Glutathione Depletion Enhances Subanesthetic Halothane Hepatotoxicity in Guinea Pigs

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Reduced glutathione has a potential role in protecting the liver against the reactive acyl acid chloride intermediate generated during the oxidative biotransformation of halothane. Glutathione is also important in maintaining the integrity of an injured cell. Thus, the effect of decreased hepatic glutathione concentrations on covalent binding of halothane metabolic intermediates to hepatic protein and lipid and the resultant hepatic injury were investigated in male, outbred Hartley guinea pigs. The animals were injected with either 1.6 g·kg^{-1} dl-buthionine-S,R-sulfoximine to deplete hepatic glutathione or vehicle-control solution 24 h before exposure to 0.1% (subanesthetic) halothane for 4 h (fractional inspired oxygen tension = 0.40). Buthionine sulfoximine pretreatment depleted liver glutathione concentrations by 85% at the time of halothane exposure, without affecting the degree of halothane biotransformation or causing hepatic injury. Glutathione depletion caused a significant increase in the level of organic fluorine covalently bound to hepatic protein but not lipid after halothane exposure. Glutathione-depleted animals also exhibited a significant enhancement of hepatotoxicity after halothane exposure; plasma isocitrate dehydrogenase activity was 25-fold greater than the increase observed 48 h after exposure in animals treated with vehicle plus halothane, and the incidence and severity of hepatic injury were significantly greater, as observed by light microscopic examination of tissue 96 h after exposure. These findings are in agreement with a previously proposed mechanism of halothane-associated hepatotoxicity in guinea pigs and indicate that hepatic glutathione status may play an important role in the susceptibility of patients to halothane-induced liver injury. (Key words: Anesthetics, volatile: halothane; subanesthetic concentration. Animals: guinea pig. Binding: reactive intermediates. Biotransformation: halothane. Glutathione: buthionine sulfoximine; depletion; hepatic. Liver: hepatotoxicity.)

CLINICALLY, halothane is believed to cause hepatic injury that can range from a mild, acute form in 20% or more of patients[1,2] to a rare, often fatal, fulminant hepatitis in 1:7,000–30,000 uses.[3,4] The mild, acute injury has been proposed to result from direct subcellular damage caused by the covalent binding of reactive halothane biotransformation intermediates to subcellular macromolecules.[1] The fulminant disease, however, is believed to be an idiosyncratic autoimmune reaction resulting from neoantigens produced by the covalent binding of halothane biotransformation intermediates to hepatic proteins.[5]

To provide a better understanding of the mechanisms involved in acute halothane-associated liver injury, a sensitive animal model has been developed in the guinea pig.[6–9] Hepatic damage, ranging from scattered foci of necrosis to confluent centrilobular necrosis, is produced consistently after 4 h of halothane (1%) anesthesia. Even exposure to a subanesthetic concentration of halothane (0.1%) causes hepatic injury.[10] The spectrum of liver injury in guinea pigs has been found to closely resemble that observed in nonfatal halothane hepatitis in humans.[8,11] However, fatal hepatic injury has not been reported in the guinea pig.

During the oxidative biotransformation of halothane by the hepatic cytochrome P-450 enzyme system, a reactive trifluoroacetyl acid chloride intermediate is formed that covalently binds to free amino groups on proteins or reacts with water within the hepatocyte, producing the metabolite trifluoroacetic acid (TFA).[5,12] Through use of the deuterated analog of halothane and hypoxic exposure conditions to alter the oxidative biotransformation of halothane, we recently have been able to associate binding of this reactive intermediate to hepatic protein with the acute liver injury observed in the guinea pig.[9] Of course, halothane also can undergo reductive metabolism, producing free radicals that primarily bind to lipid and release free fluoride ion as a metabolite.[13] However, this pathway is minor and does not appear to be critical in the development of necrosis in the guinea pig.[9]

Glutathione is a known protectant against damage caused by chemicals that produce acid chloride metabolic intermediates by reacting with the intermediates before they can bind covalently to vital subcellular constituents.[14] This investigation evaluates the effect of decreasing hepatic glutathione concentrations on the bioactivation of halothane and the degree of resultant hepatic injury. Buthionine sulfoximine (BSO), an inhibitor of γ-glutamyl cysteine synthetase, the enzyme responsible for the first step in glutathione synthesis, was used to decrease hepatic glutathione concentrations.[15] With the use of a subanesthetic concentration (0.1%) of halothane to produce a lesser degree of hepatic injury,[10] any exacerbating effect

of glutathione depletion could be observed more readily, and, it is hoped, adverse effects caused by higher concentrations of halothane avoided.[16–18]

