Hepatotoxicity and Immunotoxicity of 1-Bromohexane and Its Glutathione Conjugation in Female BALB/c Mice

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Hepatotoxic and immunotoxic effects of 1-bromohexane (1-BH) and its conjugation with glutathione (GSH) were investigated in female BALB/c mice. The animals were treated once orally with 1-BH at 500, 1000, and 2000 mg/kg in corn oil for a dose-response study or treated orally with 1-BH at 2000 mg/kg for 6, 12, 24, and 48 hr for a time-course study. Treatment with 1-BH increased serum activities of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) dose dependently. The hepatic contents of thiobarbituric acid reactive substances were significantly increased at 2000 mg/kg of 1-BH from 12 to 24 hr after the treatment. Oral 1-BH at 2000 mg/kg significantly suppressed production of splenic intracellular interleukin (IL)-2 in response to concanavalin A. Following treatment with 1-BH, three GSH conjugates such as *S*-hexyl GSH, *S*-hexyl cysteine, and hydroxyhexyl mercapturic acid were identified in livers by liquid chromatography-electrospray ionization tandem mass spectrometry. The hepatic contents of GSH were maximally decreased 6 hr after treatment with 1-BH. GSH conjugates were also detected maximally in livers 6 hr after treatment. These results suggest that 1-BH could cause hepatotoxicity and immunotoxicity as well as depletion of GSH content due to the formation of GSH conjugates with 1-BH in female BALB/c mice.

Key words —— 1-bromohexane, glutathione conjugation, hepatotoxicity, immunotoxicity

INTRODUCTION

Short-chain halogenated alkanes such as 1-bromohexane (1-BH) have been used industrially as chemical intermediates, extraction solvents, and co-polymer cross-linking agents. 1-BH, a clear slightly yellow liquid, has also been used to manufacture pharmaceuticals and organic chemicals. It is popularly used as a cleaning agent and adhesive solvent in workplaces because of its low ozone-depleting potency.[1,2]

Many halide compounds including 1-bromopropane (1-BP), 1,2-dibromopropane (1,2-DBP), 2,3-dibromopropene, and 1,2-dibromo-3-chloropropane could be metabolized by conjugation with glutathione (GSH).[3–7] Some haloalkanes capable of forming GSH conjugates may cause toxicity related directly or possibly with GSH conjugation. For example, mice treated orally with 1-BP exhibit hepatotoxicity and immunotoxicity following depletion of GSH in liver and spleen.[8] In addition, hepatotoxicity and immunotoxicity of 2-BP, 1,2-DBP, and 1,3-DBP were also observed by formation of conjugates with GSH.[9–11]

The intracellular content of GSH in liver and spleen is a very important factor to prevent hepatotoxicity and immunotoxicity induced by certain chemicals. GSH depletion *in vivo*, in cells or homogenates by the substrates of GSH *S*-transferase can result in lipid peroxidation-associated cytotoxicity, which can be prevented or delayed by antioxidants or ferric chelators.[12–15] It has therefore been suggested that GSH depletion promotes endogenous oxidative stress and that GSH plays an antioxidant role in cells. In addition, the presence of GSH is re-

quired to maintain normal function of the immune system.[16–18]

In the present study the formation of GSH conjugates was investigated following single treatment of mice with 1-BH by liquid chromatography/electrospray ionization-tandem mass spectrometry (LC/ESI-MS). In addition, the dose-response and time-course effects of 1-BH on hepatotoxicity and immunotoxicity parameters were determined to characterize whether GSH conjugation might be an important factor in 1-BH-induced toxicity.

MATERIALS AND METHODS

Materials —— 1-BH ($>99\%$; molecular weight, 165.07) was obtained from Acros Organics (Geel, Belgium). *S*-Hexyl GSH, bovine serum albumin, and reagents for GSH quantitation were obtained from Sigma Chemical Company (St. Louis, MO, U.S.A.). BD Cytofix/Cytoperm Plus[TM] with GolgiPlug[TM] kit was obtained from BD Biosciences (San Diego, CA, U.S.A.). All antibodies used for flow cytometry were purchased from Pharmingen (San Diego, CA, U.S.A.). The kits for alanine aminotransferase (ALT) and aspartate aminotransferase (AST) assay were purchased from Asan Pharm. Co. (Hwasung, Korea). All other chemicals used were of reagent grade commercially available and used as received.

