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# Prevention of Halothane-Induced Hepatotoxicity by Hemin Pretreatment

## PROTECTIVE ROLE OF HEME OXYGENASE-1 INDUCTION

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**ABSTRACT.** Reductive metabolism of halothane in phenobarbital-pretreated rats is known to increase free radical formation that results in hepatotoxicity. It also is associated with a marked induction of microsomal heme oxygenase-1 (HO-1), suggesting that there is an alteration in heme metabolism. In this study, we examined heme metabolism in rats pretreated with phenobarbital, followed by exposure to halothane–hypoxia. In this model, there was a significant decrease in microsomal cytochrome P450 content in the liver, followed by a rapid increase in free heme concentration and a decrease in the level of mRNA for the nonspecific δ-aminolevulinate synthase. A transient but dramatic induction of HO-1 mRNA and a prolonged induction of heat shock protein 70 mRNA also occurred. The HO-1 protein was detected principally in the hepatocytes around the central vein. Serum alanine transaminase (ALT) activity, an indicator of hepatic dysfunction, increased continuously throughout the experiment. Hemin pretreatment induced hepatic HO-1 with abrogation of the halothane-induced hepatotoxicity in this model, as judged by ALT activity and normal histology. Our findings in this study thus indicate that halothane-induced hepatotoxicity is due not only to its reductive metabolite formation, but also to an increase in hepatic free heme concentration, which is a potent prooxidant; HO-1 induction is an important protective response against such changes. This is also the first study to demonstrate that hemin pretreatment, which induces HO-1 prior to exposure to halothane, effectively prevents halothane-induced hepatotoxicity. BIOCHEM PHARMACOL **59**;7:871–880, 2000. © 2000 Elsevier Science Inc.

**KEY WORDS.** halothane; hepatotoxicity; heme; heme oxygenase-1; δ-aminolevulinate synthase; heat shock protein 70

Halothane anesthesia is known to cause hepatic injury, such as severe hepatitis [1, 2], but, more commonly, mild hepatic injury [3, 4]. Despite many studies, however, the mechanism(s) of halothane-induced hepatotoxicity is not well understood. To study this question, male rats pretreated with phenobarbital followed by exposure to halothane under hypoxia have been used as an experimental model of halothane-induced hepatotoxicity [5–8]. In this halothane–hypoxia model, there is an increase in phenobarbital-inducible cytochrome P450s, which under hypoxia promote increased reductive metabolism of halothane [7, 8]. The reductive halothane metabolism generates free radical intermediates, which are capable of initiating lipid peroxidation and are thought to account for halothane toxicity [9, 10].

Microsomal HO-1[‖] is the rate-limiting enzyme in heme catabolism [11], as well as being heat shock protein 32 [12]. HO-1 is induced not only by its substrate, hemin [13], but also by oxidative stress, and is thought to play an important protective role against oxidative injuries [14–17]. In this study, we examined heme metabolism in the halothane–hypoxia model in the rat by examining changes in mRNAs encoding HO-1, HSP70 [18] and ALAS-N, the rate-limiting enzyme in heme biosynthesis [19]. Changes in serum ALT (EC 2.6.1.2) activity were examined as a measure of hepatocellular toxicity, and the extent of heme saturation of tryptophan pyrrolase (tryptophan 2,3-dioxygenase, EC 1.13.11.11), which reflects hepatic free heme concentration [20], was determined. We report here that there was a rapid increase in hepatic free heme concentration, followed by a marked increase of HO-1 expression

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‖ *Abbreviations*: HO-1, heme oxygenase-1; HSP70, heat shock protein 70; ALAS-N, nonspecific δ-aminolevulinate synthase; and ALT, alanine transamimase.

both at its mRNA and protein levels, after halothane–hypoxia exposure of phenobarbital-pretreated rats. In contrast, pretreatment of these animals with hemin, which induced HO-1 prior to exposure to halothane under hypoxia, effectively prevented the development of hepatotoxicity by halothane. Thus, HO-1 gene expression may occur not only by halothane-mediated oxidative injury, but also by an increase in hepatic free heme concentration; HO-1 induction may play an important role in protecting cells from oxidative injury. Interestingly, preinduction of HO-1 by hemin confers protection against oxidative damage in the liver cells, indicating the critical role of HO-1 induction in the protective response.