Materials and Methods

ANIMALS

Male outbred Hartley guinea pigs (600–700 g) were obtained from Sasco, Inc. (Omaha, NE) and treated according to a protocol approved by the University of Arizona Animal Care Committee. Housing was in stainless steel cages with a 12-h light/dark cycle. Food and water were provided *ad libitum*. All treatments were performed at the same time of day to avoid diurnal variations.

GLUTATHIONE DEPLETION

Depletion of hepatic glutathione was accomplished by intraperitoneal injection of 1.6 $g \cdot kg^{-1}$ dl-buthionine-S,R-sulfoximine (BSO) (Chemical Dynamics Corporation, South Plainfield, NJ) dissolved in water ($pH = 8.5$) and administered at a volume of 10 $ml \cdot kg^{-1}$.[19] Vehicle-control guinea pigs received an equal volume of water containing sodium hydroxide equivalent to that required for dissolution of BSO, adjusted to a pH of 8.5 with hydrochloric acid. In guinea pigs, BSO causes a prolonged (48–72 h) depletion of hepatic glutathione. This permitted administration of the drug 24 h before inhalation exposure to halothane.[19]

EXPOSURE CONDITIONS

Groups of guinea pigs were exposed to subanesthetic (0.1%) halothane concentrations (Halocarbon Laboratories, Inc., North Augusta, SC). Exposures were performed in a 180-l Plexiglas exposure chamber for 4 h with 40% O_2 (balance N_2), at a flow rate of 8 $l \cdot min^{-1}$. Halothane and oxygen concentrations were monitored by gas chromatography and polarographic oxygen electrodes, respectively.[20] The guinea pigs remained awake and active throughout the exposure period.

SAMPLE COLLECTION

Animals were killed by cervical dislocation immediately after halothane exposure. Blood was collected by cardiac puncture. Livers were removed and sections were fixed in 10% neutral buffered formalin, with the remainder being frozen immediately on dry ice. All animals killed at other times received lethal intraperitoneal injections of pentobarbital. Blood and liver samples then were collected as described above. Blood samples collected at time points other than the time when the animals were killed were obtained by toenail bleedings, with small volumes removed (1–2 ml immediately after halothane exposure,

0.3–0.5 ml at other sampling times) to avoid effects on blood volume.

SAMPLE ANALYSIS

Plasma isocitrate dehydrogenase (ICDH) activity was measured spectrophotometrically (Procedure 153-UV; Sigma Chemical Company, St. Louis, MO). Concentrations of the halothane metabolites, TFA and fluoride ion, were determined by a gas chromatographic headspace technique[21] and with specific ion electrodes,[7] respectively. Hepatic glutathione concentrations were measured by the spectrofluorometric method of Hissin and Hilf.[22] Covalent binding of halothane biotransformation intermediates to hepatic protein and lipid was evaluated with our sodium fusion technique to release fluoride ion from bound organic fluorine.[9]

HISTOPATHOLOGIC EVALUATION

A randomly selected section of formalin-fixed liver tissue was processed and stained with hematoxylin and eosin. Slides were coded before submission to the pathologist (P.H.) for light microscopic evaluation of hepatic injury. Liver injury was graded as follows: nonresponding—normal histologic appearance; mild—occasional to multiple foci of necrosis of single or small groups of hepatocytes scattered throughout the liver lobules; moderate—areas of necrosis involving some but not all of the centrilobular region; and severe—confluent necrosis of the entire centrilobular region.

EXPERIMENTAL PROTOCOL

Groups of guinea pigs (n = 16) were injected with either BSO or vehicle-control solution. Twenty-four hours after injection, one fourth of the animals in each group (n = 4) were killed so that the effect of BSO pretreatment on hepatic glutathione concentrations could be measured. The remaining animals in each group (n = 12) were exposed to 0.1% halothane at this time (24 h after injection). Of these animals, one third (n = 4) were killed immediately after halothane exposure (0 h), one third were killed 10 h after exposure, and the remaining third were killed 96 h after. Blood samples were collected from all animals at the end of halothane exposure and at the time they were killed. In addition, at 24, 48, and 72 h after exposure, blood samples were obtained from the groups allowed to survive until 96 h after exposure.