Animal Treatment —— Specific pathogen-free female BALB/c mice (19–21 g) were obtained from the Orient (Seoul, Korea). The animals received at 5 weeks of age were acclimated for ≥ 2 weeks. Upon arrival, animals were randomized and housed five per cage. The animal quarters were strictly maintained at $23 \pm 3°C$ and $50 \pm 10\%$ relative humidity. A 12-hr light and dark cycle (07:00–19:00) was used with an intensity of 150–300 lux. All animals' procedures were followed based on a guideline recommended by the Society of Toxicology (Reston, VA, U.S.A.) in 1989. This study was approved by the Institutional Review Board of the College of Pharmacy, Yeungnam University (Gyeongsan, Korea).

1-BH in corn oil was given once orally to animals at 500, 1000, and 2000 mg/kg. Control animals received 10 ml/kg corn oil. The doses were selected from a dose range-finding study and our previous studies for some haloalkanes.[8–10] For the dose-response study, animals were euthanized with ether 24 hr after the treatment. For the time-course study, 1-BH at 2000 mg/kg was treated orally once. Animals were subjected to necropsy 6, 12, 24, and 48 hr after the treatment. Following blood collection, liver, spleen, and thymus were removed and the liver was homogenized with 4 volumes of ice-cold 0.1 M potassium phosphate buffer, pH 7.4 for determination of GSH and conjugates with GSH. Aliquots of tissue homogenates and sera were stored at $-80°C$ until use.

Hepatotoxicity Parameters —— For assaying the activities of ALT and AST, serum was prepared by centrifugation of blood at $2500 \times g$ at $4°C$ for 15 min. The activities of enzymes were determined by assay kit according to the manufacturer's instructions.

Parameters for Oxidative Stress and Content of Reduced GSH —— The contents of thiobarbituric acid-reactive substances (TBARS) were determined as previously reported.[19] Liver GSH levels were determined by Ellman's method.[20] The content of liver homogenate protein was determined using bovine serum albumin as standard.[21]

Quantitation of Lymphocyte Subpopulations —— Using the Becton Dickinson FACSCalibur, the lymphocyte subpopulations in spleen and thymus were quantitated. To enumerate B cells, T cells, CD4^{+} and CD8^{+} T cell subsets, and macrophages splenic cells were collected and suspended in staining buffer containing 2% fetal bovine serum (FBS) and 0.1% sodium azide in phosphate-buffered saline (PBS), pH 7.4, to a cell density of 1.0×10^6/tube. The cells were incubated with anti-mouse CD16/CD32 Fc receptor (1 μg/tube, clone 2.4G2) at $4°C$ for 15 min to prevent nonspecific binding and then labeled with the appropriate monoclonal antibody (mAb) conjugated to a fluorescent probe. Anti-mouse CD3e mAb (clone, 145-2C11) conjugated to peridinin chlorophyll-a was used to enumerate T cells. For T cell subsets, fluorescein isothiocyanate (FITC)-conjugated anti-mouse CD4 (clone, GK1.5) and phycoerythrin (PE)-conjugated anti-mouse CD8a (clone, 53-6.7) were used. The same mAbs were used for staining thymocytes. B cells and macrophages were stained with anti-mouse CD45R/B220 mAb (clone, RA3-6B2) conjugated to PE and FITC-conjugated anti-mouse CD11b (clone, M1/70), respectively. An isotype control was used for each antibody. Cell suspensions were incubated with appropriate antibodies at 1 μg/tube for ≥ 30 min on ice in the dark. After incubation, 1x FACS Lysing solution (Becton Dickinson, San Jose,

CA, U.S.A.) was treated for 10 min for hemolysis. The cells were washed twice, resuspended in PBS containing 2% FBS and 0.1% sodium azide in the total volume of 0.5 ml, and analyzed by FACSCalibur® flow cytometry with CellQuest® software (Becton Dickinson).

