Determination of Urine 3-HPMA, a Stable Acrolein Metabolite in a Rat Model of Spinal Cord Injury

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Abstract

Acrolein has been suggested to be involved in a variety of pathological conditions. The monitoring of acrolein is of significant importance in delineating the pathogenesis of various diseases. Aimed at overcoming the reactivity and volatility of acrolein, we describe a specific and stable metabolite of acrolein in urine, N-acetyl-S-3-hydroxypropylcysteine (3-HPMA), as a potential surrogate marker for acrolein quantification. Using the LC/MS/MS method, we demonstrated that 3-HPMA was significantly elevated in a dose-dependent manner when acrolein was injected into rats IP or directly into the spinal cord, but not when acrolein scavengers were co-incubated with acrolein solution. A nonlinear mathematic relationship is established between acrolein injected directly into the spinal cord and a correlated dose-dependent increase of 3-HPMA, suggesting the increase of 3-HPMA becomes less apparent as the level of injected acrolein increases. The elevation of 3-HPMA was further detected in the rat spinal cord injury, a pathological condition known to be associated with elevated endogenous acrolein. This finding was further validated by concomitant confirmation of increased acrolein-lysine adducts using established dot immunoblotting techniques. The noninvasive nature of measuring 3-HPMA concentrations in urine allows for long-term monitoring of acrolein in the same animal and ultimately in human clinical studies. Due to wide spread involvement of acrolein in human health, the benefits of this study have the potential to enhance human health significantly.

Key words: liquid chromatography; lipid peroxidation; mass spectrometry; oxidative stress

Introduction

ACROLEIN, an α , β -unsaturated aldehyde and product of lipid peroxidation, has been suggested to play a key role in various pathological conditions, including spinal cord injury, multiple sclerosis, and cancer.^{1,2} This is based on its high direct toxicity to various cells by attacking DNA, proteins, lipids, and mitochondria,^{3–6} perpetuating oxidative stress by further stimulating the production of free radicals,^{1,5} and having an extended presence within biological systems to exert its prolonged toxicity.^{7,8} In light of the pathological importance of acrolein, the detection and quantification of acrolein is of significant importance in delineating the pathogenesis of various diseases. In this regard, we aim to utilize acrolein as a biomarker for making diagnosis, assessing diseases progression, and validating treatments that specifically target acrolein.

Acrolein is a small molecule that is extremely reactive and volatile, properties which make quantification a major challenge due to the complexity of various techniques.³ The existing methods either require complicated immunological analysis such as Western

blotting and dot blotting, or derivatization in the case of HPLC.^{6,8–10} Recently, it has been shown that a stable and specific metabolite of acrolein, N-acetyl-S-3-hydroxypropylcysteine (3-HPMA), produced when reacted with glutathione, can be measured in urine and is a reliable biomarker for quantitation of acrolein.^{11–13} For example, in urine, 3-HPMA was the major metabolite following intravenous or oral administration of acrolein in animal studies.¹⁴

Due to the noninvasive nature of measuring 3-HPMA in urine, this method has been used in estimating acrolein intake through tobacco exposure and food consumption in humans.^{11,12,15} Based on this method, it was revealed that acrolein is significantly elevated in smokers as compared to nonsmokers, and decreased following the cessation of cigarette smoke.¹¹ However, such a method has not been widely used in human and animals models in which endogenous acrolein are elevated due to central nervous system damage or disease.

The purpose of this investigation is to validate the utility of urine 3-HPMA sampling methods in animal models of spinal cord injury (SCI), which is known to be associated with elevated levels of acrolein based on a previously established dot immunoblot method.

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By performing dot blot in parallel with 3-HPMA measurement, we intended to further validate the overall reliability of 3-HPMA concentrations in urine by correlating the results with dot blot measurements of acrolein levels in the tissue. Furthermore, through such study, we intend to establish a quantitative relationship between these two metrics and therefore enable an estimation of tissue acrolein concentration based on urine 3-HPMA measurement. Our data indicates a significant increase of 3-HPMA in SCI which is in agreement with the measurements of acrolein-lysine adducts using the dot blot. The noninvasive nature of 3-HPMA measurement and its quantitative relationship with tissue acrolein is expected to enhance the long-term study of neurotrauma where acrolein is suspected to play a role.

