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·Report·

Acute systemic accumulation of acrolein in mice by inhalation at a concentration similar to that in cigarette smoke

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ABSTRACT

Cigarette smoke is an important environmental factor associated with a wide array of public health concerns. Acrolein, a component of tobacco smoke and a known toxin to various cell types, may be a key pathological factor mediating the adverse effects linked with tobacco smoke. Although acrolein is known to accumulate in the respiratory system after acute nasal exposure, it is not clear if it accumulates systemically, and less is known in the nervous system. The aim of this study was to assess the degree of acrolein accumulation in the circulation and in the spinal cord following acute acrolein inhalation in mice. Using a laboratory-fabricated inhalation chamber, we found elevated urinary 3-HPMA, an acrolein metabolite, and increased acrolein adducts in the spinal cord after weeks of nasal exposure to acrolein at a concentration similar to that in tobacco smoke. The data indicated that acrolein is absorbed into the circulatory system and some enters the nervous system. It is expected that these findings may facilitate further studies to probe the pathological role of acrolein in the nervous system resulting from smoke and other external sources.

Keywords: tobacco; lipid peroxidation; urine; liquid chromatography; mass spectrometry

INTRODUCTION

Cigarette smoking is one of the most prominent and preventable causes of illness in the United States[1]. It is associated with a wide array of public health concerns including cancer[2], cardiovascular disease[3], and pulmonary disease[4]. Consequently, much effort has been made to identify compounds responsible for the adverse effects of smoking and the list continues to grow[5]. Acrolein is a known cytotoxin produced by burning tobacco, and is capable of inflicting serious harm in the respiratory system[6-8]. Long-term (years) cigarette smoking can result in significant systemic accumulation of acrolein in humans[9]. However, it is not yet clear if acute smoking leads to a similar buildup of acrolein. In addition, the degree of adverse effects of cigarette-derived acrolein on the nervous system, either acute or long-term, has not yet been fully elucidated; only correlative clinical studies are available, and animal models have been insufficiently investigated. This uncertainty has hindered the understanding of the adverse effects of smoking on neurodegenerative diseases and trauma.

Acrolein, a well-known component of tobacco smoke[10], is a pro-inflammatory and pro-oxidative stress aldehyde[11, 12], and is known to damage proteins, DNA, and lipids in various cell types including neurons, hepatocytes, epithelial cells, cardiac muscle cells, and kidney cells[4, 12-18]. In addition, acrolein has been linked to cancer[8, 19-22], spinal cord trauma[11, 23-26], Alzheimer's disease[27-29], amyotrophic lateral sclerosis[30], multiple sclerosis[31], and even aging[32] and pollution[33, 34]. Considering this, we speculated that acrolein generated by cigarette smoking could serve as a significant neuropathological factor. Along with other known toxins, either synergistically or additively, acrolein could

adversely influence the function of the nervous system, as has already been demonstrated in a variety of other organs such as the heart, kidney, lung, and liver[15, 16]. To discern the specific pathological role of cigarette-derived acrolein in the nervous system, it is critical to elucidate the efficiency of its systemic absorption with respiratory exposure. However, despite the clinical observations of an increased acrolein metabolite among smokers[9, 35], and the acute accumulation of acrolein in the respiratory system in both human and animal studies[36-38], no animal or clinical correlative study has been conducted linking acute exposure to systemic accumulation. Consequently, quantification of systemic absorption and accumulation of acrolein following inhalation has yet to be ascertained.

To this end, the current study tested the hypothesis that acute nasal exposure to acrolein can cause its accumulation within the circulatory system in a mouse model, with a particular interest in the possibility of acrolein accumulation in the nervous system in light of its emerging recognition as a pathological factor in both neuronal trauma and degenerative diseases[11, 12].

MATERIALS AND METHODS

Experimental Animals

This study was approved by and carried out in accordance with the guidelines established by the Purdue University Animal Care and Use Committee. Female C57BL/6 mice were purchased from Harlan Laboratories (Indianapolis, IN) and managed by the laboratory animal housing facilities.

