression analysis using carboxypeptidase B standard curve data (Fig 5A). Activity was below the limit of detection (BLD) in all sample types except 18-hour CEES-exposed rat BALF samples. Data represent means ($\pm$ SD) of two determinations for each individual BALF sample ($n = 3$ rats per 4-h CEES group or 6 for EtOH- and 18-h CEES-exposed group).

exposure, samples were incubated with a range of plasmin concentrations for 20 minutes, followed by Western blotting for α 2AP (Figure 7). BALF incubated in the absence of plasmin demonstrated two major bands: a 70-kD band corresponding to α 2AP, and a larger 130-kD band consistent with the plasmin/ α 2AP complex. Addition of plasmin caused a concentration-dependent disappearance of this lower band, and a concomitant

increase in the higher molecular weight complex, confirming the identity of the two bands. Similarly, the addition of rat uPA to BALF from rats 18 hours after CEES exposure decreased the amount of detectable PAI-1, as detected by ELISA (Figure 8). These findings demonstrate that airway surface liquid is capable of inhibiting fibrinolysis by simultaneously neutralizing both plasmin and plasminogen activator activity.

Tissue-Specific mRNA Expression of PAI-1, α 2AP, and TAFI

Quantitative RT-PCR was performed to determine whether antifibrinolytic proteins in CEES-exposed airways are a manifestation of edematous leak, or may derive from increased expression by the lung. As shown in Figure 9A, TAFI mRNA was readily detected in liver from 18-hour CEES-exposed rats, whereas its expression in lung, kidney, or cells collectively obtained from BALF was extremely low (TAFI steady-state mRNA levels were 0.1, 0.1, and 0.5%, respectively, compared with that of the liver). Expression of α 2AP mRNA (Figure 9B) was found to be at high levels in liver and kidney tissue (kidney steady-state mRNA was 76.1% of liver), whereas negligible expression was observed in ethanol- or CEES-exposed lung (0.09 and 0.21% of liver), or cells in the BALF pellet (0.96% of liver). On the other hand, marked extrahepatic expression of PAI-1 was observed in CEES-exposed rats. PAI-1 mRNA levels for lung, BALF cell pellet, and kidney were found to be at 12.1-, 1.57-, and 0.21-fold the level observed in liver. These findings indicate that rat lung tissue, and, to a lesser extent, BALF cells are significant sources of PAI-1 mRNA expression after CEES exposure, whereas liver or kidney are the more important sources of TAFI and α 2AP mRNA.

Discussion

Airway obstruction due to occlusive fibrin cast formation is the defining pathophysiological feature of acute lung injury after SM analog inhalation (8), and similar observations have been made after inhalation of authentic SM (11). These mustard studies demonstrate time- and dose-dependent formation of airway casts, and establish the causal relationship between cast formation and gas exchange impairment, oxygen delivery deficits, clinical respiratory distress, and eventual death. We have also noted increased airway procoagulant activity after SM analog inhalation, including increased expression of tissue factor, leak of clotting factors into airways, and activation of the coagulation cascade (12). Furthermore, *in vivo* studies have shown that tissue factor pathway inhibitor given early after SM inhalation prevents airway obstruction and acute mortality (9), confirming airway

Figure 7. Rat α 2AP present in CEES-exposed rat BALF retains significant plasmin-neutralizing capability. CEES-exposed rat BALF samples (18 h) were separated into 25- μ l aliquots and incubated for 15 minutes with varying concentrations (0–500 ng) of rat plasmin, followed by electrophoretic separation and Western analysis under nonreducing conditions. Plasmin added to BALF reacts with the available α 2AP, resulting in the concentration-dependent formation of plasmin–antiplasmin complex.

procoagulant activity in the disease process. In addition, we have found that fibrinolytic agents like tPA, even when given 6–24 hours after SM inhalation, can reverse airway obstruction and prevent death after SM inhalation (13), indicating that fibrin casts are the structural entity responsible for obstruction and mortality. Notably, such therapy must be continued for at least 48 hours after a severe SM inhalation event to prevent death, indicating the potential for airway coagulopathy to persist long after SM inhalation. Given this information, we hypothesized that, in addition to procoagulant activities, antifibrinolytic factors might also be active in rat airways after SM inhalation.

In the present investigation, we indeed determined that three naturally occurring antifibrinolytic pathways are present and active in airways after SM inhalation, with such activities appearing in a time-

dependent manner. We found that rat airways normally possess endogenous fibrinolytic activity, and none of three native inhibitors, PAI-1, α 2AP, or TAFI, was detectable in the noninjured airways of rats exposed only to the diluent for SMs. By contrast, inhalation of the SM analog CEES, like other disease states causing edematous injury to the upper airways (14), caused progressive declines in fibrinolytic activity.