Materials

All animal procedures below were conducted in accordance with Purdue Animal Care and Use Committee approved protocols.

Urine collection

Standard metabolic cages were used for urine collection in rats. Water sources and urine collection were carefully separated to prevent urine dilution by drinking water. Regular food and water were supplied during the experiments. In order to harvest sufficient urine for subsequent measurements in the 24-hour acrolein metabolism study, 0.5 mL of purified water was administered through the peritoneum to induce urination at each collection time. Occasionally, urine was collected by manually pressing the rat bladder.

Rat contused spinal cord injury model and hydralazine treatment

Male Sprague-Dawley rats (200–230 g) were anesthetized by an intraperitoneal injection with ketamine (80 mg/kg) and xylazine (10 mg/kg) mixture. Anesthesia was considered complete when there was no withdrawal reflex in response to noxious foot pinch. When the animal was fully anesthetized, the spinous process and the vertebrae lamina were removed to expose a dorsal surface of spinal cord at the T-10 spinal level. The contusion was produced with a New York University (NYU) impactor; a 10 g rod was dropped from a height of either 25 mm or 37.5 mm onto the intact dura mater to produce moderate and severe injury. For the sham operation, only the laminectomy was performed on vertebrae T-10 without a spinal cord contusion. After surgery, the animals were allowed to recover on a heating pad and the bladder was manually expressed twice a day until the return of reflexive control of the bladder was observed. In the hydralazine-treated group, the hydralazine hydrochloride (Sigma, St. Louis, MO, USA) solution was prepared with phosphate-buffered saline and then sterilized through a filter. 5 mg/kg of hydralazine solution was administered daily for 1 week by intraperitoneal injection following spinal cord injury.

Spinal injection of acrolein

Rats (200–230 g) were anesthetized with a cocktail of xylazine (10 mg/kg) and ketamine (80 mg/kg) intraperitoneally. Anesthetic updates, if necessary, consisted of a ketamine and xylazine mixture at 50% of the initial dose to maintain anesthesia. At the 10th thoracic vertebra, a dorsal laminectomy was performed exposing the spinal cord for injection. Taking care to avoid contact with the cord, the dural sheath was perforated using a needle point to facilitate micropipette insertion. Micropipettes were pulled to a tip outer diameter of 50 μ m using a programmable puller (Model P-

80, Sutter Instruments, Novato, CA). The tip of the micropipettes was further beveled to minimize the occurrence of obstructions and to reduce mechanical insult to tissue. The pipettes were loaded manually with sterile saline, taking care to ensure no bubbles were present except the fluid level indicator. When acrolein injections were performed, the acrolein was loaded using a three-way valve and a syringe under negative pressure. Microinjections were delivered with a PMI-100 Pressure Micro-Injector (Dagan Corp., Minneapolis, MN). 1.6 μ L injections were given 0.6 mm lateral to the midline and 1.2 mm ventral to the cord surface on the right side of the spinal cord. Following injection, the micropipette was tested to ensure patency. The surgical site was flushed with sterile saline, then muscle layers were approximated with interrupted sutures. Subsequently, dermal approximation was performed in the same fashion and triple antibiotic ointment was applied to the surgical site.

Creatinine measurement

The creatinine measurement was performed with a creatinine (urinary) assay kit (Cayman Chemical Company, Item No. 500701). Briefly, creatinine was measured in urine after a 12X dilution, and again at a further dilution of 24X. Alkaline picrate solution was prepared according to the assay manual. All diluted samples and creatinine standards were incubated with the alkaline picrate solution for approximately 20 min in 96-well plates. Absorbance at 490–500 nm was measured with a standard spectrophotometry and the results were recorded as the initial reading. Five μ L of acid solution were added to each sample after the initial reading and the plates were incubated on a shaker for 20 min. Absorbance at 490–500 nm was measured again and the results were recorded as the final reading. The difference between the initial and final reading were used for quantitative analysis. A creatinine standard curve was constructed with known amount of creatinine provided with the assay kit.