Inhalation System Design and Setup

A laboratory-fabricated air-tight Plexiglas chamber (25.4 cm x 35.56 cm x 15.24 cm) was housed in a chemical ventilation hood (Fig. 1). Aerosolized acrolein was delivered to the chamber from two gas cylinders, one a ~350 ppm mixture of acrolein and air (Praxair, Geismar, LA) and one of compressed air (Indiana Oxygen, Indianapolis, IN). The desired concentration was achieved by controlling the flow from each tank using two flowmeters (Aalborg, Orangeburg, NY). Specifically, the air-flows of the acrolein and air tanks were mixed and the final concentration of acrolein in the chamber was adjusted to 1.5 ppm, a level confirmed by gas chromatography/mass spectrometry (GC/MS) based on the procedures described below. Samples from the chamber were collected into a vacutainer (BD, Franklin Lakes, NJ) and analyzed by MS to verify the concentration. Expired air from the chamber passed through an outlet valve and then through an activated-charcoal adsorption filter (VetEquip, Pleasanton, CA) into a chemical ventilation hood.

Gas Chromatography/Mass Spectrometry

Analysis was performed using a Pegasus 4D gas

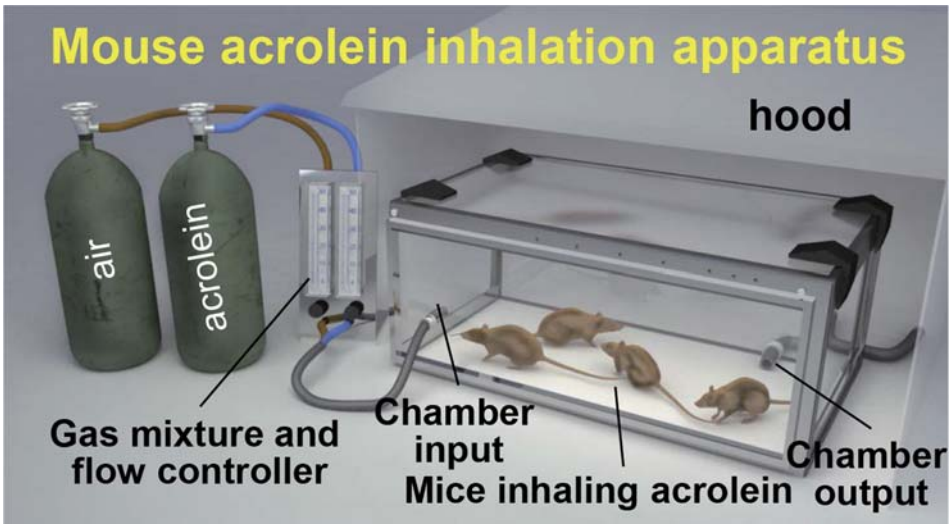

Fig. 1. The inhalation setup. Two gas cylinders, 350 ppm acrolein:air mixture and compressed air, were connected to the chamber inlet. Concentration was controlled by adjusting flow using two flowmeters. A chamber housed the mice and was placed in a chemical ventilation hood. Expired air from the chamber flowed through an outlet connected to a charcoal adsorption filter.

chromatography/gas chromatography time-of-flight mass spectrometer (GCxGC/TOF-MS, LECO Corp., St. Joseph, MI), with a CTC CombiPAL autosampler (LEAP Technologies, Carrboro, NC). The autosampler was operated in headspace mode, with a sample volume of 0.5 mL. The samples were agitated at 80°C for 5 min at 500 r/min prior to injection. The GC column was an Rtx-65 capillary column (Restek, 30 m × 0.25 mm × 0.25 μm). High-purity helium was used as the carrier gas (1.0 mL/min), with a 10:1 split ratio. The temperature program began at 40°C with a hold-time of 3 min and then increased to 140°C at a rate of 20°C/min. The injection inlet temperature was 130°C and the mass spectrometer transfer line temperature was 200°C. The electron impact ion source was held at 200°C, with a filament bias of −70 V. Mass spectra were collected from 23 to 200 *m/z* at 30 spectra/s. Acrolein standards were made between 33 and 3300 ppm. Quantification was based on the sum of mass peaks 55 and 56. The retention time for acrolein was 107 s.

