Role of Reactive Oxygen and Nitrogen Species in Olfactory Epithelial Injury by the Sulfur Mustard Analogue 2-Chloroethyl Ethyl Sulfide

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The inhalation of sulfur mustard (SM) causes substantial deposition in the nasal region. However, specific injury has not been characterized. 2-chloroethyl ethyl sulfide (CEES) is an SM analogue used to model injury and screen potential therapeutics. After the inhalation of CEES, damage to the olfactory epithelium (OE) was extensive. Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling-positive cells were present by 4 hours, and maximal at 18–72 hours. Cleaved caspase 3 immunohistochemistry (IHC) was maximal at 18 hours after the inhalation of 5% CEES. Olfactory marker protein (OMP)-positive olfactory neurons were markedly decreased at 18 hours. IHC-positive cells for 3-nitrotyrosine (3-NT) within epithelium were elevated by 8 hours, waning by 18 hours, and absent by 72 hours. AEOL 10150, a catalytic manganoporphyrin antioxidant, administered both subcutaneously (5 mg/kg) and intranasally (50 μ M, “combined treatment”), decreased OE injury. CEES-induced increases in markers of cell death were decreased by combined treatment involving AEOL 10150. CEES-induced changes in OMP and 3-NT immunostaining were markedly improved by combined treatment involving AEOL 10150. The selective inducible nitric oxide synthase inhibitor 1400W (5 mg/kg, subcutaneous), administered 1 hour after inhalation and thereafter every 4 hours (five doses), also reduced OE damage with improved OMP and 3-NT staining. Taken together, these data indicate that reactive oxygen and nitrogen species are important mediators in CEES-induced nasal injury.

Keywords: sulfur mustard; olfactory; cell death; oxidative stress; antioxidants

Sulfur mustard (SM; bis 2-chloroethyl sulfide) is a bifunctional alkylating and vesicating chemical warfare agent used in numerous military conflicts during the last century. SM poses a threat to both military and civilian populations because of its ease of synthesis and potential for terrorist deployment. SM is highly reactive, and forms stable adducts with cellular macromolecules (including DNA and proteins), resulting in an increase or decrease in function of key cellular components. The monofunctional SM analogue 2-chloroethyl ethyl sulfide (CEES) lacks one of the terminal chlorines, but retains similar alkylating and vesicating properties. CEES provides a useful model for investigating the mechanisms of, and screening therapeutic agents for, SM.

CLINICAL RELEVANCE

Humans inhaling sulfur mustard (SM) have manifested anosmia. Through the use of a rat nose-only inhalation model and the sulfur mustard analogue 2-chloroethyl ethyl sulfide (CEES), this study demonstrates that the inhalation of CEES results in oxidative damage to the olfactory neurons of the olfactory epithelium (OE). Treatment with antioxidants or the use of a selective inducible nitric oxide synthase inhibitor decreased OE damage. These data indicate that oxidative damage may play a role in the anosmia reported in humans after exposure to CEES, and provides the foundation for future studies of SM.

Exposure to SM in the battlefield or by civilian populations typically occurs via inhalation. Although notable ocular and skin injury occurs, those exposed will more likely succumb to respiratory damage (2). Interestingly, early studies of SM absorption indicated that 80–90% of the compound was absorbed through the nose (1). Even with percutaneous or intravenous administration of SM using ³⁵S-labeled SM, the highest concentrations were found, in descending order, in the nasal region, kidney, liver, and intestines (3). Numerous clinical studies also indicate that the inhalation of SM results in anosmia (2, 4, 5). Despite reports of high levels of SM absorption in the nose and of secondary anosmia, nasal injury after the inhalation of SM or CEES has not been characterized to date, to the best of our knowledge.

Oxidative stress plays a role in the pathophysiology of SM/CEES inhalation injury (6, 7). Oxidants are crucial for host defense, but an overabundance of oxidants can overwhelm repair and antioxidant adaptive responses, resulting in oxidative stress and injury. After exposure to SM, the alkylation and depletion of reduced glutathione contribute to an impaired ability to respond to oxidative stress (8–10). Treatment with exogenous superoxide dismutase (SOD), catalase, or antioxidant mimetics has proven beneficial in CEES-induced lung injury (6, 11–13). These data indicate a role for oxidative stress after exposure to CEES. What, if any, role oxidative stress plays in CEES-induced nasal injury remains unknown.