Protein immunoblotting

The spinal cord segments (1 cm long) of rats were incubated with 1% Triton solution with the corresponding amount of Protease Inhibitor Cocktails (Sigma-Aldrich, Product #: P8340) and then homogenized with a glass homogenizer (Kontes Glass Co.). The solution was then incubated on ice for at least 1 h before being centrifuged at 13,500 g for approximately 30 min at 4°C. If the experiment was not performed on the same day, the sample was then stored at –80°C and kept for no more than 2 weeks. One additional round of centrifugation at 13,500 g was performed after removal from –80°C.

Prior to the analysis, BCA protein assay was performed to ensure equal loading for all samples. Samples were transferred to a nitrocellulose membrane using a Bio-Dot SF Microfiltration Apparatus (Bio-Rad, Hercules, CA, USA). The membrane was blocked for 1 h in blocking buffer (0.2% casein and 0.1% Tween 20 in PBS) and then transferred to a solution containing monoclonal mouse anti-acrolein antibody (ABCAM). Antibodies were dissolved with a ratio of 1:1000 in blocking buffer with 2% goat serum and 0.025% sodium azide for 18 h at 4°C. Another wash of the membrane with the blocking buffer was performed before being transferred to a solution with 1:10,000 alkaline phosphatase conjugated goat anti-mouse IgG for rat tissue samples for 1 h (VECTASTAIN ABC-Amp Kit). Final washes of the membrane were performed with first the blocking buffer and followed by 0.1% Tween 20 in Tris-buffered saline. The membrane was then exposed to Bio-Rad

Immuno-Star Substrate (or the substrate of the ABC-AMP kit) and visualized by chemiluminescence. The density of bands was evaluated using Image J (NIH) and expressed as arbitrary units.

3-Hydroxypropyl mercapturic acid analysis in urine

3-Hydroxypropyl mercapturic acid (3-HPMA) was analyzed in urine according to Eckert et al.¹⁶ 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 of methanol, followed by 1 mL of water, and then 1 mL of 0.1% formic acid in water. A volume of 500 μ L of urine was spiked with 200 ng of deuterated 3-HPMA (d3-3-HPMA) (Toronto Research Chemicals Inc., New York, Ontario) and mixed with 500 μ L of 50 mM ammonium formate and 10 μ L of undiluted formic acid. This mixture was immediately loaded onto the prepared ENV+ cartridges. Each cartridge was washed twice with 1 mL of 0.1% formic acid, followed by 1 mL of 10% methanol/90% of 0.1% formic acid in water. The cartridges were dried with nitrogen gas and subsequently eluted with three volumes of 600 μ L methanol plus 2% formic acid. The eluates were combined and dried with a rotary evaporation device. Each sample was then reconstituted in 100 μ L of 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 (MS) was used to analyze 3-HPMA in each sample. A Waters Atlantis T3 2.1mm x 150 mm, 3 μ m 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: time 0 min, 0% B; time 1 min, 0% B; time 9 min, 95% B; time 10 minutes, 95% B; time 11 min, 0% B; time 15 min, 0% B. Retention time of the 3-HPMA/d3-3-HPMA was 6.2 min (Fig. 1).

FIG. 1. Metabolic pathways of acrolein in urine and examples of 3-HPMA detected in urine using a mass spectrophotometer. (A) Acrolein, once generated, is scavenged by glutathione (GSH) and then metabolized to produce 3-HPMA as one of the major metabolites. 3-HPMA is a common metabolite of GSH-acrolein metabolism shared by many different species, including rodents and humans. In contrast to acrolein, 3-HPMA is notably stable in urine. It remains stable for months if stored in -80°C freezer. (B) A typical LC/MS/MS chromatogram for 3-HPMA found in urine. The 3-HPMA elutes at 6.2 minutes. *Upper panel* depicts the chromatogram representing the 200 ng d3-3-HPMA (223 $\rightarrow$ 91 m/z transition) internal standard spiked into a urine sample. The *lower panel* shows the chromatogram for endogenous 3-HPMA (220 $\rightarrow$ 91 m/z transition) within the same sample. The absolute amount of endogenous 3-HPMA is calculated by taking the ratio of the two peak areas and multiplying by the amount of internal standard added.