## MATERIALS AND METHODS

### *Animals*

All animal experiments were carried out after approval by the Animal Care Committee of Okayama University Medical School; care and handling of the animals were in accordance with National Institutes of Health guidelines. Male Wistar rats weighing 200–220 g, purchased from Clea Limited, were used in the experiments. They were housed in a temperature-controlled (25°) room with alternating 12-hr light and dark cycles and were allowed free access to water and chow diet until the experiment. Except for the untreated control rats, they were given 0.1% (w/v) sodium phenobarbital solution instead of water for 5 days and were deprived of food on day 5 for 24 hr before halothane (1%) and/or hypoxia (14% oxygen) treatment. Animals were exposed to a premixed gas consisting of nitrogen, oxygen, and halothane for 2 hr through an Ohio anesthesia apparatus (Ohio Medical Products) at a flow rate of 6 L/min in an anesthetic box, the volume of which was approximately 6 L. The anesthetic box was placed on a heating pad to keep the rectal temperature of the animals between 36.5° and 37.5°. Halothane was delivered by a Fluotec 3 vaporizer (Cypran Limited), and oxygen concentration was controlled by mixing oxygen and nitrogen gases. Both halothane and oxygen concentrations in the anesthetic box were monitored by a multiple gas monitor (Capnomax, Datex). After a 2-hr exposure to the gas mixture, rats were allowed to recover in a normal oxygen atmosphere. After the desired period (0–24 hr), the animals were decapitated under light anesthesia with ethyl ether. Blood was collected for serum isolation, and the liver was excised. Livers were frozen immediately in liquid nitrogen and stored at −80° until used for the determination of hepatic free heme concentration and RNA preparation. For the determination of microsomal cytochrome P450 and HO activity, the liver was perfused *in situ* with ice-cold physiological saline under pentobarbital anesthesia (65 mg/kg body weight) and then removed for the preparation of microsomes. Hemin (ferriprotoporphyrin chloride), dissolved in an alkaline solution (0.1 N NaOH) and then reconstituted in physiological saline, was administered s.c. (30 μmol/kg body weight), 24 hr prior to halothane–hypoxia treatment. Control rats received the same volume of physiological saline. Both hemin-treated and untreated control rats were pair-watered and pair-fed.

### *Histological Study*

For the histological examination, liver tissue was fixed in 10% neutral buffered formalin, embedded in paraffin, and sectioned at 4- to 6-μm thickness. After deparaffinization and rehydration, the sections were stained with hematoxylin and eosin for microscopic examination. When sections were subjected to immunohistochemical analysis, endogenous peroxidases were blocked by 3% hydrogen peroxide, followed by incubation with rabbit polyclonal anti-rat HO-1 (StressGen Biotechnologies) at 37° for 3 hr. The antigen–antibody reaction was detected using anti-rabbit immunoglobulin and an avidin–biotin immunoperoxidase staining kit (DAKO). The positive reaction was visualized by 3,3′-diaminobenzidine staining. Untreated rabbit serum was used as a control for nonspecific staining. Sections were counterstained with Mayer's hematoxylin solution.

### *cRNA Probes*

Template cDNAs used were rat pRHO-1 [21] and rat pKRA2cA [19], for rat HO-1 and rat ALAS-N, respectively. Template cDNA for HSP70 was rat HSP70 cDNA corresponding to bp 163-1230, which was cloned from the rat fetal brain library [22] and constructed in pGEM-4Z vector (Promega) with *Sma*I and *Pst*I sites. All probes used for northern blot analysis were biotin–CTP-labeled antisense riboprobes prepared according to the manufacturer's instructions with a Nonradioactive RNA Labeling System (Life Technologies, Inc.).

### *Northern Blot Analysis*

Total RNA was isolated from the liver by the method of Chirgwin *et al.* [23]. Twenty micrograms of total RNA was analyzed by northern blotting as described previously [24]. After blotting onto a sheet of BIODYNE A Nylon Membrane (Pall BioSupport Division, Pall Co.), RNA samples were hybridized with biotin-labeled specific riboprobes, then treated with RNase A (1 μg/mL), followed by washing under stringent conditions. Detection of mRNAs was carried out by using a PHOTOGENE 228 Nucleic Acid Detection System, Version 2.0 (Life Technologies, Inc.). Chemiluminescent signals were visualized by exposing the membrane to x-ray film. The levels of mRNAs were quantitated by densitometry using a Bioimage Analyzer (Millipore Corp.).

### *Measurement of Microsomal Cytochrome P450 Content*

Microsomal cytochrome P450 content was determined from a CO-difference spectrum as described by Omura and Sato [25]. Microsomal protein concentration was determined by the method of Lowry *et al.* [26].