Assay of Intracellular Interleukin (IL)-2 Production—— To simplify the fixation and permeabilization of cells for immunofluorescent staining of intracytoplasmic IL-2, BD Cytofix/Cytoperm Plus™ with GolgiPlug™ kit was used. For *in vitro* activation, single cell suspensions (1×10^6 splenocytes/ml) from mice treated with 1-BH for 24 hr were cultured with 1 μg/ml of concanavalin A (Con A) in the presence of recombinant IL-2 (rIL-2, Roche Applied Science, Mannheim, Germany) at 37°C in 5% CO_2 for 12 hr. After incubation, the splenocytes were stimulated again with rIL-2 and Con A for 5 hr. Brefeldin A ($10 \mu g/10^6$ cells) was added in this stage. Then the cells were washed with staining buffer and nonspecific binding blocked by adding anti-mouse CD16/CD32 Fc receptor ($1 \mu g/10^6$ cells/tube) on ice for 20 min. CD4$^+$ cells were identified by FITC-conjugated anti-mouse CD4 antibody (0.5 μg/tube; clone, GK1.5), which was suspended in staining buffer and incubated on ice for 30 min in dark condition. To fix and permeabilize the cells, Cytofix/Cytoperm solution (100 μl/tube) was added and incubated on ice for 20 min in dark. Then, 1x Perm/Wash solution (1 ml/tube) was added for washing. To stain intracellular cytokine, fixed/permeabilized cells were incubated with 100 μl of Perm/Wash solution containing PE-conjugated anti-mouse IL-2 antibody (0.2 μg/tube; clone, JES6-5H4) on ice for 30 min in dark. Following washing with 1x Perm/Wash solution twice, cells were resuspended in the staining buffer (300 μl/tube) for flow cytometric analysis.

Sample Analysis by LC/ESI-MS—— To identify GSH conjugates, liver homogenates were extracted by addition of 2 volumes of acetonitrile. After vortex mixing for 10 min and centrifugation at 15000 $\times$ *g* at 15°C to remove the proteins, the resulting supernatant was evaporated under stream of nitrogen in a water bath maintained at 60°C. The resulting residue was reconstituted in a mixture of 50/50 of 0.1% aqueous formic acid/methanol by vortexing and subsequent centrifugation at 15000 $\times$ *g* at 15°C for 10 min. Then, a 10-μl aliquot was used for LC/MS analysis.

HPLC consisted of a surveyor system (Thermo Finnigan, San Jose, CA, U.S.A.) with the LCQ ad-

vantage trap mass spectrometer (Thermo Finnigan) equipped with an electrospray ionization source. The column used was the Atlantis dC18 (3.0 $\times$ 100 mm, 3 μm) for analysis of 1-BH conjugates. The HPLC mobile phases consisted of 100% acetonitrile in 0.1% formic acid (A) and 0.1% aqueous formic acid, pH 4.0 (B). To analyze GSH conjugates of 1-BH, a gradient program was used for HPLC separation with a flow rate of 300 μl/min. The linear gradient was as follows: 0–5 min, 5% A; 5–15 min, 5% to 70% A. Nitrogen was used both as sheath gas at 40.0 l/min and as auxiliary gas at 10.0 l/min with a capillary temperature of 300°C and the spray voltage set to 4.5 kV. The mass spectrometer was operated in the positive ion mode in *m/z* range 100–400. Helium was used as collision gas for MS experiments, followed by the isolation of ions over a selected mass window of 1 Da.

Statistics—— The mean value ± standard error (S.E.) was determined for each treatment group of a given experiment. Dunnett's *t*-test was used to compare statistical significance of data (SPSS 12.0, SPSS Institute, Chicago, IL, U.S.A.).