Nasal Acrolein Exposure

Mice were randomized into control, sham, and acrolein groups. The sham group inhaled ambient air and the acrolein group inhaled the acrolein:air mixture; each group was placed in the chamber for inhalation for 30 min, twice a day, for three weeks. The control group was not placed in the chamber and inhaled ambient air only. Urine was collected weekly for quantification of the acrolein metabolite 3-HPMA. On day 21, mice from all groups were anesthetized with a ketamine-xylazine mixture and then perfused with oxygenated Krebs solution. The spinal cord was then extracted for dot immunoblotting.

Dot Immunoblotting

The extracted spinal cord segments were incubated with 1% Triton X and protease inhibitor cocktail at a 100:1 ratio (Sigma-Aldrich, St. Louis, MO) and homogenized with a glass homogenizer (Kontes Glass Co., Vineland, NJ). The sample was then incubated on ice for at least 1 h, followed by centrifugation at 13 500g for at least 30 min at 4°C. Samples were stored at –80°C. One additional round of centrifugation at 13 500g was performed after removal from storage.

Prior to analysis, BCA protein assays were performed to ensure equal loading. Samples were transferred simultaneously to a nitrocellulose membrane using a Bio-Dot SF microfiltration apparatus (Bio-Rad, Hercules, CA). The membrane was then blocked for 1 h with 0.2% casein and 0.1% Tween 20 in PBS, and then incubated with 1:1 000 primary rabbit anti-acrolein antibody (Novus Biologicals) (in blocking buffer with 2% goat serum and 0.025% sodium azide) for 18 h at 4°C. The membrane was washed three times (10 min each) in blocking buffer before transfer to 1:10 000 secondary alkaline phosphatase-conjugated goat anti-rabbit IgG antibody (Vectastain ABC-AmP Kit, Vector Laboratories, Burlingame, CA) for 1 h at room temperature. The membrane was then washed three times (10 min each) in blocking buffer followed by 0.1% Tween 20 in Tris-buffered saline before being exposed to Bio-Rad Immuno-Star substrate and visualized by chemiluminescence. The density of dots was evaluated using ImageJ (NIH, Bethesda, MD).

3-HPMA Quantification in Urine

Urine was collected using a metabolic cage (Fig. 2B). Specifically, ~1 mL was collected in a period of 12 h once per week. The urine was then stored at –80°C until analysis. 3-Hydroxypropyl mercapturic acid (3-HPMA) was measured in urine according to the description by Eckert *et al.*[39] and our previous publication[40]. Solid phase extraction with Isolute ENV+ cartridges (Biotage, Charlotte, NC) was used to prepare each sample before LC/MS/MS analysis. Each cartridge was conditioned with 1 mL methanol, followed by 1 mL water, then 1 mL 0.1% formic acid in water. A volume of 500 μL urine was spiked with 200 ng deuterated 3-HPMA (d3-3-HPMA; Toronto Research Chemicals, Toronto, ON, Canada) and mixed with 500 μL 50 mmol/L ammonium formate and 10 μL undiluted formic acid. This mixture was immediately loaded onto the prepared ENV+ cartridges. Each cartridge was washed twice with 1 mL 0.1% formic acid, followed by 1 mL 10% methanol/90% 0.1% formic acid in water. The cartridges were dried with nitrogen gas and subsequently eluted with three volumes of 600 μL methanol + 2% formic acid. The eluates were combined and dried with a rotary evaporation device. Each sample was then reconstituted in 100 μL 0.1% formic acid before LC/MS/MS analysis.

