Airway Tissue Plasminogen Activator Prevents Acute Mortality Due to Lethal Sulfur Mustard Inhalation


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ABSTRACT

Rationale: Sulfur mustard (SM) is a chemical weapon stockpiled today in volatile regions of the world. SM inhalation causes a life-threatening airway injury characterized by airway obstruction from fibrin casts, which can lead to respiratory failure and death. Mortality in those requiring intubation is more than 80%. No therapy exists to prevent mortality after SM exposure. Our previous work using the less toxic analog of SM, 2-chloroethyl ethyl sulfide, identified tissue plasminogen activator (tPA) an effective rescue therapy for airway cast obstruction (Veress, L. A., Hendry-Hofer, T. B., Loader, J. E., Rioux, J. S., Garlick, R. B., and White, C. W. (2013). Tissue plasminogen activator prevents mortality from sulfur mustard analog-induced airway obstruction. Am. J. Respir. Cell Mol. Biol. 48, 439–447). It is not known if exposure to neat SM vapor, the primary agent used in chemical warfare, will also cause death due to airway casts, and if tPA could be used to improve outcome. Methods: Adult rats were exposed to SM, and when oxygen saturation reached less than 85% (median: 6.5 h), intratracheal tPA or placebo was given under isoflurane anesthesia every 4 h for 48 h. Oxygen saturation, clinical distress, and arterial blood gases were assessed. Microdissection was done to assess airway obstruction by casts. Results: Intratracheal tPA treatment eliminated mortality (0% at 48 h) and greatly improved morbidity after lethal SM inhalation (100% death in controls). tPA normalized SM-associated hypoxemia, hypercarbia, and lactic acidosis, and improved respiratory distress. Moreover, tPA treatment resulted in greatly diminished airway casts, preventing respiratory failure from airway obstruction. Conclusions: tPA given via airway more than 6 h after exposure prevented death from lethal SM inhalation, and normalized oxygenation and ventilation defects, thereby rescuing from respiratory distress and failure. Intra-airway tPA should be considered as a life-saving rescue therapy after a significant SM inhalation exposure incident.

Key words: tPA; airway fibrin; sulfur mustard; fibrinolysis; plastic bronchitis
intent. Therefore, the likelihood of exposure of various militaries, rebels, civilians, and/or persons in neighboring countries seems increasingly possible. In addition, the ocean floors also contain thousands of additional tons of SM, and these stores remain capable of inflicting injury upon those that attempt to handle them.

SM is a vesicant, and its principal target after inhalation is the respiratory tract, while the eyes and the skin are also greatly affected (Allon et al., 2009; Graham and Schoneboom, 2013; Kehe and Thiermann, 2009; Kehe et al., 2009; Sinclair, 1949). Degrees of respiratory compromise follow inhalation, depending on inhaled dose, with symptoms ranging from mild cough to fulminant respiratory failure (Balali-Mood and Hefazi, 2006). Although overall mortality was estimated at approximately 3% from World War I data, Willems et al. reported that humans with moderate to severe exposures airlifted to Europe for treatment during the Iran–Iraq conflict (1987–1989) had a much higher mortality rate at 15% (Willems, 1989), occurring between 3 and 14 days after exposure. More importantly, the Willems cohort study showed that those with severe respiratory involvement requiring artificial ventilation had an 89% mortality rate, all from cardiopulmonary collapse. Autopsy findings in those patients showed necrotic airways, sloughing, and formation of dense casts occluding and obliterating airways, causing asphyxia and death. Furthermore, casts were directly visualized in at least 25% of surviving patients (during bronchoscopy or upon expectoration), with another more than 40% coughing abundant white sputum that may have also contained cast material.