RESULTS AND DISCUSSION

To investigate the effect of 1-BH on general toxicity, changes in body, liver, spleen, and thymus weights were determined following single treatment of mice with 1-BH in corn oil (Table 1). Female BALB/c mice were treated orally with 1-BH at 500, 1000, and 2000 mg/kg for the dose-response study. The body weights were significantly reduced by treatment with 1-BH at 2000 mg/kg. The relative liver weight was significantly increased by treatment with 1-BH at 1000 mg/kg. When mice were treated orally with 2000 mg/kg of 1-BH once for 6, 12, 24, and 48 hr, both body and relative thymus weights were significantly decreased from 12 to 48 hr after the treatment (Table 1). The relative spleen weights were gradually decreased, and the liver weight was not changed in the time-course study.

Hepatotoxic effects of 1-BH are displayed in Table 2. Following single oral treatment with 3 doses of 1-BH for 24 hr, serum ALT and AST activities were significantly increased 1.5 and 2.4 fold, respectively, at the maximum dose of 1-BH when compared with the vehicle-treated control. In the time-course study, the activities of serum ALT and AST were maximally increased by 1-BH 6 hr af-

Table 1. Effects of 1-BH on Body and Organ Weight Changes in Female BALB/c Mice

Groups		Body weight (g)	Relative organ weight (% body weight)		
			Liver	Spleen	Thymus
Dose (mg/kg)	VH	19.7 ± 0.4	5.32 ± 0.08	0.42 ± 0.02	0.40 ± 0.02
	500	19.6 ± 0.5	5.38 ± 0.06	0.40 ± 0.01	0.40 ± 0.01
	1000	19.7 ± 0.5	5.89 ± 0.11**	0.41 ± 0.01	0.47 ± 0.04
	2000	17.8 ± 0.6*	5.42 ± 0.13	0.39 ± 0.02	0.32 ± 0.04
Time (hr)	0	20.7 ± 0.8	5.08 ± 0.05	0.45 ± 0.02	0.33 ± 0.02
	6	19.6 ± 0.6	4.63 ± 0.09	0.46 ± 0.03	0.28 ± 0.01
	12	18.7 ± 0.1*	4.91 ± 0.05	0.38 ± 0.01	0.22 ± 0.03**
	24	17.8 ± 0.3**	4.93 ± 0.12	0.39 ± 0.02	0.24 ± 0.01*
	48	18.8 ± 0.3*	5.52 ± 0.24	0.38 ± 0.02*	0.16 ± 0.01**

Each value represents mean ± S.E. of five animals. *$p < 0.05$; **$p < 0.01$ *vs.* either vehicle control (VH) or 0-hr control.

Table 2. Effects of 1-BH on Serum Activities of ALT and AST and on Production of TBARS

Groups		ALT (Karmen unit/ml)	AST (Karmen unit/ml)	TBARS (nmol/mg protein)
Dose (mg/kg)	VH	39.4 ± 3.0	66.4 ± 1.6	1.66 ± 0.08
	500	44.8 ± 2.2	106.9 ± 9.0**	2.55 ± 0.13*
	1000	54.5 ± 5.1	108.8 ± 7.2**	2.91 ± 0.32**
	2000	81.6 ± 9.6**	155.5 ± 8.2**	4.27 ± 0.29**
Time (hr)	0	39.4 ± 3.0	66.4 ± 1.6	1.66 ± 0.08
	6	194.6 ± 55.8**	244.1 ± 32.0**	2.59 ± 0.17
	12	73.9 ± 8.8	141.7 ± 18.8*	3.49 ± 0.79*
	24	81.5 ± 9.6	155.5 ± 8.2**	4.25 ± 0.26**
	48	71.8 ± 10.3	183.1 ± 33.1**	2.35 ± 0.11

Each value represents mean ± S.E. of five animals. *$p < 0.05$; **$p < 0.01$ *vs.* either VH or 0-hr control.

ter treatment. The contents of hepatic TBARS in all groups were increased in a dose-dependent manner (Table 2). Particularly, single oral treatment with 2000 mg/kg of 1-BH significantly increased in TBARS production 2.6 fold when compared with vehicle-treated control. In the time-course study, the contents of hepatic TBARS were maximally increased by 1-BH from 12 to 24 hr after treatment (Table 2).