The nasal region serves a number of key functions, including the conditioning of incoming air, filtration, and olfactory sensation. Inspired air is both heated and humidified in the nasal passages (14). Filtration is accomplished through the impaction of inhaled particles and mucociliary clearance (15). In the nasopharyngeal region, an impaction of 5–30 μ m particles occurs, with particles smaller than 5 μ m depositing in a size-dependent manner from the trachea to the alveolar spaces (16). After the inhalation of toxicants, nasal injury tends to be site-specific, with varied depositions because of airflow patterns or specific tissue susceptibilities (17). Inhalation is highly likely to affect the olfactory epithelium, given the reported anosmia. However,

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patterns of SM nasal injury, to the best of our knowledge, have not been reported. This study sought to characterize CEES inhalation injury to the nasal region, and to investigate the role of oxidative stress in the mechanisms of injury. These studies provide the foundation for investigations of actual SM-induced injury.

MATERIALS AND METHODS

Animals

We used male Sprague-Dawley rats (Harlan, Indianapolis, IN) weighing 275–350 g. Animals were provided with food and water *ad libitum*. These experiments were approved by the Animal Care and Use Committee at National Jewish Health.

Exposure

Rats were anesthetized with ketamine (75 mg/kg), xylazine (7.5 mg/kg), and acepromazine (1.5 mg/kg). Animals were exposed to CEES for 15 minutes, as previously described (6).

Treatment Protocols

Treatment with AEOL 10150. Rats received an injection of AEOL 10150 (5 mg/kg, subcutaneous) or PBS vehicle (1 ml/kg) at 1 and 9 hours after exposure to CEES. For intranasal treatment, a 25- μ l volume at concentrations of 5 mM, 500 μ M, 250 μ M, or 50 μ M was delivered to each nare through insufflation during anesthesia with isoflurane at both 1 and 9 hours after inhalation.

Treatment with 1400W. Rats received 1400W (5 mg/kg, subcutaneous) immediately after the exposure to inhalation and every 4 hours thereafter, for a total of five doses during the 18-hour study period.

Tissue Collection

At 4, 8, or 18 hours after exposure, animals were killed with 130 mg Sleepaway per animal (Fort Dodge Animal Health, Fort Dodge, IA). Heads were removed by guillotine and skinned. The lower jaw and musculature were removed, and heads were placed in 4% paraformaldehyde in PBS for 48 hours. After fixation, heads were decalcified (Immunocal; Decal Chemicals, Tallman, NY) for 7 days. Transverse sections of nasal passages were removed, based on the methods of Young (18). The section representative of olfactory epithelium, T3, was cut between the second palatal ridge and the middle of the first molars.

In Situ Cell Death Detection Using Terminal Deoxynucleotidyl Transferase–Mediated dUTP Nick-End Labeling Assay

Cell death was detected using the DeadEnd Colorimetric TUNEL Kit (Promega, Madison, WI). Briefly, antigen retrieval was performed using proteinase K (30 μ g/ml) for 10 minutes. Sections were incubated with terminal deoxynucleotidyl transferase for 1 hour at 37°C. An application of streptavidin–horseradish peroxidase (HRP) for 30 minutes was followed by detection using 3,3'-diaminobenzidine (DAB). Four fields at the entrances to the ethmoturbinates were counted. The apoptotic index was determined by counting apoptotic cells $\times$ 100 and dividing by total cells in a field.

Immunohistochemistry

Antigen retrieval was performed using a decloaking chamber (Biocare Medical, Concord, CA) in 10 mM sodium citrate buffer, pH 6.0, except for cleaved caspase 3 (CC3), which used 1 mM EDTA buffer, pH 8.0. Slides were blocked with 0.3% H₂O₂, and incubated in primary antibody to CC3 (1:50; US Biologicals, Swampscott, MA), olfactory marker protein (OMP; 1:200; Abcam ab62144; Abcam, Cambridge, MA), or 3-nitrotyrosine (3-NT; 1:300; Millipore AB5532; Millipore, Billerica, MA). Secondary antibody (biotinylated anti-rabbit IgG; Vector Laboratories, Burlingame, CA) was followed by the avidin–biotin complex (Vector Laboratories) for OMP and 3-NT, or streptavidin–HRP for CC3. IgG-negative controls were generated.

Data Analysis

Data are presented as means $\pm$ SEM. Comparisons between multiple groups were performed using one-way ANOVA and the Tukey test for

post hoc analysis (GraphPad Prism version 4.0c; GraphPad, San Diego, CA), with significance set at $P \leq 0.05$.

