Inactivation of Hepatic Enzymes by Inhalant Nitrite—In Vivo and In Vitro Studies

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Steven G. Turowski,¹ Kate E. Jank,¹ and Ho-Leung Fung¹

¹Department of Pharmaceutical Sciences, School of Pharmacy and Pharmaceutical Sciences, University at Buffalo, State University of New York, Buffalo, NY 14260-1200

ABSTRACT

We examined the effects of acute isobutyl nitrite (ISBN) exposure on the activity of several hepatic enzymes. Two strains of adult male mice (Balb/c and C57BL/6) were exposed to 900 ppm ISBN or ambient air for 45 minutes. The enzyme activity of hepatic cytochrome P450 (CYP)-mediated deethylation, glutathione S-transferase (GST), and carboxylesterase (CBE) was monitored through the substrates 3-cyano-7-ethoxycoumarin (CEC), 1-chloro-2,4-dinitrobenzene, and p-nitrophenyl acetate, respectively. Acute ISBN exposure led to a significant reduction in hepatic CYP-mediated CEC deethylation, GST, and CBE activity in Balb/c mice (of 81.5%, 74.7%, and 25.2%, respectively, vs control mice, each at $P < .05$) when livers were harvested immediately after inhalant exposure. The corresponding decreases in C57BL/6 mice were smaller (with reductions of 21.8%, 18.8%, and 13.3%, respectively, each at $P < .05$). This enzyme activity, tested in C57BL/6 mice only, returned to control values after a 24-hour period of nonexposure. Follow-up mechanistic investigations using rat liver GST indicated that ISBN-mediated enzyme inactivation was not caused by its metabolites: inorganic nitrite ion (NO_2^-) or nitric oxide. This inactivation could be prevented, but not reversed, by added glutathione, suggesting irreversible protein oxidation. Using different NO donors as comparative agents, we found that GST inactivation by ISBN was not associated with protein S-nitrosylation or disulfide formation, but with tyrosine nitration. Inhalant nitrite exposure, therefore, led to a significant reduction in hepatic enzyme activity in mice, possibly through tyrosine nitration of hepatic proteins. This effect raises the possibility of drug-drug metabolic interactions from inhalant nitrite abuse. However, determining the applicability of these findings to humans will require further study.

KEYWORDS: Inhalant nitrite, liver, enzyme, metabolism, glutathione-S-transferase, nitration, S-nitrosylation

Corresponding Author: Ho-Leung Fung, Department of Pharmaceutical Sciences, School of Pharmacy and Pharmaceutical Sciences, University at Buffalo, State University of New York, Buffalo, NY 14260-1200. Tel: (716) 645-2842, ext 222; Fax: (716) 645-3693; E-mail: hlfung@buffalo.edu

INTRODUCTION

Isobutyl nitrite (ISBN) is representative of a group of inhaled nitrites commonly called “poppers” or “snappers.” Inhaled nitrites are abused widely in the United States, particularly among male homosexuals and teenagers. These drugs are vasodilatory in action and are thought to produce aphrodisiac effects,¹ in addition to increased heart rate and decreased systolic blood pressure.² The toxicity of these agents is incompletely characterized. Recent laboratory studies have shown that acute³ and repeated⁴ nitrite inhalation alters the expression of several genes relating to cancer and angiogenesis in mouse liver and lungs, particularly those encoding vascular endothelial growth factor. Heavy abuse in HIV patients may increase the risk of developing certain types of cancer, such as Kaposi’s sarcoma.⁵

Currently, the effects of inhalant nitrite on hepatic enzyme activity are unknown. Using Western blot techniques,⁶ we showed recently that ISBN inhalation in rats led to extensive protein nitration in tyrosine residues in both liver and kidney tissues. We also found this increased protein nitration to be somewhat sustained, since hepatic nitrotyrosine was still elevated 24 hours after termination of nitrite exposure. Protein nitration has been shown to alter protein function; for instance, treatment of isolated glutathione S-transferase (GST) or rat liver homogenates with peroxynitrite, a myeloperoxidase/hydrogen peroxide/nitrite system, or tetranitromethane resulted in a loss of GST activity along with an increase in the formation of protein-associated 3-nitrotyrosine.⁷ On the other hand, Ji et al⁸ found that rat microsomal GST was activated by nitration of tyrosine 92 of the enzyme. Thus, given the observed increased protein nitration in the liver after nitrite exposure, it is possible to speculate that substantial changes in hepatic enzyme activity may occur as well. This hypothesis has not been tested.