To determine the cellular target of 1-BH-induced immunosuppression, mice were administered with the same dose of 1-BH followed by splenic and thymic lymphocyte phenotyping by flow cytometry (Table 3, Figs. 1 and 2). Mice exposed to 2000 mg/kg of 1-BH showed a significant decrease in percent macrophage cells and CD4+CD8+ cells among total splenocytes and thymocytes, respectively (Table 3). Meanwhile, the absolute numbers of splenocytes and thymocytes were reduced by 2000 mg/kg of 1-BH. Mice treated with 1-BH significantly decreased numbers of all subpopulations in spleen, and immature CD4+CD8+

cells in thymus (Figs. 1 and 2). The production of splenic intracellular IL-2 in response to Con A (1 μg/ml) was decreased significantly in 1-BH-treated mice. The number of CD4+IL-2+ cells was significantly reduced following exposure of mice to 2000 mg/kg of 1-BH (Fig. 3).

Three GSH conjugates of 1-BH were identified in liver homogenates following treatment with 1-BH (Figs. 4 and 5). The fragmentation mechanism of individual conjugates using LC/ESI-MS could be proposed for the detection of unknown GSH conjugates in complex samples of biological origin.[22] Collision-induced dissociation (CID) of parent ion species could be effective means of extracting information on the structures of GSH conjugates. Protonated molecular ions [M+H]+ of *S*-hexyl GSH, *S*-hexyl cysteine, and hydroxyhexyl mercapturic acid were observed at *m/z* 392.1, 206.0, and 264.1 in the positive ESI spectra with retention times of 14.2, 14.0, and 14.2 min, respectively (Fig. 4). *S*-Hexyl GSH was identified from the fragment ion of *m/z* 317.1 for the elimination of glycine moiety, the
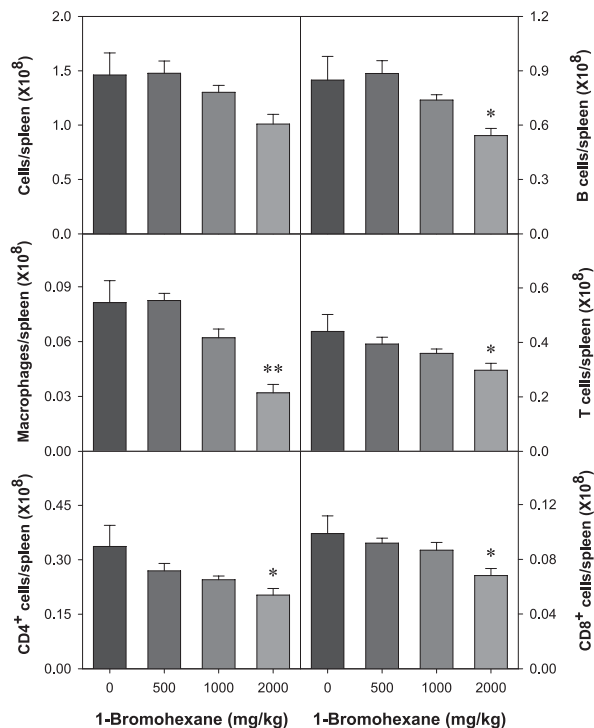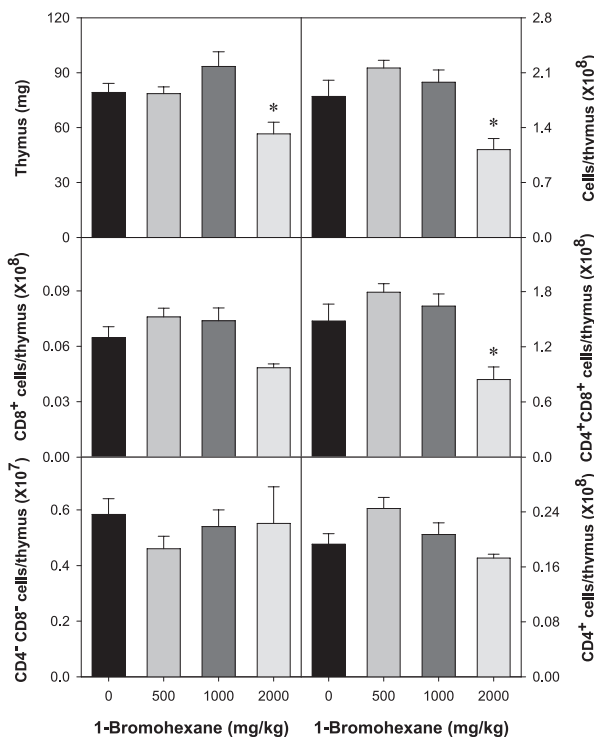
Fig. 1. Effects of 1-BH on Splenic Lymphocytes Subpopulations in Female BALB/c Mice