RESULTS

Nasal Injury and Cell Death in the Olfactory Epithelium Attributable to the Inhalation of CEES

Investigation of the T3 section of the nasal cavity indicated that damage was most prominent in the olfactory epithelium (OE) at the medial meatus of the septum, and at the entrances to the ethmoturbinates (ETs) (Figure 1, *red*). For consistency, all sections were photographed at the entrance to the ETs. Figure 2A shows a normal, organized OE layer from an animal that inhaled ethanol (EtOH) 18 hours after exposure. At 4 hours after the inhalation of CEES, the organization of the OE layer remained intact (Figure 2B), although pyknosis and nuclear clearing are evident. By 18 hours after the inhalation of CEES, marked disorganization of the full epithelial layer had occurred (Figure 2C).

Cell death in the OE was investigated using terminal deoxynucleotidyl transferase–mediated dUTP nick-end labeling (TUNEL). The OE of rats exposed to EtOH shows that a low number of TUNEL-positive, DAB-stained cells was present constitutively (see Figure E1A in the online supplement). By 4 hours after the inhalation of CEES, TUNEL-positive cells were also present (Figure E1B). TUNEL-positive cells were increased by the 18-hour time point (Figure E1C). Numbers of TUNEL-positive and TUNEL-negative cells were counted in four high-power fields at the edges of the ETs ($\times$ 400 total magnification), to calculate the percentages of TUNEL-positive cells. As shown in Figure 2D, the percentage of TUNEL-positive cells had significantly increased after the inhalation of CEES in a time-dependent manner at the 4-hour and 18-hour time points, compared with rats exposed to EtOH.

The cleavage of cysteine–aspartic acid proteases, or caspases, commits a cell to apoptosis. CC3 is a terminal caspase. Its cleavage is indicative of apoptosis, and complements TUNEL

Figure 1. Representation of nasal cross section comprised predominantly of olfactory epithelium (OE). The region in *red* shows consistently high damage after the inhalation of sulfur mustard analogue 2-chloroethyl ethyl sulfide (CEES). The septum along the middle of the nasal cavity, and especially the medial meatus of the septum (MS), exhibited marked injury. The entrances to the ethmoturbinates (ET) near E1 and E2, and the more medial E3, were regions of most severe damage.

Figure 2. Characterization of CEES-induced injury to the OE. Compared with rats inhaling ethanol (EtOH) (A), rats inhaling CEES show early cellular degeneration by 4 hours after inhalation (B), and marked apical disruption by 18 hours (C). CEES-induced cell death, as measured by TUNEL, was significantly increased over rats inhaling EtOH at 4 and 18 hours (D; $n = 3$, mean $\pm$ SEM). (E) Cleaved caspase 3 (CC3) staining was significantly increased over EtOH controls at 18 hours ($n = 3$, mean $\pm$ SEM). (F) Staining for olfactory marker protein (OMP), a specific marker for olfactory neurons, was normally distributed in the mid-OE layer (arrows) after the inhalation of EtOH. (G) Because minimal change was evident at 4 hours, an 8-hour time point was investigated and showed decreased OMP-positive staining. (H) By 18 hours, some punctate OMP staining was evident, but was in decline. 3-nitrotyrosine (3-NT) staining was absent in EtOH-exposed animals (I), but was prominent 8 hours after exposure to CEES (J). (K) By 18 hours, 3-NT staining was waning, but still present. Bars = 50 μ m.

staining as a marker of cell death. A low level of CC3 staining is present after the inhalation of EtOH (Figure E1D). Cells staining positive for CC3 were also present by 4 hours after the inhalation of CEES (Figure E1E). The number of CC3-positive cells increased by 18 hours after the inhalation of CEES (Figure E1F). The counting of CC3-positive cells indicated a significant increase at the 18-hour time point after the inhalation of CEES, compared with EtOH (Figure 2E).

Olfactory neuronal cell bodies at various levels of maturity are present below the more apical sustentacular cell bodies, or support cell layer. OMP is expressed specifically in the cytosol of olfactory neurons (19). In Figure 2F, arrows indicate that dark Nova Red-stained, OMP-positive olfactory neurons are located along the central region after the inhalation of EtOH. After exposure to CEES, the loss of this region is evident. No changes were noted at the 4-hour time point (not shown). At 8 hours after the inhalation of CEES (Figure 2G), the OMP-positive region was present but beginning to lose the structured organization seen in rats exposed to EtOH. By 18 hours after the inhalation of CEES, punctate OMP-positive regions were evident but greatly reduced in number (Figure 2H).