The main goal of the present study, therefore, was to explore the effects of in vivo exposure to inhalant nitrite on the activity of several representative hepatic enzymes, namely those mediating cytochrome P450 (CYP)-dependent deethylation, GST, and carboxylesterase (CBE). Each of these enzyme systems possesses important functions in hepatic metabolism. Hepatic CYP is a family of enzymes involved in the metabolism of numerous drugs and pharmacologically active compounds in phase 1 metabolism, and 3-cyano-7-ethoxycoumarin (CEC) deethylation has been used to denote the activity of a

variety of CYP isozymes, including 1A and 2C19.⁹ GST is an enzyme system endogenous to liver cytosol that is involved in the detoxification and metabolism of many xenobiotics and endobiotics. This family of enzymes facilitates the conjugation of glutathione (GSH) to certain electrophilic substrates to render them water-soluble for renal elimination.¹⁰ Hepatic CBE is a family of phase I enzymes involved in the detoxification of xenobiotics. While significant cocaine metabolism occurs via serum cholinesterases, a portion of the hydrolytic degradation is mediated in the liver by CBEs.¹¹ It has been reported¹² that nitrite abuse is a high risk factor for mortality in intravenous drug users of cocaine and heroin. Inhibition of cocaine metabolism through suppression of CBE activity may therefore contribute to a potential interaction between these classes of abused drugs.

In an attempt to understand the underlying biochemical mechanisms involved in the potential enzyme inactivation by inhalant nitrite, we conducted further *in vitro* investigations using rat liver GST as a representative enzyme. We first examined whether nitrite-induced enzyme inactivation could be mediated through its metabolites: inorganic nitrite ion (NO_2^-) or nitric oxide (NO). We have shown previously that GST inactivation by nitroglycerin (NTG, another NO donor) is mediated through protein thiol oxidation and not tyrosine nitration.¹³ We therefore examined the relative contributions of 3 potential mechanisms of enzyme modification—S-nitrosylation, disulfide formation, and tyrosine nitration—to inhalant nitrite-mediated GST inactivation.

MATERIALS AND METHODS

Materials

All chemicals and reagents were obtained from Sigma-Aldrich Chemical Co (St Louis, MO), except for the following: sodium nitrite (NaNO_2 , EMD Pharmaceuticals, Durham, NC), NTG sublingual tablets (ETHEX Corporation, St Louis, MO), CEC (Gentest Corp, Woburn, MA), absolute ethanol (Pharmaco Products, Inc, Brookfield, CT), polyclonal antibody against 3-nitrotyrosine (Upstate Biotechnology, Lake Placid, NY), S-Nitrosylated Protein Detection Assay Kit (Cayman Chemical Co, Ann Arbor, MI), and Silver Stain Plus kit and other chemicals for Western blot analysis (Bio-Rad Co, Hercules, CA).

ISBN Exposure

All animal studies were conducted under approved protocols by the Institutional Animal Care and Use Committee at the University at Buffalo. Mice were obtained from the Jackson Laboratories (Bar Harbor, ME). Preliminary studies were performed with male Balb/c mice, of ~30 g each, with 1 group of 3 mice exposed to ambient air (control group)

and another group of mice exposed to 900 ppm of ISBN mixed in breathing air (treated group) for 45 minutes,¹⁴ using an exposure chamber similar to that we described recently.³ Because other mechanistic studies of inhalant-induced toxicity may involve knockout mice, C57BL/6 mice were also employed to examine the effects of inhalant nitrite exposure on hepatic enzyme activity. These mice were exposed to nitrite either acutely at 900 ppm for 45 minutes, or repeatedly for 6 days, also at 900 ppm for 45 minutes per day. In both cases, the mice were either sacrificed immediately after the last exposure, or after a 24-hour washout period (ie, without drug exposure). Each treatment and control group employed 3 mice in the study. ISBN concentrations in the exposure chamber were monitored at regular intervals using a Varian Aerograph Series 2400 gas chromatograph (Palo Alto, CA) equipped with a flame ionization detector and a glass-packed column (2 m $\times$ 2 mm internal diameter; 10% silicone OV-17 on Chromosorb W-HP, 80/100).¹⁵ The following conditions were used in the analysis of vaporized ISBN in the exposure chamber: oven temperature 50°C, injection port temperature 100°C, detector temperature 90°C, and carrier gas flow (nitrogen) 12 mL/min. After exposure, the mice were anesthetized with ketamine-xylazine (90-9 mg/kg) and sacrificed by exsanguination via the aorta. Liver tissue was immediately harvested and frozen at -80°C until assay.