All blood samples were measured for plasma ICDH activity. Glutathione concentrations were measured in all livers. Liver tissue sections from all animals underwent histopathologic evaluation. So that possible effects of BSO administration on halothane biotransformation could be observed, plasma concentrations of the halothane metab-

olites, TFA and fluoride ion, were measured at 0, 10, and 24 h after exposure, as plasma volumes allowed. Effects on covalent binding of reactive halothane biotransformation intermediates were evaluated by measurement of bound organic fluorine to hepatic protein and lipid in livers collected at 0 and 10 h after halothane exposure. Baseline ICDH activity, background values for halothane metabolite concentrations, and background values for bound organic fluorine to hepatic protein and lipid were determined in the group (n = 4) of guinea pigs that received vehicle-control solution and were killed 24 h later.

An additional group of control animals (n = 3) received only BSO so that the long-term hepatotoxic potential of glutathione depletion could be evaluated. In this group, blood was collected and the animals were killed on the same schedule as the groups that were allowed to survive until 96 h after halothane exposure. Because all animals were injected 24 h before halothane exposure, the animals in the group receiving only BSO were killed 120 h (24 + 96 h) after injection.

STATISTICAL ANALYSIS

All data are expressed as mean ± standard error of the mean. Hepatic glutathione concentrations were compared with a one-tailed Student's t test. Comparisons of plasma ICDH, TFA, and fluoride ion values and bound organic fluorine to protein and lipid were made by analysis of variance with a Newman-Keuls multiple comparison test. Because of increasing standard errors with increasing means, a log transformation of ICDH values was performed before analysis.[23] Incidences of hepatic necrosis at 96 h after halothane exposure were compared by analysis of variance. $P < 0.05$ was considered significant.

Results

Pretreatment of guinea pigs with BSO led to a decrease in hepatic glutathione concentrations from 2.8 ± 0.1 to 0.5 ± 0.1 μmol/g liver (n = 4, $P < 0.01$) by 24 h after injection. When these animals were exposed to a subanesthetic concentration of halothane (0.1%), the hepatotoxicity of halothane was enhanced significantly in the glutathione-depleted animals as compared with concurrent controls. Increases in plasma ICDH activity developed in BSO-pretreated guinea pigs after halothane exposure that were significantly greater than the increases observed in vehicle-pretreated animals at all sampling times except 10 h after exposure (fig. 1). The appearance of liver tissue from both treatment groups was within normal limits immediately after halothane exposure. By 10 h after exposure, early indications of hepatic injury were evident, but there were no apparent differences between treatment groups. However, at 96 h after exposure,
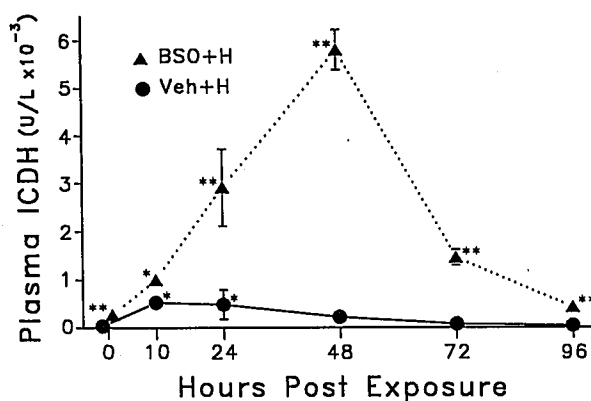

FIG. 1. Plasma isocitrate dehydrogenase activity in guinea pigs after exposure to 0.1% halothane. Exposures were at 40% O_2 for 4 h. Symbols at 0 h are offset slightly for clarity. BSO + H = 1.6 g/kg BSO ip 24 h before halothane exposure; Veh + H = vehicle control solution ip 24 h before exposure. All values are mean ± SEM; N = 12 at 0 h, N = 4 at other time points. *$P < 0.01$ versus vehicle control (34 ± 3 units/ml, N = 4) values. **$P < 0.01$ versus vehicle control values and Veh + H values at same time point.

the differences in appearance between the groups were significant. All guinea pigs treated with vehicle plus halothane exhibited mild injury (table 1, fig. 2), whereas all of the animals treated with BSO plus halothane showed severe injury (table 1, fig. 3).