Increased Oxidative Stress in the OE Is Attributable to the Inhalation of CEES

3-NT is a stable end product of the formation of peroxynitrite, and is commonly used as a marker for the formation of reactive nitrogen species (RNS). No constitutive 3-NT staining was evident in rats exposed to EtOH (Figure 2I). No changes were

evident at 4 hours after the inhalation of CEES (not shown). Eight hours after exposure to CEES, an increased deposition of 3-NT was evident in the OE (Figure 2J). The deposition of 3-NT was present 18 hours after the inhalation of CEES, but appeared to be declining as of 8 hours (Figure 2K).

Neutrophils Were Minimally Evident at Sites of Injury after the Inhalation of CEES

Polymorphonuclear leukocytes (PMNs) are commonly implicated in inflammatory tissue damage, especially when present at high levels. Esterase staining can be used to detect neutrophils. Figure E2 demonstrates the presence or absence of these cells, indicating minimal neutrophil involvement after the inhalation of CEES. After the inhalation of EtOH, no PMNs were evident (Figure E2A). No PMNs were noted near the injured OE ETs by 8 hours after exposure to CEES (Figure E2B). PMNs were observed to be extravasating by 18 hours after the inhalation of CEES in small numbers (2–6 per $\times 400$ field), as indicated by arrows in Figure E2C.

AEOL 10150 Treatments and OE Damage

Previous studies indicated that treatment with AEOL 10150 resulted in improved outcomes in lung injury markers (6). Because the maximal histopathological effect was evident at 18 hours after the inhalation of CEES, this time point was chosen to investigate the therapeutic effects of AEOL 10150 in the nose. Hematoxylin-and-eosin-stained sections indicated severe apical damage to the OE after exposure to CEES only

(Figure E3A) or CEES combined with PBS (diluent) treatment, administered both subcutaneously (1 ml/kg) and intranasally (25 μ l per nare) at 1 and 9 hours after the inhalation of CEES (Figure E3B). In contrast with previous studies in the lung, AEOL 10150 delivered only by the subcutaneous route (5 mg/kg, 1 and 9 hours after the inhalation of CEES) did not improve nasal injury (Figure E3C). Based on effective dosing *in vitro*, AEOL 10150 delivered intranasally (50 μ M, 25 μ l per nare) alone was not effective against CEES-induced nasal injury (Figure E3D) (7). A combination of subcutaneous and intranasal delivery of AEOL 10150 was then investigated, with the subcutaneous dose maintained at 5 mg/kg, and with intranasal dose concentrations of 5 mM, 500 μ M, 250 μ M, or 50 μ M. Both subcutaneous and intranasal treatments were administered at 1 and 9 hours after the inhalation of CEES. The delivery of intranasal concentrations of 5 mM (Figure E3E), 500 μ M (Figure E3F), or 250 μ M (Figure E3G) did not appear to result in improvement. Treatment with 50 μ M AEOL 10150 did result in less apical damage, and was investigated further (Figure E3H), and is hereafter referred to as “combined AEOL 10150 treatment.”

Combined AEOL 10150 Treatment Decreased Histopathology Severity Scores

The double-blind scoring of apoptosis, apical damage, basement membrane injury, the presence of edema, and overall severity of injury was measured around four regions of the entrances to the ETs. All injuries were graded as either absent (–) or present (+). Animals exposed to EtOH showed no signs of histologic injury in any of the five markers investigated. Rats exposed to CEES alone showed increased apical membrane disruption (“ruffling”), evidence of edema, areas of basement membrane disruption, regions of pyknotic cells indicative of apoptosis, and overall increased injury, evident in the four entrances to the ETs. When animals received intranasal and subcutaneous treatment with AEOL 10150, levels of apical membrane disruption showed some improvement. Pyknotic cells, indicative of apoptosis, had also decreased in some areas. No appreciable change was evident in edema as a result of AEOL 10150 combined treatment, although areas of basement membrane compromise were not evident, compared with rats only exposed to CEES. The overall severity of injury was improved with AEOL 10150 combined treatment, primarily because of the appearance of decreased apical membrane disruption and decreased apoptosis.