Preparation of Hepatic Samples

Liver microsome and cytosol samples were extracted at 4°C. Frozen liver samples were first allowed to bathe in pH 7.4 Krebs buffer and then minced on glassine paper using a sterile razor. A buffer containing Tris-HCl pH 7.4 (20 mM), KCl (150 mM), ethylenediamine tetraacetic acid (1 mM), phenylmethyl sulfonyl fluoride (1 mM), leupeptin (10 μM), bestatin (1 μM), aprotinin (0.04 unit/mL), trypsin inhibitor (1 mg/mL), and glycerol (20% [vol/vol]) was next added to the minced samples and then homogenized. The homogenates were centrifuged at 10 000 $\times$ g for 20 minutes. The supernatants were saved and then ultracentrifuged at 105 000 $\times$ g for 60 minutes. The pellet (microsome) and supernatant (cytosol) from ultracentrifugation were collected and frozen at -80°C. Protein concentrations of all samples were determined by the Bradford protein assay, standardized against bovine serum albumin (BSA).¹⁶

Ex Vivo Enzyme Activity Assays

CYP-mediated deethylation enzyme activity was monitored using CEC via a SpectraMax Gemini Microplate Fluorometer (Sunnyvale, CA). Approximately 50 μg of mouse liver microsomes was mixed with NADPH (0.5 mM) and MgCl_2 (10 mM) in sodium phosphate buffer, pH 7.5 (0.2 mM). Reactions were started with CEC (0.03 mM) dissolved in

dimethyl sulfoxide. Samples were incubated for 1 minute, and the fluorescence intensities were monitored over time at an excitation wavelength of 408 nm and an emission wavelength of 455 nm at a constant temperature of 37°C.¹⁷ GST activity was measured with a SpectraMax 340 PC Microplate Spectrophotometer (Sunnyvale, CA) using ~25 µg of liver cytosol and 1-chloro-2,4-dinitrobenzene (CDNB, 1 mM) in phosphate buffer, pH 6.5 (0.1 mM). The reactions were started with GSH (1 mM), and each sample was left to incubate for 1 minute. Absorbance changes over time were monitored at room temperature at 340 nm.¹⁸ CBE activity was monitored via hydrolysis of the substrate p-nitrophenyl acetate (PNPA). Approximately 75 µg of mouse liver microsomes was added to phosphate buffer, pH 7.0 (100 mM), and the reactions were initiated with PNPA (1 mM) dissolved in methanol. A 1-minute incubation period was allowed prior to spectrophotometric analysis, and the change in absorbance at room temperature was monitored at 410 nm.¹⁹

In Vitro GST Activity Studies

Rat liver GST (Sigma G8386, 25 mg/L in 0.1 M potassium phosphate buffer [KPB], pH 7.5) was incubated at 37°C for 60 minutes with ISBN (22 mM in absolute ethanol), NaNO₂ (22 mM in 2.2% ethanol/KPB), or the vehicle control (2.2% ethanol/KPB). Ten seconds after the reaction was initiated, half the volume was placed on ice to quench the reaction. The remaining sample was further incubated until the 60-minute end point and then placed on ice. The chilled GST samples were then separated by ultrafiltration using Nanosep 10K ultracentrifuge tubes (Pall Life Sciences, Ann Arbor, MI). GST enzyme activity was assessed spectrophotometrically using the model substrate CDNB, as described previously. Protein concentration was assessed to correct for enzyme recovery from ultrafiltration. In separate experiments, GSH (5 mM in KPB) was added before or after the reaction to examine the possible preventative and reversal effects of GSH on nitrite-induced inactivation of GST.