Mice were treated once orally with each dose of 1-BH in corn oil at 10 ml/kg. All animals were subjected to necropsy 24 hr after treatment. Each bar represents mean no. cells ± S.E. of five animals. $^{*}p < 0.05$; $^{**}p < 0.01$ *vs.* vehicle control.

Fig. 2. Effects of 1-BH on Thymocytes Subpopulations in Female BALB/c Mice

Mice were treated once orally with each dose of 1-BH in corn oil at 10 ml/kg. All animals were subjected to necropsy 24 hr after treatment. Each bar represents mean no. cells ± S.E. of five animals. $^{*}p < 0.05$ *vs.* vehicle control.
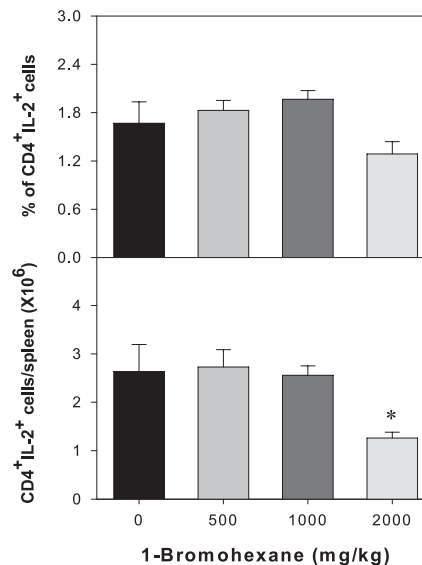

Fig. 3. Effects of 1-BH on Splenic Intracellular IL-2 Production in Female BALB/c Mice

Splenocytes isolated from 1-BH-treated female BALB/c mice for 24 hr were cultured for 12 hr in the presence of Con A. Each bar represents mean ± S.E. of five animals. $^{**}p < 0.01$ *vs.* vehicle control.

ion at m/z 263.0 for the loss of glutamate moiety, and the ion at m/z 246.0 for the loss of amino-glutamyl moiety.[2,6,23,24] *S*-Hexyl cysteine could also be identified from the fragment ion at m/z 188.9 for the loss of amine moiety. Although the right position of hydroxyl group could not be determined, a hydroxyhexyl mercapturic acid was identified from the fragment ion at m/z 246.1 for elimination of water, the ion at m/z 221.8 for loss of acetaldehyde group, and the ion at m/z 203.8 for loss of acetamide group from the metabolite (Fig. 5).

To characterize the formation of GSH conjugates *in vivo*, dose-response and time-course studies were performed in female BALB/c mice. Following treatment of mice with 500, 1000, and 2000 mg/kg of 1-BH once for 24 hr, liver homogenates were analyzed. The content of hepatic GSH following single oral treatment with 2000 mg/kg of 1-BH for 24 hr was significantly reduced to 46.6% of control (Fig. 6). Hepatic GSH was maximally reduced at 6–12 hr following treatment with 1-BH, in association with maximal production of *S*-hexyl GSH conjugates (Fig. 6).