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 $\rightarrow$ 91 with collision energy of 5 V and 220.1 $\rightarrow$ 89 with collision energy of 15 V. The d3-3-HPMA data were acquired in ESI mode by monitoring the following transitions: 223 $\rightarrow$ 91 and 223 $\rightarrow$ 89 with collision energy of 5 V and 15 V, respectively. 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.

Statistical analysis

Unless otherwise specified, one-way ANOVA and Tukey tests were used for statistical analyses. If multiple pairs of tests were performed from the same sample, a Bonferroni correction was used. In some cases, two-way ANOVA was performed to separate irrelevant blocking effects, especially with experimental data that was collected on different days. Normality assumption was tested by the Kolmogorov-Smirnov Test, and equality of variances was tested by the method of Bartlett. If normality assumption was not held, log transformation was performed before fitting the ANOVA model; $p < 0.05$ was considered statistically significant. All statistical analyses and modeling were performed with SAS 9.2, SAS institute.

Results

Increase of 3-HPMA following injection of acrolein into the peritoneum

When acrolein was injected into the peritoneum at a single bullous dosage of 0.7 mg/kg, there was a time-dependent increase of 3-HPMA as measured in the urine using the LC/MS/MS technique (Fig. 2). Specifically, when measured immediately at pre-injection and then every 3 hours up to 24 h following, the 3-HPMA values were 2.06 ± 0.13 , 239.2 ± 17.73 , 183.88 ± 10.08 , 86.74 ± 3.56 ,

FIG. 2. Time course of the changes of 3-HPMA following IP injection of 3-HPMA. Acrolein was administered into peritoneum at a concentration of 0.7 mg/1 kg body weight. Urine samples were collected at various hour time points post administration. 3-HPMA levels were measured with LC/MS/MS normalized to urinary creatinine levels that were also quantified at the same time. Therefore, 3-HPMA/creatinine ($\mu\text{g}/\text{mg}$) levels were used to represent 3-HPMA quantity. Note that the peak level of 3-HPMA was identified 3 h after administration. Within 24 h, the majority of acrolein was metabolized into 3-HPMA and excreted through urine. The *dash line* signifies the average 3-HPMA concentration following injection which is estimated at $76.63 \mu\text{g}/\text{mg}$. $N=3$ for each time point.

FIG. 3. The suppression of 3-HPMA by acrolein scavengers. Four groups of rats receive IP injection of saline, saline containing acrolein (0.7 mg/kg body weight), saline containing acrolein and hydralazine, and saline containing acrolein and phenelzine, respectively. In the latter two groups, acrolein solution was pre-incubated with either hydralazine or phenelzine at a concentration of 5 mg/kg body weight before injected into the peritoneum. The amount of 3-HPMA was measured based on the accumulative urine collected within 24 h after injection. Note the amount of 3-HPMA collected from urine was significantly increased following the injection of acrolein. However, the elevated 3-HPMA was reduced by more than 90% when either hydralazine or phenelzine was added in the solution. Specifically, the levels of 3-HPMA are $2.06 \pm 0.06 \mu\text{g/mg}$ in control group; $76.79 \pm 1.45 \mu\text{g/mg}$ in acrolein-only group; $6.84 \pm 0.23 \mu\text{g/mg}$ in acrolein + hydralazine group; and $5.03 \pm 0.86 \mu\text{g/mg}$ in acrolein + phenelzine group. ($\#p < 0.001$ when compared to control; $*p < 0.001$ when compared to acrolein only, ANOVA). $N = 3$ in each group.

75.26 ± 3.94 , 14.84 ± 0.81 , 10.17 ± 0.93 , 12.64 ± 0.7 , and $14.32 \pm 0.43 \mu\text{g/mg}$ creatinine, respectively. The average value of 3-HPMA post injection was $76.63 \mu\text{g/mg}$.