Among the potentially damaged organs, injury to airways and lung remains the principal cause of mortality from SM (Eisenmenger et al., 1991; Somani and Babu, 1989; Willems, 1989). Despite many years of research, there remain no effective antidotes for treatment of this airway injury (Graham and Schoneboom, 2013; Poursaleh et al., 2012; Razavi et al., 2013), although recent research has shown N-acetylcysteine to improve 12 h morbidity in pigs, but without significant improvement in mortality (Jugg et al., 2013). Fibrin deposition and lung coagulopathies have been reported in several lung injury states, such as in acute lung injury (Idell, 2003; Vadass and Sznejder, 2011), but airway-specific coagulopathies have been more rare. Previously we have reported an exudative airway coagulopathy in rats exposed by inhalation to the SM analog 2-chloroethyl ethyl sulfaide (CEES) (Veress et al., 2010), resulting in airway fibrin cast formation, or plastic bronchitis, that directly correlates with death due to airways obstruction. In addition, we have also previously noted that the fibrinolytic drug tPA, when administered via the airways, can relieve airway obstruction and prevent mortality due to SM analog (CEES) (Veress et al., 2013). SM is capable of causing even more devastating tissue injury than CEES, due to the presence of a second terminal chlorine molecule in the former, allowing SM to cause crosslinking of DNA with itself and other molecules such as proteins. Because it was unclear whether fibrinolytic agents could prevent lung injury and mortality after SM, we tested their capacity to reverse injury and death in rats given inhaled authentic SM.

Here, we report that intratracheally delivered tissue plasminogen activator (tPA), a potent fibrinolytic agent currently FDA-approved for intravascular clot lysis in stroke (American College of Emergency Physicians and American Academy of Neurology, 2013) and heart attack (Fitchett et al., 2011), can reverse hypoxemia, normalize gas exchange, and eliminate mortality, after SM vapor inhalation in rats. Further, an optimized regimen of the fibrinolytic agent tPA was capable of largely eliminating airway fibrin casts and optimizing cardiopulmonary performance, while completely preventing mortality after acute SM inhalation, without causing any enhancement of airway bleeding. These findings provide further support for a new, promising and potentially lifesaving rescue therapy against acute SM inhalation, via use of an intra-airway delivered fibrinolytic agent.

MATERIALS AND METHODS

An expanded methods description can be found in the online supplement.

Chemicals. SM (2,2’-dichlorodiethyl sulfide; 4 mM, vapor inhalation) was acquired from the U.S. Army Edgewood Research, Development and Engineering Center (Aberdeen Proving Ground, Maryland). tPA (intratracheal administration) was purchased from Genentech (Roche, San Francisco, California).

Study animals and animal care. Sprague-Dawley male rats (230–260 g), purchased from Charles River Laboratories (Wilmington, Massachusetts), were maintained in animal care facility fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International. The “Guide for the Care and Use of Laboratory Animals” (1996) was followed during the conduction of the research described in this report. Animals used in this study: naïve (n = 5), SM-exposed but untreated (NT, n = 12), SM-exposed and placebo treated (PBS; n = 13), and SM-exposed and tPA treated (tPA, n = 9).

SM exposure. The exposure model has been described previously (Anderson et al., 1996, 2000). Briefly, rats were anesthetized, intubated with a modified glass Pasteur pipette, and exposed to SM vapor, at 3.8 mg/kg dose (0.95 mg SM in 100 μl absolute ethanol) for 50 min. At the end of the exposure, the endotracheal tube was removed, and the rats were returned to their cage for recovery.

tPA intratracheal delivery. tPA was administered intratracheally per previously published methods (Veress et al., 2013). Briefly, animals given isoflurane anesthesia were given 0.7 mg/kg tPA (in PBS, or PBS alone) via microsprayer (Penn-Century, Wyndmoor, Pennsylvania). tPA was administered every 4 h for the entire 48 h of the study. Dose and interval of tPA dosing was based on best effective outcome from preliminary experiments.

Noninvasive oxygen saturation measurements. A MouseOx small animal oximeter (Starr Life Sciences, Oakland, Pennsylvania) in unanesthetized rats was used with large CollarClip sensor.