These results were paralleled with other bromoalkanes-induced hepatotoxicity.[2,8—11] ALT and AST activities and the content of TBARS elevated by 1-BH were proportional to the depletion of GSH and formation of GSH conjugates (Table 2 and Fig. 6). Although the direct role of GSH con-
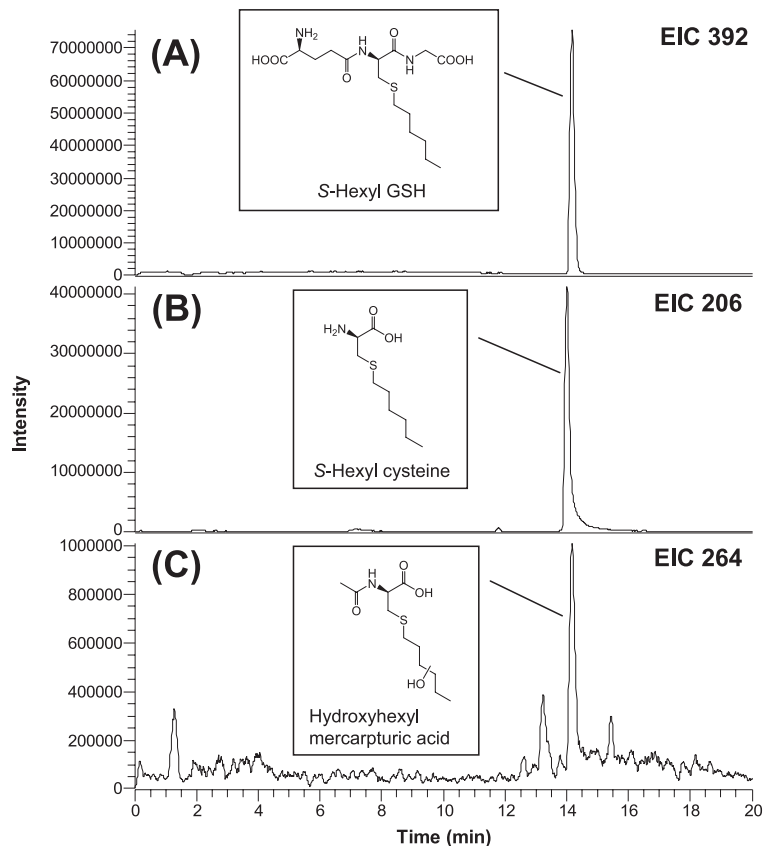
Fig. 4. Extracted Ion Chromatograms of Three Conjugates

    S-Hexyl GSH (A), *S*-hexyl cysteine (B), and hydroxyhexyl mercapturic acid (C). Female BALB/c mice were treated once orally with 2000 mg/kg of 1-BH in corn oil for 6 hr.
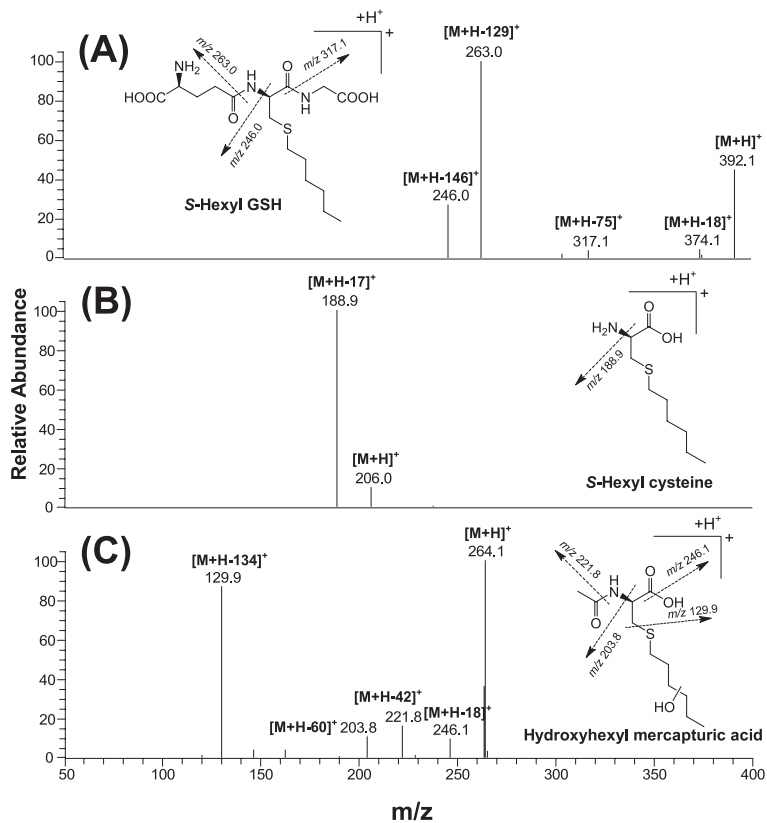