The enhancement of hepatotoxicity in BSO-pretreated animals was associated with significantly greater levels of covalent binding of halothane biotransformation intermediates to hepatic protein at both 0 and 10 h after exposure (fig. 4), whereas there were no significant differences between treatment groups in binding to lipid (fig. 5). Although the extent of covalent binding to protein in both halothane-exposed groups remained unchanged between 0 and 10 h (fig. 4), the degree of binding to lipid decreased significantly by 10 h (fig. 5). Plasma concentrations of the halothane metabolites, TFA and fluoride ion, were not different between treatment groups after exposure (fig. 6). TFA concentrations increased slightly by 10 h after exposure, whereas fluoride ion concentrations remained unchanged.

Hepatic glutathione concentrations were not affected by halothane exposure. Values obtained before halothane exposure remained unchanged from those observed both immediately after exposure (0 h) and 10 h after exposure (data not shown). By 96 h after exposure, hepatic glutathione concentrations in the animals treated with BSO plus halothane had returned to the normal range, and values in those animals that had received vehicle-control solution plus halothane remained unchanged (i.e., normal) (data not shown). Hepatic glutathione concentrations in the control group (n = 3) that received only BSO also returned to normal levels by 120 h after injection. No

TABLE 1. Incidence of Hepatic Necrosis in Guinea Pigs

Treatment	Histopathologic Evaluation			
	Nonresponding	Mild	Moderate	Severe
Vehicle control	4	0	0	0
BSO	2	1	0	0
Halothane	0	4*	0	0
BSO + halothane	0	0	0	4†

Values are no. of animals.

Vehicle control = control solution ip killed 24 h after injection; BSO (buthionine sulfoximine) = 1.6 g/kg BSO ip, killed 120 h after injection; halothane = 0.1% halothane 40% O_2, 4 h, killed 96 h after exposure; BSO + halothane = 1.6 g/kg BSO ip 24 h before 0.1% halothane, 40% O_2, 4 h, killed 96 h after exposure; nonresponding = normal histologic appearance; mild = scattered small foci of necrosis; moderate = limited centrilobular necrosis; severe = confluent centrilobular necrosis.

* $P < 0.05$ versus vehicle control and BSO treatment groups.
† $P < 0.05$ versus all other treatment groups.

increases in plasma ICDH activity were observed in any of the animals that received only BSO. Liver tissue from guinea pigs that were killed 24 h after receiving either BSO or vehicle-control solution was normal in appearance. In one animal from the control group (n = 3) that received only BSO, a few scattered single-cell foci of necrosis were displayed in the liver at 120 h after dosing (table 1). However, this group was not significantly different from the vehicle-control group.

Discussion

Depletion of hepatic glutathione concentrations by 85% in guinea pigs, which resulted from pretreatment with BSO, caused a significant potentiation of halothane-as-
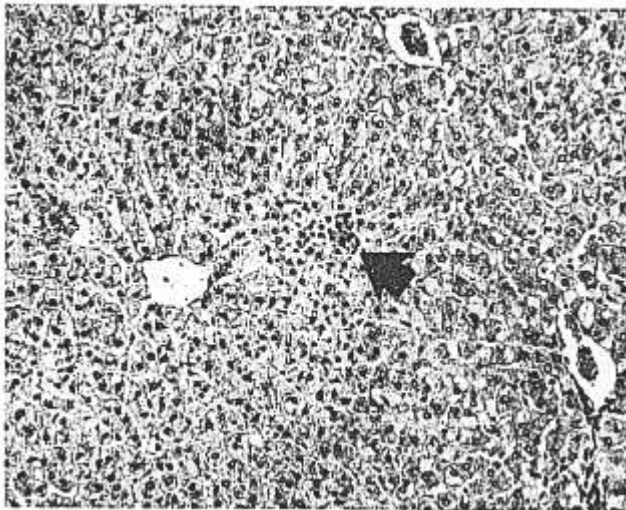