To confirm that acrolein within the injected solution was the cause of the observed increase in 3-HPMA, we conducted additional experiments using acrolein solution with the addition of two known acrolein scavengers, hydralazine and phenelzine (Fig. 3). These two compounds have repeatedly demonstrated their ability to neutralize acrolein in both *in vitro* and *in vivo* conditions (Fig. 3).¹⁷ Either hydralazine (5 mg/kg body weight) or phenelzine (5 mg/kg body weight) was added to the solution 60 min before the injection for the purpose of scavenging acrolein. As indicated in Figure 3, such treatment significantly reduced 3-HPMA levels in urine. Specifically, the average 3-HPMA levels within 24 h post injection in rats given saline (control), acrolein only, acrolein plus hydralazine, and acrolein plus phenelzine were 2.06 ± 0.06 , 76.79 ± 1.45 , 6.84 ± 0.23 , and $5.03 \pm 0.86 \mu\text{g/mg}$, respectively. It is clear that peritoneum injection of the acrolein-containing solution with the addition of hydralazine or phenelzine significantly reduced the 3-HPMA in the urine when compared to the acrolein only group ($p < 0.001$).

Increase of 3-HPMA following injection of acrolein into the spinal cord

Next we were interested in urine 3-HPMA levels resulting from spinal cord acrolein exposure. Acrolein was injected at three different dosages, 40 nmol, 160 nmol, and 1600 nmol into the spinal cord. As indicated in Figure 4A, the majority of acrolein injected was metabolized into 3-HPMA and excreted through urine within the first 2 days. By day 4 post injection, the urinary 3-HPMA level returned to normal ($\sim 2 \mu\text{g/mg}$). The graph in Figure 4B displays the total accumulative amount of 3-HPMA excreted within 4 days after injection. Specifically, following injection of three different dosages of acrolein into the spinal cord, we detected significant and graded increases of urine 3-HPMA: 352 ± 32 , 788 ± 122 , and $2354 \pm 395 \text{ nmol}$, respectively. The overall correlation between acrolein injected to the spinal cord and the resulting increase in

urine 3-HPMA is plotted in Figure 4C. The data suggest that acrolein inside the spinal cord tissue is capable of diffusing out to the circulation and is secreted as 3-HPMA in the urine. The fitted curve between injected acrolein dosage and urine 3-HPMA corresponds to the following relationship:

$$Y = 52.242 \times 0.5158$$

where Y represents the amount of urine 3-HPMA and x denotes the amount of acrolein within the spinal cord.

Elevation of urine 3-HPMA levels results from endogenous acrolein generated in injured rat spinal cord and its reduction by IP injection of hydralazine

In order to detect changes of 3-HPMA derived from acrolein generated endogenously, we examined the level of urine 3-HPMA in rats subjected to spinal cord injury—a condition in which acrolein is known to be elevated.^{8,17} As indicated in Figure 5, 3-HPMA is significantly increased following a moderate spinal cord injury in the first 3 days post injury with the value of 1-day post injury being the highest. Specifically, the levels of 3-HPMA at 1–3 days post SCI are 3.51 ± 0.24 , 2.96 ± 0.20 , and $2.49 \pm 0.12 \mu\text{g/mg}$, respectively. All initial values were significantly higher than the control (pre-injury), but are closer to normal 4–7 days post injury. In a separate group, rats were treated with hydralazine. Treatment occurred immediately following SCI and through daily IP injections at a dosage of 5 mg/kg (b.w). As a result of the treatments, the levels of 3-HPMA decreased significantly at 1 day post-injury. No significant differences were detected in the remaining period, up to 7 days post injury, between SCI and SCI plus hydralazine group (Fig. 5).