Impact of Combined AEOL 10150 Treatment on OE Cell Death, Neuronal Loss, and Nitrotyrosine

Hematoxylin-and-eosin–stained sections were obtained after treatment with CEES alone (Figure 3A), after treatment with CEES combined with PBS (Figure 3B), or after treatment with CEES combined with AEOL 10150 (Figure 3C). TUNEL-positive cells had increased 18 hours after exposure to CEES alone (Figure 3D) and after exposure to CEES combined with PBS, 1 and 9 hours after exposure (Figure 3E), and appeared to decrease with combined AEOL 10150 treatment (Figure 3F). The quantitation of DNA nick-end labeling shows that AEOL 10150 combined treatment, administered 1 and 9 hours after the inhalation of CEES, resulted in decreased TUNEL-positive staining, compared with CEES alone or combined treatment with CEES and PBS (Figure 3G).

A similar concentration of DAB-stained, CC3-positive cells was evident 18 hours after the inhalation of CEES with either CEES alone (Figure 3H) or CEES with PBS, 1 and 9 hours after exposure (Figure 3I). CEES with combined AEOL 10150

treatment, 1 and 9 hours after exposure, appeared to result in decreased CC3 staining (Figure 3J). The quantitation of CC3-positive cells showed a significant decrease with AEOL 10150 combined treatment administered 1 and 9 hours after the inhalation of CEES, compared with CEES alone or combined treatment with CEES and PBS (Figure 3K).

A decrease in OMP-positive staining cells was evident 18 hours after the inhalation of CEES with either CEES alone (Figure 3L) or CEES combined with PBS, 1 and 9 hours after exposure (Figure 3M). CEES with combined AEOL 10150 treatment (Figure 3N) preserved olfactory neurons, as evidenced by OMP staining in the OE.

3-NT is a marker of reactive nitric oxide metabolites, including peroxynitrite. CEES alone or CEES combined with PBS (Figures 3O and 3P, respectively) produced similar levels of 3-NT staining, whereas CEES with AEOL 10150 combined treatment produced decreased 3-NT staining in the OE (Figure 3Q).

Treatment with the Selective Inducible Nitric Oxide Synthase Inhibitor 1400W Improved OE Damage 18 Hours after the Inhalation of CEES

N-(3-(aminomethyl) benzyl) acetamidine (1400W) is a novel selective inhibitor of inducible nitric oxide synthase (iNOS) (20). Figure 4 demonstrates the impact of iNOS inhibition with 1400W treatment 18 hours after the inhalation of CEES, compared with rats receiving PBS after the inhalation of CEES. When rats inhaled CEES and received PBS (1 ml/kg) immediately and then every 4 hours after exposure to CEES, the loss of OE structural integrity was apparent (Figure 4A). In contrast, when rats received 1400W (5 mg/kg) immediately after exposure to CEES and then every 4 hours, the structural integrity of the OE was maintained (Figure 4B).

Cell death was measured by both TUNEL staining and the more apoptosis-specific CC3 immunohistochemistry staining. The inhalation of CEES with subsequent PBS treatment resulted in the presence of TUNEL-positive cells 18 hours after the inhalation of CEES (Figure 4C). TUNEL positivity was reduced when exposure to CEES was followed by 1400W treatment (Figure 4D). The quantitation of DNA nick-end labeling shows that treatment with 1400W resulted in decreased TUNEL-positive staining, compared with CEES combined with PBS (Figure 4E).

CC3 positive cells were evident after the inhalation of CEES followed by treatment with PBS, as detected 18 hours after exposure (Figure 4F). CC3-positive cells were evident with 1400W treatment, but were less prominent in the region near the basement membrane (Figure 4G). As shown in Figure 4H, treatment with 1400W after the inhalation of CEES resulted in a significant decrease in CC3-positive cells, compared with CEES combined with PBS.

Representative OMP staining after treatment with either PBS or 1400W is also shown. The darker Vector Red–stained regions of OMP staining were evident at the center of the OE when rats inhaled EtOH (Figure 4I, arrow). When rats inhaled CEES and then received PBS at 1 hour and then every 4 hours, areas of decreased olfactory neurons, as indicated by reduced OMP staining, were evident (Figure 4I). In contrast, when rats received 1400W in the same dosing protocol, the olfactory neuronal layers were visible and were more organized 18 hours after the inhalation of CEES (Figure 4J).