FIG. 2. Photomicrograph of liver from a guinea pig that received vehicle control solution 24 h before 0.1% halothane. Exposure was at 40% O_2 for 4 h. Tissue was obtained 96 h after exposure. A small foci of necrosis (arrow) accompanied by a mononuclear cell infiltrate, "mild injury," is evident (hematoxylin and eosin, original magnification X340).
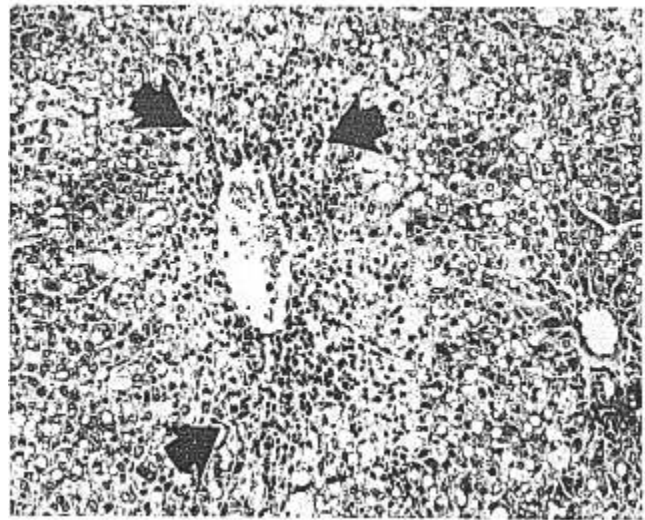

FIG. 3. Photomicrograph of liver from a guinea pig that received BSO 24 h before 0.1% halothane. Exposure was at 40% O_2 for 4 h. Tissue was obtained 96 h after exposure. There is confluent centrilobular necrosis (arrows) and an associated lymphohistiocytic infiltrate. Fatty change is also prominent (hematoxylin and eosin, original magnification X340).

sociated hepatic injury after a 4-h inhalation exposure to a subanesthetic (0.1%) concentration. With glutathione depletion, there was a 25-fold greater increase in plasma ICDH activity 48 h after halothane exposure and a much more severe degree of hepatic necrosis observed at 96 h. This was not a result of altered halothane biotransformation because BSO pretreatment did not affect metabolite production. Glutathione depletion did cause a 60% or greater enhancement of covalent binding of reactive halothane biotransformation intermediates to hepatic protein while not affecting binding to hepatic lipid.

To better observe any potential effect of BSO administration on biotransformation and binding, we measured
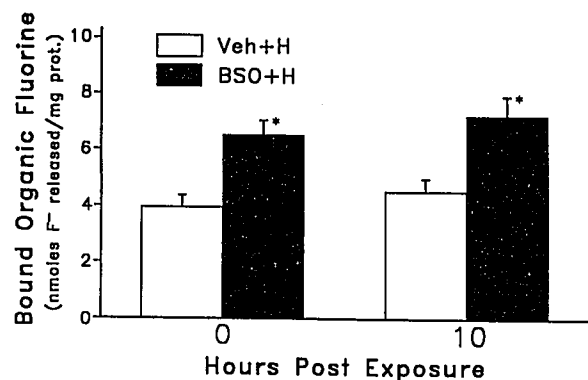

FIG. 4. Covalent binding of halothane biotransformation intermediates to hepatic protein in guinea pigs after exposure to 0.1% halothane. Exposures were at 40% O_2 for 4 h. Veh + H = vehicle control solution ip 24 h before exposure; BSO + H = 1.6 g/kg BSO ip 24 h before exposure. All values are mean ± SEM, N = 4. *$P < 0.05$ versus Veh + H group at same time point.
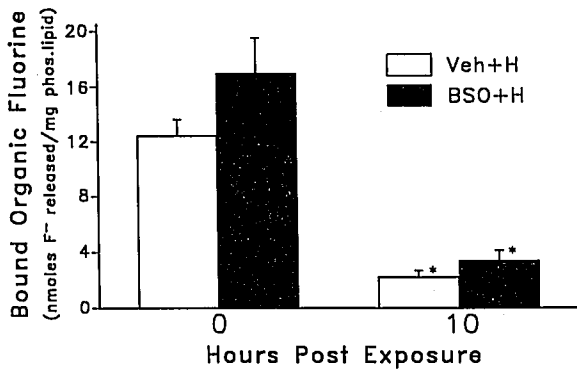
FIG. 5. Covalent binding of halothane biotransformation intermediates to hepatic lipid in guinea pigs after exposure to 0.1% halothane. Exposures were at 40% O_2 for 4 h. Veh + H = vehicle control solution ip 24 h before exposure; BSO + H = 1.6 g/kg BSO ip 24 h before exposure. All values are mean ± SEM, N = 4. *P < 0.01 versus same treatment group at 0 h.