The rise in high-molecular weight IgM suggests that CEES induces a form of vascular leak that is relatively nonselective, given that high-molecular weight proteins are allowed to exit the vasculature and enter the airways. Just as clotting factors (Figures 2B and 2C) can enter the airways after SM inhalation injury (12), increased permeability of the airway–capillary barrier likely plays a role in allowing fibrinolysis inhibitors to enter the airways. For example, α 2AP protein was detected in

naive rat plasma, but not in the BALF from naive rats. However, it entered the airway over time after SM inhalation, and complexed with plasmin. Likewise, a latent form of TAFI protein also was detectable in naive plasma from rats exposed to SM diluent. It was not detected in BALF from these animals. However, TAFI was increasingly detected over time in BALF from half-mustard-exposed rats, and, by 18 hours, a band representing activated TAFI had also appeared. Finally, PAI-1 was not detectable in BALF from naive rats. However, it appeared after SM exposure in a time-dependent fashion. Quantification of the tissue-specific mRNA expression of each fibrinolytic inhibitor (Figures 9A–9C) provided mechanistic insight (see Figure 10) into the cause of the elevated levels of antifibrinolytic molecules in the airways. Such data indicate that extrapulmonary organs are the predominant source of TAFI and α 2AP, with minimal synthesis occurring in the lung. The lack of any significant mRNA production by lung tissue or hematopoietic cells in BALF provides a strong inference that both TAFI and α 2AP enter the airways via leakage from lung vasculature. PAI-1 mRNA, however, is readily detected in lung tissue and BALF cells, an indication that its airway expression could be occurring independently of vascular leak.

Fibrinolysis in the airways is initiated when plasminogen is activated to plasmin by the action of localized uPA, or by introduction of tPA from the systemic circulation. Fibrin clots contain C-terminal lysine residues that permit binding of plasminogen and plasminogen activators to the surface of the structure. Binding facilitates proteolysis of plasminogen by uPA or tPA, maintains plasmin in close proximity to fibrin substrate, and protects it from inactivation by α 2AP (15). Thus, rates of plasmin generation and fibrinolysis are determined by the level of C-terminal lysine residues on fibrin (16). Carboxypeptidases, like TAFI, remove C-terminal lysines, and, in so doing, modulate plasminogen binding and fibrinolytic rate (17–19). In plasma, the rate of TAFI formation is important in regulating clot breakdown. Fibrinolysis is attenuated as long as TAFI concentrations remain above a certain threshold value, whereas fibrinolysis is accelerated in response to increased numbers of C-terminal lysine residues when TAFI drops below threshold.

Figure 8. Effect of exogenous uPA treatment on BALF PAI-1 activity. The 18-hour CEES-exposed rat BALF samples (200 μ l) were incubated with PBS (2 μ l) containing BSA (100 ng) or uPA (1–100 ng). After 20 minutes, each sample was split into duplicate 100- μ l samples and tested for active PAI-1 protein by ELISA ($n = 4$ per group). Error bars represent SD. * $P < 0.05$ compared with BALF plus BSA.

Figure 9. RT-PCR analysis of fibrinolytic inhibitor mRNA expression in various rat tissues after EtOH or CEES inhalation. Rats were exposed to vehicle or CEES, followed by total RNA isolation (18 h after exposure) from liver, kidney, lung, and the collective cell pellet recovered from BALF. Transcript abundance for TAFI (A), α 2AP (B), or PAI-1 (C) are presented relative to values from lung tissue (18-h CEES). Values were normalized to 18S used as a housekeeping gene. Data are expressed as mean ($\pm$ SD). * $P < 0.05$ compared with CEES lung (ANOVA on ranks, Dunn's test); $n = 5$ rats per group.

Less is known about the presence and impact of TAFI in the airways. Mice deficient in TAFI are protected against vascular remodeling in the lung associated with pulmonary hypertension (20). During

LPS-induced acute lung injury, TAFI has been shown to be protective, possibly by regulation of the complement pathway (21). However, in a model of abdominal sepsis, TAFI-deficient mice did not show any

decrease in fibrin accumulation in the lung (22). So the relative impact of TAFI on fibrinolysis of airway casts is unclear.

In summary, we found that the activities of three independently regulated

Figure 10. Schematic representation of the pathways involved in the recruitment of fibrinolytic inhibitors to airways after CEES exposure. Mustard inhalation causes lung injury, which facilitates leakage of serum protein into the airways. Egress/influx of coagulation factors, including prothrombin and fibrinogen, leads to the formation of fibrin casts that are capable of airway obstruction. Plasmin-dependent fibrinolysis is the physiologic mechanism for removal of airway casts and restoration of airway patency. Mustard-induced lung injury also gives rise to elevated airway levels of antifibrinolytic molecules, which collectively inhibit the plasminogen activation pathway. PAI-1 is the principal inhibitor of tissue plasminogen activator (tPA) and uPA. TAFI also restricts plasmin formation by removal of C-terminal lysines from fibrin, which are binding sites for plasminogen. α 2AP provides a third level of fibrinolytic suppression by covalently binding any plasmin that is generated. RT-PCR analysis indicates that the lung produces negligible amounts of TAFI or α 2AP mRNA, an indication that their presence in airways is due to vascular permeability and leak. PAI-1 mRNA expression, however, does occur at high levels in the lung, an indication that its presence may be leak independent.

antifibrinolytic pathways were increased over time in airway fluids from rats after exposure to SM by inhalation. Because all three were active, it seems likely that more than one pathway is required to impact native uPA- or tPA-driven plasminogen activation. We speculate that these antifibrinolytic pathways could contribute to the persistence of airway-

obstructive lesions after SM inhalation, and to the eventual outcome of bronchiolitis obliterans observed in many survivors of such attacks (3). Finally, the fact that airway vascular permeability may also be increased in patients with airway fibrin casts, causing plastic bronchitis, such as those with complex congenital heart disease and palliative

surgeries (e.g., Fontan procedure [23]), these patients might also be investigated for the presence and activation of the antifibrinolytic pathways identified in this toxic inhalation model. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

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