An Agilent 1200 Rapid Resolution liquid chromatography (LC) system coupled to an Agilent 6460 series QQQ mass spectrometer was used to analyze

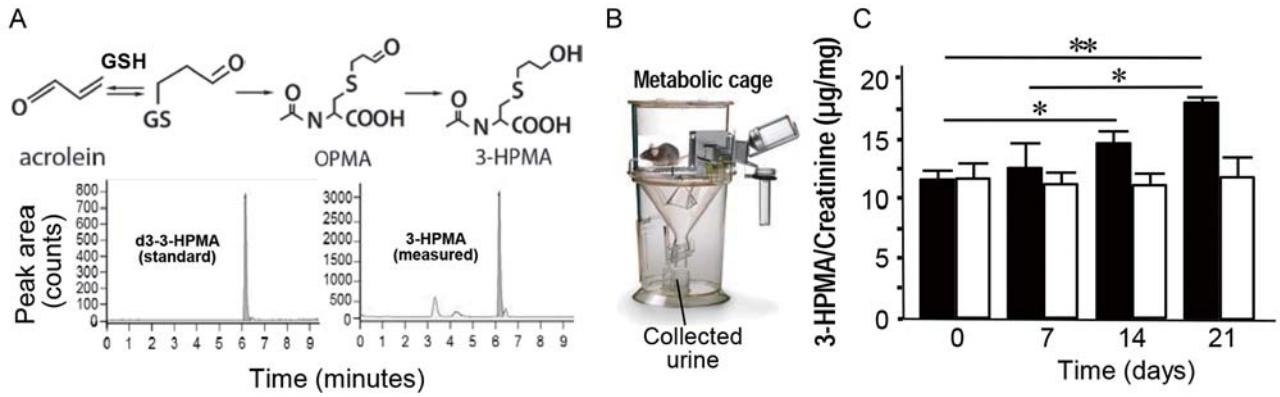

Fig. 2. Change of urine 3-HPMA in response to acrolein inhalation. (A) Upper panel, schematic of the acrolein reaction with glutathione and production of the metabolites OPMA and 3-HPMA. Lower panel, mass spectra outputs of the d3-3-HPMA (standard) and 3-HPMA measurements from mouse urine. (B) Image of the metabolic cage used for urine collection. (C) Bar graph of the ratio of 3-HPMA to creatinine in urine on days 0 (11.46 ± 0.50 μg/mg), 7 (12.41 ± 1.85 μg/mg), 14 (14.43 ± 0.84 μg/mg), and 21 (17.82 ± 0.33 μg/mg) of acrolein exposure (filled bars, acrolein group, *n* = 5). The histogram also shows the sham group on days 0 (11.80 ± 1.57 μg/mg), 7 (11.29 ± 1.17 μg/mg), 14 (11.09 ± 1.34 μg/mg), and 21 (12.02 ± 2.02 μg/mg) (open bars, sham group, *n* = 10). ANOVA comparisons among the acrolein groups yielded **P* <0.05 between days 0 and 14, and days 7 and 21; ***P* <0.01 between days 0 and 21. No significant differences were found within the sham group. Values are expressed as mean ± SEM.

3-HPMA in each sample. A Waters Atlantis T3 2.1 mm × 150 mm, 3 μm column was used for LC separation. The buffers were (A) water + 0.1 % formic acid and (B) acetonitrile + 0.1% formic acid. The linear LC gradient was as follows: 0 min, 0% B; 1 min, 0% B; 9 min, 95% B; 10 min, 95% B; 11 min, 0% B; 15 min, 0% B. The retention time of the 3-HPMA/d3-3-HPMA was 6.8 min. Multiple reaction monitoring was used for MS analysis. The 3-HPMA data were acquired in negative electrospray ionization (ESI) mode by monitoring the following transitions: 220.1 → 91 with collision energy of 5 V and 220.1 → 89 with collision energy of 15 V. The d3-3-HPMA data were acquired in negative ESI mode by monitoring the following transitions: 223 → 91 with collision energy of 5 V and 223 → 89 with collision energy of 15 V. The jet stream ESI interface had a gas temperature of 325°C, gas flow rate of 8 L/min, nebulizer pressure of 40 psi, sheath gas temperature of 250°C, sheath gas flow rate of 7 L/min, capillary voltage of 4000 V, and nozzle voltage of 1000 V.