Detection of S-Nitrosylated GST Formation

NTG solutions were prepared from the sublingual tablets, which were dissolved in KPB and adjusted for the appropriate concentration after removal of the insoluble materials by filtration. Rat liver GST (25 mg/L in KPB) was incubated at 37°C with NTG, S-nitroso-N-acetyl penicillamine (SNAP, both at 220 µM in 2.2% ethyl alcohol/KPB for 60 minutes), or tetranitromethane (TNM, 100 µM in 2.2% ethyl alcohol/KPB) for 45 minutes. After protein separation by ultrafiltration and assay for protein recovery, as described previously, the treated GST samples were tested with the S-Nitrosylated Protein Detection Assay Kit. The original protocol from the manufacturer was modified as follows: GST protein (2 µg)

was incubated with 7 volumes of buffer A containing the blocking reagent with constant agitation at 4°C for 2 hours. The blocking reagent was removed by ultrafiltration using the Nanosep 10K ultracentrifuge tubes. Ice-cold acetone (4 volumes) was added, and the mixture was incubated at –20°C for 1 hour. A pellet was formed by centrifugation at 3000 × g for 10 minutes at 4°C and resuspended in 0.5 mL of buffer B containing the supplied reducing and labeling reagents. Following 1 hour of incubation at room temperature, ice-cold acetone (4 volumes) was added and incubated at –20°C for 1 hour. The samples were centrifuged again to produce a pellet, which was resuspended in cold wash buffer (0.1 mL). GST-treated samples were dotted (900 ng) onto a prewashed nitrocellulose membrane (Invitrogen, Carlsbad, CA). Nonspecific sites on the membrane were blocked with 1% BSA in phosphate-buffered saline, pH 7.4, containing 0.05% Tween 20 (PBS-T) for 1 hour at room temperature. After being washed with PBS-T, the membrane was probed with the S-Nitrosylation Detection Reagent I HRP (1:75 dilution) at 4°C overnight with constant agitation. The immunoreactive bands were detected with an enhanced chemiluminescence system (GE Healthcare BioSciences Corp, Piscataway, NJ), and densitometry analysis was performed on each of the dot blot samples (n = 3) by Kodak ID Image Analysis Software (Eastman Kodak Co, New Haven, CT). Final net intensity values were calculated as the net intensities observed divided by the respective background intensities and were reported in arbitrary units.

Measurement of Disulfide Bond Formation and Tyrosine Nitration

A known amount of GST protein from the treatment samples described above was separated on 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel under nonreducing conditions. The proteins were detected with the Silver Stain Plus kit. Tyrosine nitration of GST was assessed by Western blot, as described previously.¹³

Statistical Analysis

All data were reported as mean ± SD. Changes in ex vivo enzyme activity were compared against the respective controls using the Student *t* test. Data from multiple treatment groups were compared using 1-way analysis of variance (ANOVA) followed by the Tukey HSD post hoc test. A *P* value of less than .05 was considered to be statistically significant.

RESULTS

In Vivo Exposure Studies

As we reported previously,²⁰ ISBN concentrations in the air of the exposure chamber can be readily monitored throughout

the study period. Figure 1 shows the measured ISBN concentrations in the chamber typically observed before and during an exposure. Prior to placing the animals in the chamber, the system was primed with ISBN, and the input rate was adjusted to reach ~900 ppm in the breathing air. This priming typically took ~2 hours to reach the desired ISBN concentration (left panel, Figure 1). The door of the chamber was then opened and mice were introduced into the exposure system. The desired ISBN exposure concentration (ie, 900 ppm) was usually regained after ~15 to 20 minutes (right panel, Figure 1).

With Balb/c mice, ISBN exposure led to a significant reduction in CEC deethylation activity in treated mice compared with that observed in control mice (by 81.5%, $P < .0001$, Table 1). GST activity in hepatic cytosol of Balb/c mice was found to be significantly lowered (by 74.7%, $P < .05$, Table 1) when compared against that in control mice ($P < .05$). Lastly, ISBN-treated mice had a 25.2% decrease in hepatic CBE activity ($P < .05$), when compared with control mice (Table 1).