Respiratory distress clinical scoring and euthanasia. Clinical scoring parameters were modified from previously published methods (Veress et al., 2013). Respiratory distress quality (0–6) and activity depression (0–3) were assessed then added to obtain a cumulative score (0–9; see Supplementary Table 1). Animals were euthanized at 48 h or earlier if criteria for severe distress was met, as dictated by the Institutional Animal Care and Use Committee. Criteria for early euthanasia was oxygen saturation less than 70%, plus respiratory distress score of 7 or greater, as previously published (Veress et al., 2013).

Airway cast scoring and lung fixation. Lungs were fixed with 4% paraformaldehyde in situ, removed, and cast scoring performed using a modified technique described previously (Veress et al., 2013).
per previously published methods (Veress et al., 2013).

Statistical analysis. Prism 6 software (GraphPad, La Jolla, California) was used, with one-way analysis of variance (ANOVA) followed by Tukey’s post hoc analysis, unless otherwise indicated. All mean values are reported with standard error of the mean (SEM). A P < 0.05 was significant. Survival data were analyzed via Mantel–Cox Log-rank test.

RESULTS

Improved Survival with tPA

Arterial blood gas measurements. Descending aorta blood was collected under ketamine/xylazine (1:1) anesthesia, immediately prior to euthanasia by exsanguination plus bilateral thoracotomy. Blood was then placed into a precalibrated test card (EPOC-BGEM Test Card), and analyzed using the EPOC-Vet Blood Analysis system (Epocal Inc, Ottawa, Ontario, Canada).

In order to test the maximum efficacy of tPA to rescue from the severest of acute respiratory injuries due to SM inhalation, we exposed sedated and intubated rats to a previously determined lethal SM dose of 3.8 mg/kg in ethanolic vapor (LD_{50} by 12 h, see Supplementary Fig. 1). Animals were extubated and awakened after exposure, monitored hourly for oxygen saturation, then randomized into 1 of 3 groups when their oxygen saturation dropped below 85% (average time to randomization, or mean time to first treatment, was 6.5 h post-exposure). The 3 groups evaluated were: (1) treatment group, given SM and intratracheally delivered tPA (0.7 mg/kg/dose, n = 9), (2) placebo group, given SM and intratracheally delivered diluent for tPA (PBS, n = 13), and (3) non-treated, but SM-exposed, group (NT, n = 12).

At this dose of SM, we observed a 100% mortality by 48 h in all control groups, with a median survival of 9.25 h and 8 h in NT and PBS groups, respectively. Remarkably, we found that tPA completely eliminated mortality due to SM exposure, with 100% survival maintained to study end point of 48 h (P < 0.0001, Mantel–Cox Log-rank test). Thus, tPA therapy effectively rescued lives of animals that otherwise would have died due to SM-related pulmonary injury.

Improved Oxygen Saturations with tPA

In order to determine tPA’s effects on SM-associated hypoxemia, we measured oxygen saturation (SpO\textsubscript{2}) using a pulse oximetry collar after tPA treatment in SM-exposed animals. SpO\textsubscript{2} was monitored hourly until randomization and treatment start, then every 2 h thereafter, with continued treatment every 4 h. At time of randomization, all 3 groups had very similar oxygen saturations, with mean SpO\textsubscript{2} of 91% in all groups (see Supplementary Fig. 2). When comparing SpO\textsubscript{2} taken immediately prior to euthanasia, tPA treatment resulted in mean saturations of 92.7 ± 1.3% in the active treatment animals, whereas those of controls were 58.8 ± 2.8% for the PBS group, and 60.5 ± 2.9% for the NT group (P < 0.0001, Fig. 2). Oxygen saturation at euthanasia was not significantly different between the two control groups.