Creatinine Measurement

Creatinine was measured using a creatinine (urinary) assay kit (Cayman Chemical Co., Ann Arbor, MI) described in detail in a prior publication[40]. Briefly, urine samples were diluted up to 24-fold before quantification. Alkaline picrate solution was prepared according to the manual. All diluted samples and creatinine standards were incubated with the alkaline picrate for ~20 min in a 96-well plate. Absorbance at 490–500 nm was quantified using a standard spectrophotometer and the results were recorded as the initial reading. Five microliters of acid solution was added to each sample after acquiring the initial reading and the plate was then incubated on a shaker for 20 min. Absorbance at 490–500 nm was determined again and the result was recorded as the final reading. The difference between the initial and final readings was used for quantitative analysis. A creatinine standard curve was constructed with known amounts of creatinine provided with the assay kit.

Statistical Analysis

Unless otherwise specified, one-way ANOVA followed by *post-hoc* Tukey's test was used for statistical analyses. $P < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Urine 3-HPMA/Creatinine Levels Increase Following Acrolein Inhalation

Urine samples were obtained at 0 (before inhalation), 1, 2, and 3 weeks of inhalation to ascertain whether acrolein

was systemically absorbed and accumulated following nasal exposure. LC/MS/MS revealed a direct relationship between urinary levels of 3-HPMA and progressive acrolein exposure in the acrolein-treated group (Fig. 2C). A significant elevation was found at both week 2 (14.43 ± 0.84 μg/mg, P <0.05) and week 3 (17.82 ± 0.33 μg/mg, P <0.01), compared with baseline (11.46 ± 0.05 μg/mg). An increase was also detected at week 3 compared with week 1 (12.41 ± 1.85 μg/mg, P <0.05). However, in the sham group where mice inhaled air only in the chamber, there was no difference in urine 3-HPMA among weeks 0, 1, 2, and 3.

Nasal Acrolein Exposure Elevates Acrolein-Lysine Adducts in Spinal Cord

Acrolein-lysine adduct levels in the spinal cords of mice after 3 weeks of nasal exposure to acrolein [10.56 ± 0.59 arbitrary units (a.u.)] were markedly increased compared to the sham group (3.71 ± 0.58 a.u., P <0.05) and the control group (4.52 ± 1.97 a.u., P <0.05), while no significant difference was found between the control and sham groups.

To the best of our knowledge, this is the first evidence that acute acrolein inhalation at a level corresponding to cigarette smoke results in circulatory accumulation of acrolein, absorption by the nervous system, and excretion in urine as 3-HPMA in mice. These data are consistent with previous observations that human smokers tend to have higher levels of urinary 3-HPMA[9, 35]. Considering the established toxicity of acrolein in animal studies and its close association with human pathological conditions[12, 15, 16, 26, 41], these findings facilitate future investigations of its adverse effects on various functions resulting from smoke and other external sources.

3-HPMA is elevated in human smokers to a level that is 5–10 times of that of non-smokers[9, 35]. However, most participants in a clinical study have a smoking history significantly longer than that mimicked in the current study, years *versus* weeks. Here, we demonstrated that acrolein accumulation through nasal absorption does not have a lengthy latency. It is likely that if the concentration of acrolein administered by the respiratory route was reduced, the latency would lengthen and a reduced elevation of urine 3-HPMA would be detected. This would not only substantiate the hazardous effect of acrolein in chronic smokers, but also underscore the potential