When C57BL/6 mice were acutely exposed to 900 ppm ISBN and sacrificed immediately after the 45-minute exposure period, the activity of all 3 enzymes was also significantly inhibited (Table 1). Interestingly, however, the ISBN-mediated suppression of hepatic enzyme activity was considerably smaller in C57BL/6 mice than in Balb/c mice. For example, the CEC deethylation activity was reduced by only 21.8% in C57BL/6 mice, compared with an 81.5% reduction in Balb/c animals. Correspondingly, GST activity was suppressed by only 18.8% in C57BL/6 mice, vs 74.7% in Balb/c mice. Interestingly, the suppression of liver CBE activity in both strains of mice was modest, at ~13.3% for C57BL/6 and 25.2% for Balb/c mice. However, after a subsequent 24-hour exposure-free period, values for the 3 types of hepatic enzyme activity in C57BL/6 mice were not different from control values ($P > .05$).

Figure 1. ISBN concentrations in exposure chamber before (left panel) and during (right panel) nitrite exposure to mice (mean $\pm$ SD, $n = 7$ experiments). ISBN indicates isobutyl nitrite.

Table 1 additionally shows that, in C57BL/6 mice, repeated ISBN exposure led to a similar degree of inhibition of hepatic enzyme activity, when compared with the results obtained after a single exposure. Again, when a nonexposure washout period of 24 hours was instituted, the suppressed enzyme activity was not different from that of control samples.

In Vitro GST Inhibition by ISBN

Figure 2 shows that in vitro exposure of GST to 22 mM ISBN, but not to 22 mM NaNO_2 , for only 10 seconds resulted in a significant and immediate inactivation of GST activity. The activity was further decreased by ISBN over a longer incubation period (60 minutes). However, NaNO_2 did not exhibit any inhibitory effect on GST activity even with this longer exposure period.

Figure 3 shows that addition of the antioxidant GSH prior to exposure to ISBN prevented ISBN-induced inhibition of GST activity, whether inhalant nitrite exposure was short term (10 seconds) or longer term (60 minutes). Under the conditions studied, GSH addition, by itself, did not alter GST activity.

We then examined whether added GSH was able to reverse ISBN-induced inhibition of GST activity. Figure 4 shows that addition of GSH after ISBN exposure, for either 10 seconds or 60 minutes, was unable to reverse ISBN-mediated enzyme inhibition.

The extent of protein S-nitrosylation was quantified using a dot blot. Incubation of GST with ISBN (22 mM), NaNO_2 (22 mM), NTG (220 μM), SNAP (220 μM), or TNM (100 μM) produced significantly more S-nitrosylation (dot intensity values of 251 ± 22 , 471 ± 18 , 405 ± 32 , 591 ± 58 , and 258 ± 36 , respectively) when compared with the vehicle control (50.2 ± 2.2 intensity units, $P < .001$ for ANOVA and post hoc test for each NO donor vs control). Based on these findings, it is clear that while all of the NO donors examined caused significant S-nitrosylation in GST, not all of them inactivated GST. Thus, in this study, we found that ISBN inactivated GST, while NaNO_2 did not. In a previous study,¹³ we also found that NTG and TNM inactivated GST but SNAP did not. Thus, GST S-nitrosylation did not appear to be associated with enzyme inactivation by these NO donors.

Figure 5 shows a representative comparison of the extent of GST-disulfide formation by silver staining on a 12% SDS-PAGE gel. Samples exposed to the vehicle control, 22 mM ISBN, or 22 mM NaNO_2 produced bands at 25 kDa and 50 kDa, consistent with the approximate molecular weight of GST monomer and dimer, respectively. However, no apparent difference was observed in the extent of GST dimerization between ISBN- and NaNO_2 -treated GST samples.