plasma concentrations of the halothane metabolites up to 24 h after exposure and covalent binding to protein and lipid up to 10 h after exposure. No large differences in plasma metabolite concentrations and binding to protein were observed between the time points. A large decrease in covalent binding to hepatic lipid did occur by 10 h after exposure, indicating a rapid turnover of these moieties and an apparent lack of their significance in producing necrosis. This would be in agreement with our previous work that showed binding of halothane metabolites to protein but not lipid, correlated with the extent of hepatic injury in the guinea pig after a 4-h exposure to 1.0% halothane.[9] We also have found similar results in preliminary studies with glutathione-depleted guinea pigs anesthetized with 1.0% halothane.[24]

By use of subanesthetic concentrations of halothane in this study, it was hoped physiologic effects of anesthesia, such as decreased hepatic blood flow,[16] could be avoided. In addition, the lesser degree of injury caused by the subanesthetic concentration of halothane allowed for a clearer demonstration of potentiation of hepatotoxicity. In fact, the animals that received vehicle-control solution plus halothane developed only scattered foci of necrosis (mild injury), whereas we recently reported limited centrilobular necrosis (moderate injury) developing in 3 of 8 naive guinea pigs subsequent to exposure to 0.1% halothane.[10] This variability is not surprising considering the wide range in hepatotoxic responses to halothane normally observed in the guinea pig,[6-10] the small treatment group size used in this study, and the lesser degree of hepatotoxicity occurring with 0.1% halothane.[10] Combined data from these two studies show that none of 12 animals exposed to the 0.1% concentration have shown the extensive centrilobular necrosis (severe injury) that occurs regularly with higher (0.25–1.0%) concentrations of halothane.[6-10]

It is well known that acid chloride metabolic intermediates react with glutathione as a means of detoxification and, in the process, cause a decrease in cellular glutathione concentrations.[14] Because depletion of glutathione enhanced binding of halothane intermediates to hepatic protein, it would appear that glutathione plays at least a partial role in preventing the trifluoroacetyl acid chloride intermediate generated by halothane biotransformation from reacting with protein. However, it has been shown previously in rats[25-27]—and we have shown this with guinea pigs—that halothane exposure does not appear to cause a decrease in hepatic glutathione concentrations. One possible explanation for this lack of a decrease would be rapid hydrolysis or enzymatic degradation of the potential trifluoroacetyl–glutathione thioester derivative, leading to the regeneration of glutathione and production of the halothane metabolite, TFA.

Because glutathione is not depleted by halothane exposure, it would be expected that augmentation of glutathione would not provide protection from halothane hepatotoxicity. Initial studies have shown this to be true. We have administered either N-acetylcysteine or glutathione ethyl ester to guinea pigs both before and after exposure to 1% halothane, with no protective effect. These and other compounds available to enhance hepatic glutathione concentrations have serious drawbacks that limit their use. N-acetylcysteine may not increase glutathione concentrations under normal conditions[28] and can cause convulsions and death at high doses.[29] On deesterification, glutathione ethyl ester releases ethanol within the hepatocyte[28] and produces only transient (2–6 h) increases in hepatic glutathione concentrations.[30] Another compound available to increase hepatic glutathione concentrations, oltipraz, has been shown to increase hepatic cytochrome P-450 levels by 60%.[31] Constant efflux of glutathione from the liver and feedback inhibition of glu-
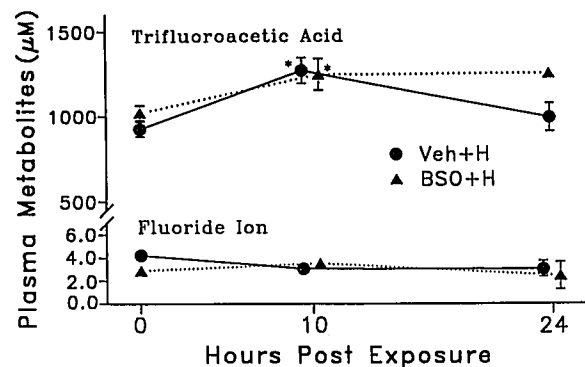