We also found that tPA treatment resulted in improved oxygenation from a nadir mean SpO\textsubscript{2} of 81% at randomization (average of 6 h post-exposure) to 88% after induction dose alone, and to more than 90% within 2 h after initiating maintenance therapy (Fig. 3A). Although tPA-treated animals had a sustained SpO\textsubscript{2} of more than 90% throughout the remainder of the study, NT and PBS controls showed a progressive and rapid decline in oxygenation from time of randomization to death mostly within 12 h of exposure (Fig. 3B). Two PBS-treated animals with less severe respiratory injury (noted by delayed randomization, with delayed signs of SM-associated hypoxemia) showed a more gradual decline in oxygenation until death by 41 h. With tPA administration, we have noted oxygen saturations improvement within 5 min of treatment, often from less than 60% to more than 90% within this timeframe (data not shown). Thus, intratracheal tPA can rapidly and sustainably rescue from severe respiratory compromise due to lethal SM injury, as indicated by efficient resolution of significant SM-associated hypoxemia after tPA treatment.

**FIG. 1.** Effect of tPA treatment on mortality after SM inhalation. Survival curves for rats exposed to SM vapor (3.8 mg/kg) and given (1) no treatment (NT, n = 12, black line), (2) PBS diluent “placebo” treatment (PBS, n = 13, green line), or (3) tPA treatment (tPA, n = 9, red line). Both control groups had 0% survival at 48 h in this lethal SM exposure model (median time of death was 9.25 h with NT and 8 h with PBS). tPA treatment resulted in 100% survival at 48 h (P < 0.0001, via Long–rank, Mantel–Cox test).

**FIG. 2.** Effect of tPA treatment on tissue oxygenation in unanesthetized rats at time of euthanasia after SM vapor inhalation. Noninvasively acquired oxygen saturations (SpO\textsubscript{2}) measured immediately before euthanasia (at 12 h or earlier if euthanized early due to meeting euthanasia criteria) in rats exposed to SM (3.8 mg/kg) vapor and given (1) no treatment (NT, n = 12, black bar), (2) PBS diluent (PBS, n = 13, dark gray bar), or tPA (0.7 mg/kg, n = 12, light gray bar). Significant improvement in SpO\textsubscript{2} was noted with tPA relative to controls (P < 0.0001, ANOVA for repeated measures, with Tukey’s post hoc analysis), with tPA treatment group resulting in near baseline oxygen saturations (>90%) at time of euthanasia (12 h). Values represent means ± SEM. ***P < 0.001.
Reduction of Airway Obstruction and Improved Clinical Distress with tPA

To assess effects of tPA on fibrin casts within airways after SM exposure, we scored total airway obstruction of rat lungs using quantitative morphometric methods previously published by our group (Veress et al., 2013). We found that SM exposure caused severe airways obstruction from casts (Fig. 4), with a total major airway cast score in NT group of 4.05 ± 0.39, corresponding to 50%–65% of total airflow obstruction (median time to euthanasia of 9.25 h). Similarly, PBS treatment did not alleviate airway obstruction by casts, resulting in a total major airway cast score of 4.67 ± 0.19 (median euthanasia time of 8 h). The worst affected lung lobes were right accessory and right middle lobe bronchi, both with 100% obstruction at time of euthanasia, followed by right lower lobe main bronchus with more than 70% obstruction. This degree of obstruction paralleled severity of clinical respiratory distress and severity of hypoxemia of the animals, resulting in progression to imminent death without tPA treatment. Conversely, airway obstruction by casts was drastically diminished after tPA treatment, with a major airway cast score of 0.88 ± 0.17 (at 48 h; P < 0.0001), and this was associated with a significant reduction in respiratory distress.