Figure 6 shows the extent of nitrotyrosine formation on GST when it was exposed to various NO donors. Panel A shows

Table 1. Enzyme Activity for Mice Sacrificed After ISBN Exposure*

Mouse Strain	Treatment [†]	Time of Sacrifice After Nitrite Exposure	Enzyme Activity		
Cytochrome P450–Mediated Deethylation (nmol/mg protein/min)	Glutathione S-Transferase (μmol/mg protein/min)	Carboxylesterase (μmol/mg protein/min)			
Balb/c	Control	Immediate	0.313 ± 0.004	4.64 ± 0.70	0.127 ± 0.013
	Acute ISBN	Immediate	0.058 ± 0.008 ^{††}	1.17 ± 0.08 [§]	0.095 ± 0.012 [§]
C57BL/6	Control	Immediate	0.522 ± 0.021	1.05 ± 0.11	0.248 ± 0.012
	Acute ISBN	Immediate	0.408 ± 0.016	0.85 ± 0.04 [§]	0.215 ± 0.015 [§]
	Control	24 hours later	0.471 ± 0.010	1.10 ± 0.03	0.245 ± 0.016
	Acute ISBN	24 hours later	0.473 ± 0.011	1.01 ± 0.10	0.254 ± 0.029
C57BL/6	Control	Immediate	0.503 ± 0.025	1.01 ± 0.06	0.252 ± 0.018
	Repeated ISBN	Immediate	0.360 ± 0.026	0.86 ± 0.03 [§]	0.215 ± 0.007 [§]
	Control	24 hours later	0.524 ± 0.033	1.04 ± 0.06	0.258 ± 0.023
	Repeated ISBN	24 hours later	0.506 ± 0.016	1.01 ± 0.04	0.242 ± 0.006

*ISBN indicates isobutyl nitrite. In each study, n = 3.

[†]Acute ISBN is 900 ppm for 45 minutes once; repeated ISBN is 900 ppm for 45 minutes each day for 6 days.

^{††}*P* < .0001, [§]*P* < .05, ^{||}*P* < .01.

that nitrotyrosine formation was observed for only ISBN- and TNM-treated samples (lanes 2 and 3, respectively), while samples treated with vehicle control (lane 1), NaNO₂ (lane 4), or SNAP (lane 5) did not exhibit significant tyrosine nitration. In panel B, the effect of GSH pretreatment (lane 2) and posttreatment (lane 3) of GST samples exposed to ISBN was compared with that of the vehicle control (lane 1, no GSH added). It was observed that addition of GSH prior to enzyme exposure to inhalant nitrite abrogated nitrotyrosine formation. In contrast, addition of GSH after GST exposure to ISBN did not reverse tyrosine nitration.

DISCUSSION

In the present study, we examined whether acute or repeated ISBN exposure in vivo at 900 ppm for only 45 minutes (for

1 or 6 days, respectively) might lead to significant decreases in the activity of several hepatic enzymes in mice. We chose the exposure conditions based on those established by Soderberg and Barnett,²¹ who used them to examine the immunomodulating effects of inhalant nitrite. The actual dose for human exposure to inhalant nitrite during abuse situations is not known but has been estimated to be as high as 7000 ± 260 ppm.²² Under our stated exposure conditions, all 3 enzymes monitored in this study, namely CEC-deethylase, GST, and CBE, demonstrated inhibition after both acute and repeated inhalant nitrite exposure (Table 1).

CEC deethylation is mediated by an array of CYP isozymes. Donato et al⁹ showed recently that in immortalized human liver epithelial cells expressing individual human P450

Figure 2. GST enzyme activity after exposure to ISBN (22 mM) and NaNO₂ (22 mM) for 10 seconds (A) or 60 minutes (B). GST activity was expressed as μmol 1-chloro-2,4-dinitrobenzene conjugated/min/mg protein (U/mg). * *P* < .05 and *** *P* < .001 vs control. GST indicates glutathione S-transferase; ISBN, isobutyl nitrite.

Figure 3. Effect of 5 mM GSH preincubation on the inactivation of GST by exposure to 22 mM ISBN for 10 seconds (A) or 60 minutes (B). GST activity was expressed as μmol CDNB conjugated/min/mg protein (U/mg). * *P* < .05 and *** *P* < .001 vs control. GST indicates glutathione S-transferase; ISBN, isobutyl nitrite; GSH, glutathione.