Due to our interest of whether more severe SCI would result in higher corresponding levels of 3-HPMA, we conducted a separate group of experiments with rats subjected to severe contusion injury. As indicated in Figure 6A, it is obvious that control, moderate, and severe injuries resulted in graded and significantly higher levels of 3-HPMA when measured 24 hours post injury. Specifically, the

FIG. 4. Detection of urine 3-HPMA as a result of acrolein injection to the spinal cord. Acrolein solutions (1.6 µL) of 3 different dosages (1600 nmol, 160 nmol, and 40 nmol) were injected to rat spinal cord. (A) Urines samples were collected daily for up to 4 days. It is evident that the majority of acrolein was metabolized into 3-HPMA within the first 2 days, and the urinary 3-HPMA level return to a normal level ($\sim 2 \mu\text{g}/\text{mg}$) within 4 days. The *dash line* indicates the baseline value of $2.06 \mu\text{g}/\text{mg}$ in control animals. $N=3$ in each group. (B) The graded changes of 3-HPMA are expressed as the values accumulated within 4 days following acrolein injections of 1600, 160, and 40 nmol, respectively. The values are as follows (in nmol): 2354 ± 395 , 788 ± 122 , and 352 ± 32 . A baseline level of acrolein was subtracted from the samples before estimating the total accumulative value ($*p < 0.01$ when compared to 1600 nmol, $\#p < 0.05$ when compared to 160 nmol, ANOVA). $N=3$ in each group. (C) The correlation of the amount of acrolein injection into spinal cord with the 3-HPMA measured in urine. Four different levels of acrolein (0, 40, 160, 1600 nmols) that were injected were plotted against various concentrations of 3-HPMA determined in urine. This resulted in a nonlinear fit as expressed by the equation displayed.

3-HPMA levels correspondent to control, moderate, and severe injuries are 1.99 ± 0.21 , 3.51 ± 0.24 , and $5.51 \pm 1.13 \mu\text{g}/\text{mg}$. While the level of 3-HPMA in moderate injury is higher than that in control ($p < 0.05$), the severely injured rats have a higher level of 3-HPMA than that of the moderate SCI ($p < 0.05$). In addition to the correlation between 3-HPMA levels and the severity of SCI, we have shown that escalated dosages of hydralazine, 0, 5, and 25 mg/kg also resulted in a significant augmented reduction of 3-HPMA, from 3.51 ± 0.24 (0 mg/kg), to 3.08 ± 0.11 (5 mg/kg), and 2.17 ± 0.13 (25 mg/kg) ($p < 0.05$), when measured 24 h post injury and treatment. No significant hypotension was detected after hydralazine treatment at doses of both 5 and 25 mg/kg. Specifically, the average systolic pressure without and with hydralazine is 110 ± 7 and 95 ± 6 mmHg when hydralazine was applied at 5 mg/kg ($N=3$, $p > 0.05$), and 103 ± 10 and 87 ± 9 mmHg when hydralazine used at a dose of 25 mg/kg ($N=3$, $p > 0.05$).

Correlations between acrolein measured in the tissue versus 3-HPMA quantified in urine in rat SCI

In order to understand the correlation between direct measurements of elevated acrolein from the spinal cord tissue and the increased 3-HPMA levels in urine, we performed dot immunoblotting (detect acrolein-lysine adduct in spinal cord tissue) and LC/MS/MS (detect 3-HPMA in urine) simultaneously in the same animals subjected to SCI. As indicated in Figure 7, when using dot immunoblotting, acrolein-lysine adducted was elevated by a factor of 4.5

(from 8.79 to 39.8 au, $p < 0.005$) 1 day post SCI. At the same time point, the 3-HPMA in the urine was also increased significantly by a factor of 1.8 (from 1.99 to $3.51 \mu\text{g}/\text{mg}$, $p < 0.05$). In both measurements, spinal cord and urine, there were significant reductions in values following the application of hydralazine.