Indeed, clinical distress scoring greatly improved with tPA treatment, with a score of 2.1 ± 0.2 at euthanasia (average of 2.2 ± 0.1 over the entire 48 h), compared with severe distress scores of 7.5 ± 0.3 and 7.6 ± 0.3 for NT and PBS control groups. Note that this figure shows data from the time of exposure, and not from randomization, thereby each animal receiving therapies at different times on this graph. tPA treatment (n = 9) resulted in saturation improvement from nadir of less than 81% to above 90% by 12 h after exposure, with continued overall saturations more than 90%–92% for the remainder of the study (red solid line). NT controls (n = 12) had severe oxygen saturation deterioration within hours of exposure, resulting in eventual death (black solid line). PBS-treated animals with similar SM-associated injury (green solid line, n = 11) also had a rapid decline in oxygenation until death by 12 h, whereas 2 PBS-treated animals with less severe respiratory injury (noted by delayed randomization, green dashed line) showed a more gradual decline in oxygenation until death by 41 h. (Orange vertical line: median time of randomization and start of treatment.)

Improved Blood Acidosis and Ventilation with tPA

To evaluate tPA efficacy on morbidity, we analyzed arterial blood gas (ABG) values obtained at euthanasia after SM exposure (Figs. 6A–C). We found profound acidosis in both SM-exposed control groups (Fig. 4), with arterial pH of 7.12 ± 0.02 in NT, and 7.05 ± 0.03 in PBS-treated animals (naïve rats had pH = 7.35 ± 0.01). tPA treatment given every 4 h normalized arterial pH at euthanasia (P < 0.0001), with an arterial pH of 7.39 ± 0.01, a value comparable to that seen in naïve rats (pH = 7.35 ± 0.01).

Similarly, arterial partial pressure of carbon dioxide (pCO2) at euthanasia was normalized to naïve levels after tPA treatment (Fig. 6B), with pCO2 of 51.3 ± 3.1 mm Hg in the tPA-treated group (naïve rats had pCO2 of 54.6 ± 1.5 mm Hg). Controls
showed significant ventilation disturbance, with $p_{aCO_2}$ of 104.4 ± 4.7 mm Hg in NT and 117.4 ± 7.8 mm Hg in PBS-treated groups ($P < 0.0001$). The marked hypercarbia in control animals indicated a severe ventilation defect, which would usually require mechanical ventilator support if it occurred in human patients. tPA treatment resulted in normalization of ventilation, despite a severe SM inhalation exposure. Circulating bicarbonate levels were not significantly different between the groups, and values of 30–33 mmol/l were measured in all naïve and exposed groups (data not shown). Thus, the severe respiratory acidosis in this model was completely eliminated with tPA treatment.

Finally, we also observed an improvement in circulating blood lactate with tPA treatment (Fig. 6C). SM exposure caused an increase of blood lactate in both control groups at euthanasia compared with naïve (naïve lactate of 1.4 ± 0.1 mmol/l), with
lactate values of 3.6 ± 0.7 mmol/l in NT, and 4.7 ± 0.7 in PBS groups. Animals after tPA treatment showed an improved blood lactate, with values of 2.2 ± 0.5 mmol/l (P = 0.02), comparable to air-breathing naive controls. Therefore, tPA treatment resulted in restoration of tissue perfusion and oxygen delivery in a model of lethal SM exposure.

**DISCUSSION**

In the present study, we utilized a previously described model of SM inhalation toxicity (Anderson et al., 1996, 2000), but we employed a far greater inhaled dose of the agent than has been described previously. This was done to ensure a scenario in which the resulting injury would cause a profound and overwhelming inhalation insult likely to produce fulminant critical illness rapidly. These studies were initiated in order to examine the efficacy of an intratracheally administered tPA treatment regimen previously optimized in a severe airway injury model due to SM analog (CEES) exposure (Veress et al., 2013). Because SM is both an alkylating agent and crosslinking agent for DNA and proteins, whereas CEES is an alkylating agent without crosslinking capability, we anticipated that SM-related lung injury might not respond as impressively to fibrinolytic therapy as did injury due to CEES. That was not the case. The optimized tPA regimen completely prevented mortality, whereas mortality was 100% in rats receiving no treatment, or in those given placebo intratracheal injections under anesthesia.