Fig. 5. CID Spectra of Protonated *S*-Hexyl GSH (A), *S*-Hexyl Cysteine (B), and Hydroxyhexyl Mercapturic Acid (C)
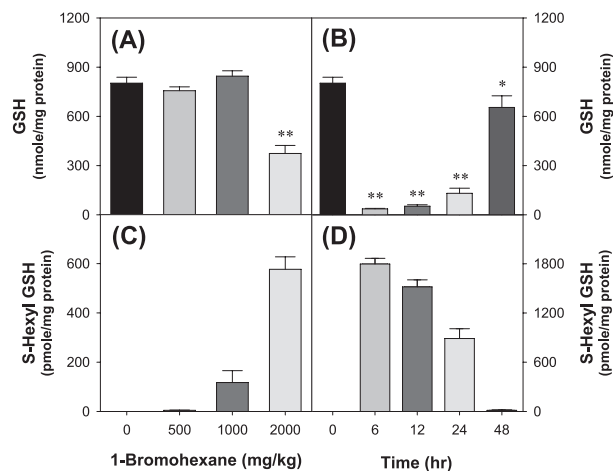
Fig. 6. Effects of 1-BH on Content of GSH (A and B) and Formation of *S*-Hexyl GSH (C and D) in Liver: Dose-response and Time-course Studies

For dose-response study, 1-BH was treated orally at 500, 1000, and 2000 mg/kg for 24 hr. Mice were treated once orally with 1-BH at 2000 mg/kg in corn oil at 10 ml/kg for the time-course study. All animals were subjected to necropsy at 6, 12, 24, and 48 hr after treatment. Each bar represents mean ± S.E. of five animals. $^*p < 0.05$; $^{**}p < 0.01$ *vs.* control.

jugates in 1-BH-induced toxicity was not studied in the present investigation, it seems clear that the hepatotoxicity by 1-BH might be closely related to increased production of reactive oxygen species following depletion of GSH due to the formation of GSH conjugates in female BALB/c mice.

Although depletion of GSH in spleen was not investigated, 1-BH could suppress immune function at the dose showing GSH depletion in liver (Figs. 1 and 2). Indeed, there are reports that immune function could be affected by the contents of GSH.[16–18] In many previous studies, depletion of GSH induced by formation of GSH conjugate with 1-BP and 1,3-DBP severely induced immunotoxicity in spleen and/or thymus.[8, 11, 17, 25] The function of T cells would also be affected by the content of GSH in intracellular environment, so that the condition to suppress GSH content might result in inhibition of IL-2-dependent T cell growth and suppression of DNA synthesis.[17, 26–28] In addition, intracellular GSH levels have a strong influence on the T cell system in human immunodeficiency virus-infected persons who generally show decreased levels of intracellular GSH.[29]

Besides *S*-hexyl GSH conjugate, some other metabolites were identified in livers by LC/ESI-MS. From the present studies, *S*-hexyl cysteine and hydroxylhexyl mercapturic acid conjugates were detected in mice treated orally with 1-BH. All these conjugates could, at least in part, contribute to re-

duction of intracellular content of GSH, the primary defensive material against oxidative or electrophile-induced stresses.

In conclusion, the present studies demonstrated that 1-BH might induce hepatotoxicity and immunotoxicity, and that GSH could play an important role in metabolism of 1-BH. Moreover, the formation and structures of GSH conjugates with 1-BH and some other metabolites were identified by LC/ESI-MS. Although the toxic potential of GSH conjugates with 1-BH was not investigated in the present studies, it was clear that depletion of hepatic GSH through conjugate formation is closely associated with oxidative stress induced by 1-BH.

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