Figure 4. Effect of 5 mM GSH added after GST exposure to 22 mM ISBN for 10 seconds (A) and 60 minutes (B). The results for the 2 sets of studies were obtained with different lots of GST enzyme. GST activity was expressed as μmol 1-chloro-2, 4-dinitrobenzene conjugated/min/mg protein (U/mg). * $P < .05$, ** $P < .01$, and *** $P < .001$ vs the respective control. GST indicates glutathione S-transferase; ISBN, isobutyl nitrite; GSH, glutathione.

isozymes, CEC metabolite formation is primarily mediated through 1A2, and partially through 2C19 and 2D6. Using isolated perfused rat liver preparations, Vuppugalla and Mehvar^{23,24} observed that short-term infusion of sodium nitroprusside, another NO donor, caused time-dependent and isozyme-selective inhibition of CYP enzymes. Thus, the strain-dependent inhibition of CYP activity observed in this study could well be explained by the different isozyme compositions in the livers of these animals, and the differential sensitivity of each isozyme toward inhalant nitrite-mediated inhibition.

GST is known to eliminate drugs and toxic chemicals found in foods. However, GST is also thought to play a physiologic role in the activation of certain antineoplastic prodrugs, such as cis-3-(9H-purin-6-ylthio)acrylic acid, which is a structural analog of azathioprine and a prodrug of the antitumor and immunosuppressive drug 6-mercaptopurine.²⁵ The pharmacokinetics and pharmacodynamics of these agents may therefore be altered in nitrite abusers. GST is a hepatic enzyme that protects against many harmful xenobiotics, and reduction in hepatic GST activity may therefore impair

Figure 5. SDS-PAGE of glutathione S-transferase samples treated with the vehicle control, 22 mM ISBN, and 22 mM NaNO_2 . Representative of triplicate runs. ISBN indicates isobutyl nitrite.

Figure 6. Western blots for nitrotyrosine presence in GST-treated proteins. Panel A: GST was treated with vehicle control (lane 1), 22 mM ISBN (lane 2), 100 μM tetranitromethane (lane 3), 22 mM NaNO_2 (lane 4), or 220 μM S-nitroso-N-acetyl penicillamine (lane 5). Panel B: GST was treated with vehicle control (lane 1), pretreated with 5 mM GSH before ISBN exposure (lane 2), or treated with 5 mM GSH after ISBN exposure (lane 3). Representative of triplicate runs. GST indicates glutathione S-transferase; ISBN, isobutyl nitrite; GSH, glutathione.

the physiological protection mechanism of the liver in nitrite abusers. Nitrite abuse was found to be a high risk factor for morbidity in intravenous drug users.¹² Our finding that CBE activity was reduced after acute nitrite exposure could suggest the possibility of reduced cocaine or heroin metabolism, and thereby a greater potential for toxicity. However, the relevance of this interaction in humans will need to be demonstrated.

The underlying biochemical mechanisms for ISBN-induced enzyme inactivation were further investigated in vitro using rat liver purified GST as a model. It was difficult to estimate the ISBN concentrations that were exposed to hepatic enzymes in vivo. ISBN is highly soluble in both water and lipids, and its volume of distribution ($\sim 1 \text{ L/kg}$) far exceeds plasma or blood volume,²⁰ suggesting the possibility of intracellular sequestration. In a previous study, we used concentrations of 100 to 220 μM of various NO donors (NTG, SNAP, and TNM) to examine the mechanism of GST inactivation by NTG.¹³ However, at these concentrations, we did not observe substantial inactivation of GST by ISBN. Preliminary studies (data not shown) indicated that a concentration of 22 mM or higher reproducibly inactivated GST at both 10 seconds and 60 minutes, and this concentration was therefore selected for further study to gain some mechanistic insights into the nature of the biochemical reaction between the enzyme and the inhalant nitrite.

Figure 2 shows that ISBN-mediated GST inactivation was probably not monophasic: a significant inhibitory effect could be observed after only 10 seconds of exposure, but this fast rate of reaction was not maintained, and only a moderate additional decrease in GST activity was observed at 60 minutes. This nonlinear rate of inactivation was probably mediated through reactions with different accessible

site(s) and/or isozyme(s), which possess different sensitivity toward nitrite inactivation. Nevertheless, irrespective of the sites of inactivation, the reaction appeared to be mediated through intact ISBN, rather than its metabolic product, inorganic NO_2^- ion, since the latter (at equimolar concentration when compared with ISBN) had no effect on GST activity.