Representative 3-NT staining after the inhalation of CEES with either PBS or 1400W is also shown. When rats inhaled CEES and then received PBS at 1 hour after inhalation injury

Figure 3. Treatment with AEOL 10150 decreased CEES-induced injury to the OE. Rats receiving CEES alone (A) or CEES combined with PBS (both subcutaneously and intranasally) (B) caused extensive epithelial apical disruption. (C) AEOL 10150 combined treatment (both subcutaneously and intranasally) resulted in decreased apical disruption. (D and E) TUNEL staining was evident in both CEES and CEES combined with PBS. (F) A reduction in TUNEL-positive cells was evident with AEOL 10150 combined treatment. (G) This decrease was significant ($n = 3$, mean $\pm$ SEM). (H and I) CC3-positive cells were similarly evident after both CEES alone and CEES combined with PBS. (J) CC3-positive cells were evident after AEOL 10150 combined treatment. (K) CC3-positive cells were significantly decreased by AEOL 10150 versus CEES alone ($n = 3$, mean $\pm$ SEM). (L and M) OMP staining was punctate and disorganized with either CEES alone or CEES combined with PBS. (N) OMP staining, indicating olfactory neurons, was better preserved when treatment with AEOL 10150 followed the inhalation of CEES. 3-NT staining was also evident after CEES alone (O) and CEES combined with PBS (P). (Q) 3-NT staining was reduced in the epithelium with AEOL 10150 combined treatment, although 3-NT staining remained evident in the lamina propria. Bars = 50 μ m.

and then every 4 hours, diffuse areas of 3-NT staining were evident in the epithelium and lamina propria (Figure 4K). When rats received 1400W in the same dosing protocol as the vehicle, areas of 3-NT staining were less evident in the OE 18 hours after the inhalation of CEES (Figure 4L).

Plasma Concentrations of Nitrate and Nitrite after Inhalation of CEES Alone or in Conjunction with AEOL 10150 or 1400W Treatment

As illustrated in Figure E4, plasma concentrations of nitrate and nitrite after the inhalation of CEES were significantly increased over the concentrations in control rats exposed to EtOH. Treatment with AEOL 10150, both intranasally and subcutaneously, did not significantly alter CEES-induced concentra-

tions of nitrate and nitrite. Treatment with 1400W significantly decreased plasma concentrations of nitrate and nitrite, compared with animals that only inhaled CEES.

DISCUSSION

Previous studies showed that the accumulation of SM is greatest in the nasal region after exposure to inhalation (1). 35 S-labeled SM, delivered percutaneously or intravenously, also resulted in high levels of deposition in the nasal region (3). Despite this knowledge, specific injury to the nasal region has never been characterized, to the best of our knowledge. The present study indicates that inhalation of the SM analogue CEES resulted in marked damage to the OE, which may explain the SM-induced

Figure 4. The selective inducible nitric oxide synthase (iNOS) inhibitor 1400W decreased CEES-induced injury to the OE. (A) After the inhalation of CEES, apical epithelial disorganization was evident in the OE of rats receiving PBS. (B) In contrast, rats inhaling CEES and receiving treatment with 1400W maintained their OE structural organization. (C) TUNEL-positive cells were present after CEES inhalation with PBS treatment. TUNEL-positive cells were also present when the inhalation of CEES was followed by treatment with 1400W (D), and TUNEL-positive were decreased with 1400W treatment compared with PBS treatment (E; $n = 3$, mean $\pm$ SEM). (F) CC3-positive cells were evident throughout the OE with PBS treatment. CC3-positive cells were still apparent when the inhalation of CEES was followed by treatment with 1400W (G), but CC3-positive cells were decreased with 1400W versus PBS treatment (H; $n = 3$, mean $\pm$ SEM). (I) OMP-stained OE was disorganized by the inhalation of CEES followed by treatment with PBS. (J) OMP staining indicated that olfactory neurons were preserved by treatment with 1400W. 3-NT staining was evident with the inhalation of CEES and treatment with PBS (K), whereas the inhalation of CEES and treatment with 1400W resulted in reduced, but not absent, 3-NT staining in OE (L). Bars = 50 μ m.

anosmia after exposure. Treatment with AEOL 10150, delivered as a combination of intranasal and subcutaneous dosing, improved nasal injury indices. The inhibition of iNOS, using the selective inhibitor 1400W, also reduced cell damage in the OE, indicating that peroxynitrite and/or other toxic metabolites of NO may play a role in nasal injury. Taken together, these data

confirm that oxidative stress plays a role in CEES-induced nasal pathology.