Discussion

In the current study, we presented data that indicate 3-HPMA to be a stable and specific metabolite of acrolein and therefore may be a reliable biomarker and indicator of acrolein levels in animals and potentially humans. This is based on the observation that when acrolein was injected into rats IP or directly into the spinal cord, we were able to detect and correlate dose-dependent changes in 3-HPMA. Moreover, when acrolein was co-incubated with known scavengers, hydralazine or phenelzine, urine 3-HPMA levels decreased. The elevation of 3-HPMA was further detected in SCI, a pathological condition known to be associated with elevated acrolein. Specifically, we observed a correlation between the levels of urine 3-HPMA and the severity of SCI. The detection of urine 3-HPMA in SCI was further validated by concomitant confirmation of increased acrolein-lysine adducts using established dot immunoblotting techniques.

Thus far, the majority of cases where urine 3-HPMA has been measured involve detection of acrolein accumulation in human smokers and individuals consuming acrolein-rich foods.^{11,15} Obviously, the main systems involved in acrolein uptake in these two

FIG. 5. Elevation of 3-HPMA in urine and its suppression by hydralazine following spinal cord contusion injury in rats. Urines samples from rats subjected to moderate SCI with or without hydralazine (5 mg/kg body weight) treatment were collected daily for 7 days to determine 3-HPMA contents. In the treated group (SCI+hydralazine), hydralazine was administrated daily through IP injections immediately after SCI. For SCI injury only group, 3-HPMA levels increased significantly for up to 3 days after SCI: Control: 1.99 ± 0.21 µg/mg, Day 1: 3.51 ± 0.24 µg/mg, Day 2: 2.96 ± 0.20 µg/mg, and Day 3: 2.49 ± 0.12 µg/mg (* $p < 0.05$ when compared to control, ANOVA). In addition, hydralazine treatment significantly reduced the urinary 3-HPMA level on day 1 following injury (# $p < 0.05$ when compared to SCI only). $N = 4$ in each group.

situations are the respiratory and digestive systems, respectively. Based on our experimental findings from injection of acrolein into the peritoneum and directly into spinal cord, it is clear that 3-HPMA may also serve as a reliable biomarker when acrolein is introduced in either the abdominal cavity or spinal cord. Additionally, the observation that 3-HPMA levels were elevated in SCI in a severity-dependent manner indicates that our method is effective in detecting acrolein either generated endogenously or administered from exogenous sources.

FIG. 6. Correlation of injury severities and the levels of urine 3-HPMA and the dose-dependent reduction of urine 3-HPMA by systemic application of hydralazine. (A) Urine samples from rats subjected to either control, moderate, or severe SCI were collected 24 h post-injury to determine 3-HPMA contents. As indicated in the bar graph, the graded injuries produced correspondent increases of 3-HPMA among control (sham injury), moderate, and severe injury. $N = 4-6$ for each group. * $p < 0.05$. (B) Intraperitoneal application of hydralazine at either 0, 5, or 25 mg/kg, resulted in a graded decrease of urine 3-HPMA in moderate SCI when assessed 24 h post injury. $N = 3-5$ for each group. * $p < 0.05$. All statistics, A and B, were performed using ANOVA.

FIG. 7. Comparison of acrolein concentrations in tissue and urine with and without hydralazine 1 day post SCI. The tissue acrolein-lysine adducts were determined by immunoblotting, while 3-HPMA levels were quantified in urine using LC/MS/MS. Immunoblotting was conducted using antibody against proteins that were bound to acrolein to confirm the increase of acrolein-lysine adduct in the injured spinal cord tissue. Note the increase of either acrolein-lysine adducts, or 3-HPMA in urine 1 day following SCI and their suppression by hydralazine treatment. (** $p < 0.005$ or * $p < 0.05$ when compared to control; # $p < 0.05$ when compared to SCI group, ANOVA). $N = 4$ in each group of acrolein measurement in tissue and 3-HPMA measurement in urine.