Protein S-nitrosylation has been shown to alter enzyme activity,²⁶ but in the present case, we did not observe any parallelism between the degree of S-nitrosylation of GST among those NO donors that inactivated the enzyme (ISBN, NTG, and TNM) and those that did not (NaNO_2 and SNAP). Thus, it can be concluded that S-nitrosylation is unlikely to be the mechanism responsible for ISBN-mediated inactivation of GST. These results also indicated that the responsible molecular species of enzyme inactivation is not NO, since the NO generator SNAP did not inactivate GST, as we have reported previously.¹³

The inactivation of rat liver GST by ISBN was prevented by 5 mM GSH (Figure 3), but 5 mM GSH could not reverse the reaction (Figure 4). These results strongly indicated that the inactivation mechanism likely involved irreversible protein oxidation. Since sulfhydryl oxidation to form the disulfide was usually reversible, this mechanism was unlikely to be applicable. Results shown in Figure 5 supported this view, since enhanced dimer formation by ISBN was not observed when compared with NaNO_2 exposure, which did not inactivate GST.

Finally, results shown in Figure 6 implicated tyrosine nitration as the possible mechanism for inhalant nitrite-mediated inactivation of GST. Nitrotyrosine formation in GST was observed with ISBN treatment but not after treatments with NaNO_2 and SNAP, neither of which inactivated the enzyme. Additionally, the absence and presence of nitrotyrosine residues upon GSH intervention (by pretreatment and post-treatment, respectively) were consistent with its effects on enzyme activity (Figures 3, 4, and 6), that is, nitrotyrosine formation was consistently observed when GST activity was decreased by ISBN, and it was not observed when enzyme inactivation was absent. These *in vitro* results are highly consistent with our previous *in vivo* observation that ISBN exposure led to extensive protein nitration in rat livers.⁶

Results of the present study regarding TNM-mediated inactivation of GST were consistent with those that we reported earlier¹³ indicating that tyrosine nitration was the applicable mechanism. We showed here, however, that the mechanism of ISBN-mediated inactivation of GST differed from that involving NTG, which reduced GST activity through protein sulfhydryl oxidation. These results underscore the important point that although all nitrovasodilators (eg, ISBN, NTG, and SNAP) are believed to cause vascular relaxation through the release of NO, their reac-

tions with cellular proteins, and their various sites, need not be identical.²⁷⁻²⁹

Although it was recognized that the GST enzyme source used in this study contained a mixture of isozymes, it is commercially available in sufficient quantities at reasonable costs, and a previous study indicated that similar mixed GST sources can produce meaningful mechanistic insights.¹³ Based on the present work, a detailed study of a highly purified GST (single isozyme) is in progress to determine the exact protein location(s) that might be involved in tyrosine nitration (T. Kamei et al, unpublished data, July 2007).

Although our data indicated that ISBN exposure led to marked decreases in the enzyme activity studied when the mice were immediately sacrificed after exposure, the enzyme activity returned to control values when the animals were allowed to recover for 24 hours after exposure. This apparent regeneration in enzyme activity suggests that the inhibitory effects of nitrite exposure are reversible within a 24-hour period. We showed previously⁶ that increased protein tyrosine nitration was still evident in rat livers 24 hours after nitrite exposure.⁶ The combined results suggest that new protein synthesis, rather than enzyme-mediated back-conversion of nitrotyrosine to tyrosine,³⁰ may be responsible for the return of enzyme activity. A similar rapid recovery in enzyme activity was observed even when mice were repeatedly exposed to ISBN for 6 days, suggesting that the effects of nitrite exposure on liver enzyme activity may not be cumulative. However, the relationships among exposure dose, frequency, and duration of enzyme inhibition were not defined by the present studies.

CONCLUSION

In conclusion, we showed for the first time that inhalant nitrite exposure led to a significant reduction in hepatic enzyme activity in 2 different strains of mice. The degree of enzyme inactivation appeared to be nonselective and highly strain-dependent. The likely underlying biochemical mechanism appeared to be tyrosine nitration of the affected protein. Determining the applicability of these animal results to humans will require further investigation.

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