In recent years, investigations of the histopathology of nasal injury have increased, with reports of both inhaled and systemically delivered compounds resulting in nasal injury. For example, systemically delivered cancer chemotherapeutics such as

vincristine sulfate were shown to induce the selective apoptosis of olfactory neurons, with subsequent atrophy of the OE (21). Systemic methimazole, an antithyroid drug, also results in extensive damage to the OE sustentacular cells and to Bowman's glands in the lamina propria, because of the high concentrations of cytochrome P450 in these cell types and the P450-mediated metabolism of methimazole (22). The inhalation of a mixture of dibasic esters also selectively targets sustentacular cells, although exposure ultimately results in the denudation of OE 24 hours after exposure (23). The intranasal instillation of a single low dose of the tricothecene mycotoxin satratoxin G (derived from the black mold *Stachybotrys chartarum*) resulted in apoptosis of the olfactory neurons (24). Non-cell type-specific damage to the OE was also evident after the inhalation of chlorine or sulfur dioxide (25, 26). All of these compounds result in varying degrees of cell death to various cell types in the OE.

Because olfactory sensory neurons (OSNs) are in direct contact with the environment, inhaled toxins such as CEES can directly induce injury or cell death (27). Marked changes were evident in the OE after the inhalation of CEES. Although changes by 4 hours after inhalation were minimal according to histology, by 18 hours after inhalation, marked changes in OE structural integrity were noted. Cell death in the OE occurred after the inhalation of CEES. This result was demonstrated using both the histochemical assessment of the cleavage of caspase 3, and the detection of DNA fragmentation using TUNEL staining. In the present study, fragmented DNA, as measured by TUNEL staining, was significantly increased at 4 and 18 hours after the inhalation of CEES, compared with the inhalation of EtOH. In contrast, staining for CC3, an early-phase apoptosis marker, had significantly increased only 18 hours after inhalation. Cytosolic olfactory marker protein concentrations were decreased 18 hours after the inhalation of CEES, indicating damage to the OSNs. Cell death and the specific loss of OSNs after the inhalation of CEES may provide an explanation for how anosmia occurs after the inhalation of SM.

Reactive oxygen species (ROS), derived from high concentrations of inflammatory cells such as PMNs, are often contributing factors in oxidative stress. However, an investigation of the nasal region at multiple time points indicated that few PMNs were present in the injured area, and are thus not primarily responsible for oxidative stress in nasal injury. Given the extensive cell death in the OE, and the possibility that not all cell death is attributable to controlled apoptosis, mitochondrial dysfunction attributable to dying cells may constitute a major contributor to OE oxidative stress. To investigate further the role of oxidative stress in CEES-induced damage to the OE, studies with the catalytic antioxidant manganoporphyrin AEOL 10150 were undertaken.

AEOL 10150 is an antioxidant effective in scavenging superoxide, hydrogen peroxide, peroxynitrite, and lipid peroxides (28–31). The formation of 3-NT after 18 hours was decreased by AEOL 10150, suggesting that the scavenging of superoxide and/or peroxynitrite may have played an important role in the improved outcomes. Another possible mechanism by which manganoporphyrins confer protection may involve the inhibition of NO production (32). AEOL 10150 was shown to redox-cycle with flavin-dependent oxidoreductases, including nitric oxide synthases (33). Although redox cycling is generally considered deleterious, AEOL 10150 and similar compounds can scavenge superoxide produced at the site and thus may reduce injury. In addition, AEOL 10150 may mitigate leak of superoxide attributable to mitochondrial dysfunction. Previous *in vitro* studies indicated that exposure to CEES results in decreased mitochondrial potential (7). Insofar as measureable cell death occurs in the OE, mitochondrial dysfunction and the

resultant production of ROS may also have occurred *in vivo* in the present model. iNOS may constitute another source of ROS, because that enzyme is known to produce superoxide under basal conditions (34).