Associated with elimination of mortality was a steady improvement in tissue oxygenation with tPA therapy. Improvements in oxygenation could be noted within minutes after each dose of tPA was administered, and these improvements were still present at 2-4 h after dosing. Further, oxygen saturations generally were above 90% in the treated animals, a level at or above which oxygen supplementation is generally not required in patient care settings. This was in stark contrast to the continued decline in oxygenation in the control groups. This assessment, made in conscious rats, reflects overall improvement in cardiopulmonary function (pulmonary gas exchange and/or cardiac output). These improvements in survival and oxygenation were associated with a dramatic decrease in obstruction of the conducting airways by fibrin casts, as assessed by airway microdissection and morphometric assessment of airway occlusion. At the time of euthanasia, well in excess of 50%-65% of the total luminal areas of central conducting airways were obstructed with fibrin casts in both the non-treated and placebo-treated (PBS) rats, whereas the degree of airway obstruction was nonsignificant in tPA-treated animals. Clinical respiratory distress was also markedly decreased, and activity scores markedly improved, in tPA-treated versus rats in both control groups. Alleviation of airway obstruction was further evidenced by markedly improved alveolar ventilation, as shown by greatly decreased pCO2 in ABGs obtained under anesthesia, just prior to euthanasia. This reduction in circulating carbon dioxide was associated with normalization of arterial pH. Although mainly attributable to better ventilation, improved arterial pH may also have been related to improved cardiopulmonary performance, oxygen delivery, and metabolic efficiency, as indicated by normalization of circulating plasma lactate in tPA-treated versus control groups.

We observed an overwhelming exudative airway coagulopathy induced by SMs, as shown in previous SM analog studies, leading to occlusion of airways by fibrin-containing casts (Veress et al., 2010). Previous studies by our group have also indicated that SMs may initiate this airway coagulation, via both the intrinsic (Houin et al., 2014) and extrinsic (Rancourt et al., 2012, 2013) coagulation pathways. Anticoagulants such as TF pathway inhibitor and heparin, when administered intratracheally very early (1 h) after SM analog exposure, were capable of decreasing airway cast formation and prevent 12 h mortality (Jugg et al., 2013; Rancourt et al., 2013), but could be associated with sporadic pulmonary hemorrhage with heparin. Pulmonary hemorrhage was never noted in present or previous studies of intratracheally delivered tPA given after SM exposures. In contrast to anticoagulants, which work via preventing cast formation, tPA therapy works via breaking up already formed casts. Thus, tPA treatment could be delayed for hours to even days after SM exposure, depending on level of exposure, and treatment with tPA can be initiated at the time when airway cast formation reaches the critical level causing signs and symptoms of airway obstruction. Given that victims of SM attack or accidental exposure may not be made aware of their situation for hours to days, as shown by blistering of the skin and/or ocular symptoms, tPA may be a more realistic "rescue" agent for many scenarios, where patients have a delayed presentation after exposure.

Outcome studies of humans surviving the immediate interval after SM attacks have shown that airway obstruction is the primary cause of death, and that such deaths occur usually 3–5 days, but can be delayed as much as 7–14 days, after exposure (Willems, 1989). Thus, a therapy that can be administered very late after exposure should be advantageous for saving lives of victims. Up until now, no such therapy has ever been reported. Based on our findings here, we propose airway delivered fibrinolytic drugs, like tPA, as potential rescue agents for acute SM toxicity from respiratory injury. As efficacy testing of tPA in human subjects after exposure to SM warfare agent is neither ethical nor possible, we also believe that the use of fibrinolitics after SM exposure warrant further advanced study, particularly in larger animal species with airways similar to those found in humans.

**SUPPLEMENTARY DATA**

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

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**REFERENCES**