FIG. 6. Plasma trifluoroacetic acid and fluoride ion concentrations in guinea pigs after 0.1% halothane. Exposures were at 40% O_2 for 4 h. Some data points offset slightly for clarity. Veh + H = vehicle control solution ip 24 h before exposure; BSO + H = 1.6 g/kg BSO ip 24 h before exposure. All values are mean ± SEM, N = 3–12. *P < 0.05 versus same treatment group at 0 h.

tathione synthesis[28] are formidable obstacles to significant enhancement of hepatic glutathione concentrations over long periods of time and to additional elucidation of its potential role in halothane-associated hepatotoxicity.

Untoward side effects also are found with most agents that deplete hepatic glutathione, which led to our choice of BSO for this study. The glutathione depletors, phorone and 2-cyclohexene-1-one, have been shown to cause hyperglycemia and decrease brain glutathione concentrations, whereas BSO did not.[32] Diethylmaleate has seen wide use in decreasing glutathione concentrations. However, it is lethal in male guinea pigs at doses tolerated in other species.[33] We also have found this to be true.§ In addition, it has been shown to inhibit protein synthesis,[34] cause hypothermia,[34] and affect cytochrome P-450 activity,[35] whereas BSO did not.[34,36] Additional evidence that BSO does not affect cytochrome P-450 activity is shown by its lack of effect on halothane biotransformation in this study. A series of studies have shown no apparent toxicity resulting from BSO administration,[37] and it has been reported to be under investigation as an adjuvant chemotherapeutic agent for human malignant neoplasms.[28]

Although enhanced covalent binding of halothane biotransformation intermediates to hepatic protein that occurred in the glutathione-depleted animals is consistent with our proposed mechanism for halothane hepatotoxicity in guinea pigs,[9] other effects of a prolonged reduction in glutathione concentrations may have contributed to the observed potentiation of injury. Previous work in guinea pigs has shown that BSO produces a decrease in hepatic glutathione concentrations of 48–72 h in duration.[19] Consistent with these findings, the BSO-induced decrease of glutathione concentrations in our guinea pigs was observed to remain 10 h after halothane exposure, with a return to normal levels by 96 h after exposure. Because BSO was administered 24 h before halothane exposure, it could be expected that low hepatic glutathione concentrations continued for 24–48 h after halothane exposure. During this period, the function of cellular processes dependent on glutathione may have been compromised. For example, glutathione peroxidase plays an important role in protecting the cell from peroxidation of membrane lipid by activated oxygen and peroxides.[28] A lack of glutathione thus would increase susceptibility to the adverse effects of activated oxygen and peroxides released during the necrotic process by stimulated Kupffer cells and subsequently recruited inflammatory cells.[38] Glutathione is also important in maintaining a proper thiol–disulfide ratio for key metabolic enzymes that are regulated by the degree of oxidation of their cysteinyl residues.[28] Some of the enzymes involved in glucose me-

tabolism, such as glycogen phosphorylase phosphatase, phosphofructokinase, and pyruvate kinase, are among those that are up or down regulated, as may be the case, by increased disulfide formation due to a lack of glutathione.[28] Problems with glucose metabolism could hamper the ability of an injured hepatocyte to produce the energy for repair and recovery. However, the lack of glutathione did not cause significant hepatic injury in and of itself because the animals that received only BSO demonstrated no increases in plasma ICDH activity. Although one third of the animals had a few small scattered foci of necrosis within the liver, this finding was not significant and thus cannot be attributed to low glutathione concentrations.

Regardless of the mechanism involved in the observed potentiation of halothane-associated hepatic necrosis by glutathione depletion, these findings may hold implications for patients receiving halothane anesthesia. If a similar mechanism of injury is involved in the minor, acute hepatic dysfunction that occurs in 20% or more of patients anesthetized with halothane,[1,2] then patients with reduced liver glutathione concentrations because of excessive acetaminophen ingestion[39] or exposure in the workplace or environment to chemicals that deplete glutathione[40] could be at risk for a more severe hepatic injury.

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