Treatment with AEOL 10150, when administered only subcutaneously or intranasally, was not protective against CEES-induced nasal injury. When subcutaneous treatment was bolstered with intranasal treatment, a relatively low (50 μ M) concentration (AEOL 10150 combination treatment) was effective in reducing nasal injury. As the concentration of AEOL 10150 administered via the intranasal route increased, no visible protection was afforded. This finding is consistent with those in previous studies reporting a hormetic, bell-shaped curve, with beneficial tissue-protective effects increasing and then waning as exogenously administered concentrations of SOD increased (35). Although we did not demonstrate that the effect of higher intranasal concentrations of AEOL 10150 was toxic above and beyond CEES-induced injury, we did see a clear lack of efficacy. The 50- μ M concentration was also most effective at preventing CEES-induced epithelial cell injury in a previous *in vitro* model (36). OE histologic markers were significantly decreased with AEOL 10150 combination treatment, compared with either CEES alone or CEES combined with PBS. In addition, AEOL 10150 combination treatment decreased cell death in the OE. Olfactory neurons appeared to be protected 18 hours after the inhalation of CEES when combined AEOL 10150 treatment was administered, and 3-NT staining was reduced. These data indicate that damage to the OE was decreased through the combination of subcutaneous and intranasal treatments with AEOL 10150.

The generation of even a moderate level of ONOO[−] over extended periods of time may result in cellular dysfunction, the disruption of cell signaling pathways, and the induction of cell death through both apoptosis and necrosis (37). Previous studies demonstrated an increase in iNOS activity after the administration of SM in keratinocytes in a time-dependent and dose-dependent manner (38). In an *in vitro* model of exposure to SM in primary chick neurons, the nonspecific NOS inhibitor L-NAME conferred significant protection, implying a role for NO (39). In the present study, the formation of 3-NT increased in the OE after the inhalation of CEES, indicative of increased production of RNS. Therefore, we investigated whether the inhibition of iNOS would result in improved outcomes in the OE.

N-(3-(aminomethyl)benzyl) acetamidine (1400W) is a tightly binding, extremely slowly reversible inhibitor of iNOS. 1400W has a 1,000-fold greater selectivity for iNOS than for eNOS (20). In contrast to bithioureas (shown to be biologically inactive) and arginine analogues (which are nonspecific for the various NOS isoforms), 1400W is both biologically active *in vivo* and selective for iNOS (20). 1400W significantly decreased TUNEL-positive and CC3-positive cells. Olfactory neurons, as indicated by OMP staining, were substantially better preserved by treatment with 1400W compared with PBS after the inhalation of CEES, suggesting that a decrease in iNOS activity is beneficial to the survival of olfactory neurons (40). Although 3-NT staining was still evident after treatment with 1400W, it appeared to have decreased in the epithelium. Overall, the inhibition of iNOS by treatment with 1400W decreased apparent nasal injury, implying a significant role for RNS in CEES-induced pathology.

Previous studies indicated that a metalloporphyrin compound similar to AEOL 10150 was effective at reducing the activity of NOS (32). The present study indicated, at least systemically, that treatment with AEOL 10150 was not effective at decreasing CEES-induced concentrations of nitrate and nitrite. This finding indicates that mechanistically, any improvement in nasal injury

Figure 5. Potential mechanism of CEES-induced OE injury and protection by AEOL 10150 or 1400W. CEES-induced OE injury may result from enhanced activation of iNOS or superoxide release because of mitochondrial dysfunction, or both. AEOL 10150 can detoxify superoxide and peroxynitrite. 1400W can inhibit iNOS, decreasing concentrations of nitric oxide and potentially superoxide, which could decrease the production of peroxynitrite.

derived from treatment with AEOL 10150 was likely related to either superoxide or peroxynitrite scavenging. In contrast, 1400W did decrease systemic CEES-induced nitrate and nitrite concentrations, indicating that a reduction of iNOS activity was effective and caused beneficial effects in protecting nasal epithelium. A schematic diagram of the potential modes of action of AEOL 10150 and 1400W in the reduction of CEES-induced nasal injury is presented in Figure 5.

The interpretation of these data in the context of human inhalation must be performed with caution. In contrast to humans, who have a poorly developed sense of smell (microsmatic), rodents have a highly developed sense of smell (macrosmatic) (27). These differences are especially obvious when considering the percentage of total nasal epithelium committed to olfaction in humans (3%), compared with that in rats (~50%) (27, 41). Although differences exist between species, the loss of sense of smell after the inhalation of SM in humans is likely attributable to a loss of olfactory neurons. Our data provide strong support for the concept that RNS and/or ROS are critical in the pathogenesis of injury to the OE after inhalation of the SM analogue CEES.

Author Disclosure: B.J.D. served as a consultant for and owns stock in Aeolus Pharmaceuticals, has received reimbursement for lectures from Kyowa Hakko, and has a patent with National Jewish Health for treatments that rescue injury from alkylating species. None of the other authors have a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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