In experiments where acrolein was injected directly into the spinal cord, we noticed a correlated dose-dependent increase of 3-HPMA, although the relationship is nonlinear (Fig. 4). This indicates that, as the level of injected acrolein increases, the increase of 3-HPMA becomes less apparent. This suggests a decreasing ability of the rat to convert acrolein to 3-HPMA when acrolein concentrations rise. There may be several factors that dictate this relationship. 3-HPMA is the metabolite produced when acrolein binds to glutathione, a major endogenous antioxidant that is responsible for keeping acrolein and overall oxidative stress at a nonpathological level.^{15,18,19} However, it is well known that with an increase in oxidative stress (and consequently acrolein elevation), the levels of glutathione may not be able to compensate due to its rapid consumption.²⁰ Consequently, lesser amounts of 3-HPMA are produced as acrolein further increases. In summary, it is likely that higher levels of acrolein expressed in the tissue, and the quicker generation of acrolein in the spinal cord, leads to a greater degree of underestimation of acrolein using urine 3-HPMA sampling. This suggests that the estimation of acrolein based on urine 3-HPMA is more linear and accurate in the low range of acrolein. This finding may prove more useful as lower levels of acrolein ($\sim 1-25$ µM) are more likely to be pathologically relevant in the clinic.^{1,5,21}

One interesting phenomena is that overall the number of 3-HPMA molecules produced was greater than the amount of acrolein injected to the spinal cord (Fig. 4B, C). Since the ratio of acrolein and 3-HPMA is 1:1, this indicates that there are more endogenous acrolein molecules produced in response to the exogenous acrolein application. This phenomenon is consistent with the understanding that acrolein is both the product and the catalyst of oxidative stress.^{1,3,17} Acrolein has the ability to further stimulate the production of free radicals, and consequently lipid peroxidation—a process that leads to even more acrolein production.^{5,21-23} Therefore, acrolein is known to be self-regenerative that could

perpetuate the oxidative stress. Thus, this study could serve as quantitative evidence to support the notion of acrolein self-amplification in an experimental animal model.

Although similarities exist between 3-HPMA (in urine) and acrolein-lysine (in spinal cord) following spinal cord injury, there are also key differences in the current study. For example, it is known from our previous findings that the increase of acrolein-lysine lasted at least 1 week in SCI.⁸ However, in these experiments the increase of 3-HPMA lasted only 3 days post-SCI. This suggests there may be a temporal window using the 3-HPMA method, possibly caused by the diffusion characteristics of acrolein *in vivo*. In this sense, running two methods of quantification, that is, using both urine and spinal cord tissue, provided complementary data and a more systematic view of acrolein dynamics following SCI.

In addition to the discrepancy of the acrolein elevation time course post SCI, there is also a conspicuous difference in the degree of elevation between acrolein in the spinal cord and 3-HPMA in urine when measured 1 day after injury. As indicated in Figure 7, the increase of acrolein-lysine in the spinal cord is 4.5 times greater, while 3-HPMA increased only 1.8 times. Again, this is likely due to the limited diffusion of acrolein into the blood stream. Another factor is that acrolein estimation based on dot blot is tissue specific, because only the spinal cord tissue of the injury site was used. However, it is understandable that the acrolein concentration is significantly diluted when it leaked systemically. This dilution factor will predictably influence the level of 3-HPMA and provides an explanation of the disproportionate increase following SCI.

In summary, we have presented evidence to indicate that 3-HPMA is a reliable biomarker to estimate the acrolein concentration in animal model of SCI. The noninvasive nature of measuring urine 3-HPMA permits the long-term monitoring of acrolein in the same animal and ultimately in human clinical studies. Since acrolein has been implicated in the pathology of SCI,^{17,24} and furthermore, severity-dependent elevation of 3-HPMA and dose-dependent reduction of 3-HPMA by hydralazine can be detected in SCI, its quantification could benefit the diagnosis and prognosis of SCI or help assess the effectiveness of potential anti-acrolein therapies. Taken together, measurement of urine 3-HPMA may represent a novel theranostic approach for SCI. Since acrolein may potentially be linked to many other conditions, such as cancer,^{2,25–28} aging,²⁹ pollution,^{30,31} and smoking,^{32,33} the benefits of this study has the potential to significantly enhance human health.

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Author Disclosure Statement

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Abbreviations Used

ESI = negative electrospray ionization

3-HPMA = *N*-acetyl-S-3-hydroxypropylcysteine

LC = liquid chromatography

MS = mass spectrometer

SCI = spinal cord injury