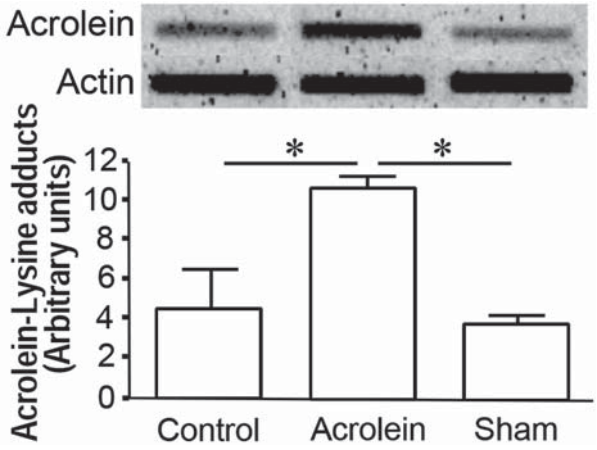

Fig. 3. Accumulation of acrolein-lysine adducts in spinal cord following nasal exposure to acrolein. Upper panel: Representative dot blots for control, acrolein, and sham groups. Actin served as a loading control. Lower panel: Quantification of the band intensities (n = 4 in each group, $^{*}P$ <0.05, ANOVA). Values are expressed as mean ± SEM.

detrimental consequences of short-term smoking on human health.

The use of 3-HPMA to monitor systemic acrolein changes provoked by smoking has been mainly limited to human studies[9, 35]. Compared to the constraints of clinical studies consisting mainly of retrospective cohort investigations, we used an animal model that allows for accurate control of the experimental parameters, such as the concentration used and the duration of inhalation. In addition, changes of 3-HPMA in urine can be independently confirmed using dot immunoblotting to measure acrolein adducts in tissue, further validating acrolein absorption, which is only possible in an animal study. Therefore, while clinical studies are indispensable, the animal acrolein inhalation model can be used to expand this area of research and supplement human investigations.

We used an acrolein concentration of 1.5 ppm which is well below the upper limit estimated for tobacco smoke (50–70 ppm)[42, 43], and within the well-established dose range used in previous studies[37, 43-45]. We also used a paradigm of two 30-min exposures per day. Assuming each cigarette lasts for 5 min, a 60-min acrolein exposure is equivalent to consuming 12 cigarettes per day. According to the Center for Disease Control, an average smoker consumes 20 cigarettes per day[46]. So, the time of

exposure in this study was below the average exposure. Future studies to quantify acrolein accumulation at an increased frequency and longer duration of inhalation are crucial to provide further insight regarding its pathological impact and absorption, and the subsequent occurrence of related diseases in the nervous system.

Although absorbed through the respiratory tract in the current study, an increase in acrolein concentration was not limited to urine, but was also found in the spinal cord where acrolein is associated with pathological events[11, 12, 23-26, 40, 47]. Since spinal cord-related neurodegenerative disease and trauma have been linked to acrolein[12], its increase due to smoke could play an important role in the pathology of these conditions and this deserves further investigation. Here, we demonstrated local acrolein absorption into the spinal cord after inhalation. Studies may further elucidate the differential accumulation of acrolein in other tissues *in vivo*, including but not limited to the brain, the peripheral nervous system, and other organs such as the heart, liver and kidney.

The importance of acrolein accumulation after short-term inhalation is not clear. Acrolein increased two weeks after respiratory exposure and remained high for at least another week before the experiment was terminated. Based on our *in vitro* study, at a low micromolar level, acrolein causes time-dependent toxicity and more importantly, evident cellular damage of neurons results from only a few hours of exposure[47-52]. Therefore, it is likely that acute accumulation of acrolein through inhalation can inflict significant cellular damage. It is also reasonable to assume that the longer the insult persists, the more likely acrolein damage is to progress and eventually lead to cell death. If so, a longer duration of smoking may correspond with more severe neural injury.

In summary, the current results show the time-dependent systemic accumulation of acrolein evoked by weeks of exposure to aerosolized acrolein. The data support the notion of effective systemic absorption of acrolein from short-term exposure. Serving as a viable supplement to clinical studies, the animal model described here could contribute to future investigation of the quantitative relationship between acrolein absorption and respiratory exposure. Consequently, endorsement of smoking cessation with the aim of improving the quality of life and public health could be further validated. Additional utility of these studies may lie in potential contribution to future investigations aimed at revealing the molecular mechanisms and strategies to curtail the absorption and toxicity of acrolein.

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