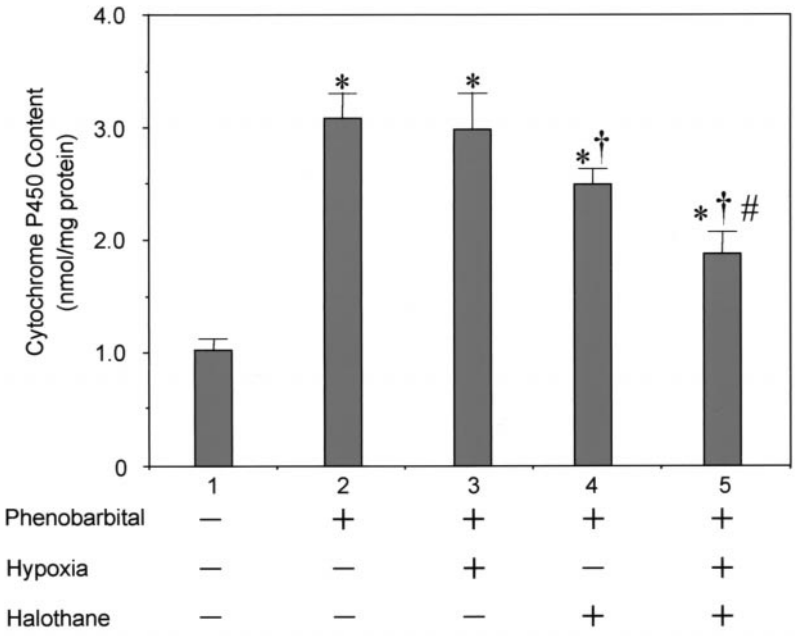


**FIG. 1. Effect of hypoxia and/or halothane on hepatic microsomal cytochrome P450 content in phenobarbital-pretreated rats. Phenobarbital pretreatment and hypoxia and/or halothane gas treatment were performed as described in Materials and Methods. At 0 hr after gas treatment, the liver was removed for the measurement of cytochrome P450 content as described in Materials and Methods. Bar 1, untreated control; bar 2, phenobarbital-pretreated; bar 3, phenobarbital-pretreated and 14% $O_2$; bar 4, phenobarbital-pretreated and halothane (1% halothane under 21% $O_2$); and bar 5; phenobarbital-pretreated and halothane–hypoxia (1% halothane under 14% $O_2$). Data are expressed as means ± SD (N = 6). Key: (*) $P < 0.01$ vs untreated control; (†) $P < 0.01$ vs phenobarbital-pretreated; and (#) $P < 0.01$ vs phenobarbital-pretreated and halothane (1% halothane under 21% $O_2$).**

### *Determination of Hepatic Free Heme Concentration*

Hepatic free heme concentration was estimated as the extent of saturation of tryptophan pyrrolase activity, as described previously [27]. The degree of saturation of the enzyme with heme was expressed as the percentage of the holoenzyme activity (determined without exogenous hemin addition) to the total activity (determined after the addition of 1.2 μM hemin).

### *Hepatic HO Activity*

Livers were homogenized in 3 vol. of 0.1 M potassium phosphate buffer (pH 7.4) containing 0.25 M sucrose, and were centrifuged at 10,000 *g* for 30 min at 4°, followed by a 104,000 *g* centrifugation of the supernatant for 60 min at 4° to obtain the microsomal fraction. HO (EC 1.14.99.3) activity in the microsomes was measured spectrophotometrically as the amount of bilirubin formation, as described previously [28]. The cytosolic fraction prepared from the livers of adult untreated rats served as a source of biliverdin reductase. HO activity was expressed as nanomoles of bilirubin formed per 60 minutes per milligram of protein.

### *Assay of Serum ALT Activity*

Serum was separated from whole blood by centrifugation at 1600 *g* for 10 min, and serum ALT activity was measured with a Du Pont Automatic Enzyme Analyzer calibrated with quality control standards (E.I. Du Pont de Nemours & Co.).

### *Statistical Analysis*

Statistical analysis was made by ANOVA with Tukey's test using StatView software (Abacus Concepts). Differences were designated as significant when $P < 0.05$.

## RESULTS

### *Effect of Halothane and/or Hypoxia on Hepatic Microsomal Cytochrome P450 Content*

Since hepatic cytochrome P450 is believed to be the key enzyme for halothane metabolism [29, 30], we examined its level immediately after a 2-hr halothane–hypoxia exposure in phenobarbital-treated animals (Fig. 1). Rats treated with 0.1% phenobarbital in the drinking water for 5 days showed an ~3-fold induction of cytochrome P450 as compared with untreated controls. Hypoxia (14% oxygen) alone had little effect on cytochrome P450 levels induced by phenobarbital, whereas halothane treatment (1% halothane under 21% oxygen) decreased the level to 84% compared with that of the phenobarbital-induced control. Combined treatment with halothane and hypoxia (1% halothane under 14% oxygen) markedly decreased cytochrome P450 content to 56% of that of the phenobarbital-induced control. Decreases in cytochrome P450 content induced by halothane treatment or by combined treatment with halothane and hypoxia were statistically significant ($P < 0.01$) compared with those in the phenobarbital-treated controls, or with those subjected to hypoxia without halothane.

### *Effect of Halothane and/or Hypoxia on the Expression of Hepatic HO-1 mRNA, and on HSP70 mRNA and Serum ALT Activity*

We examined the effect of halothane and/or hypoxia treatment on the expression of hepatic HO-1 mRNA, HSP70 mRNA, and serum ALT levels, immediately or 6 hr after each treatment in phenobarbital-treated rats (Fig. 2). Although HO-1 mRNA and HSP70 mRNA were only barely detectable in untreated control animals, under normoxia (21% oxygen), hypoxia (14% oxygen), or halothane (1% halothane under 21% oxygen)-treated rat livers, their

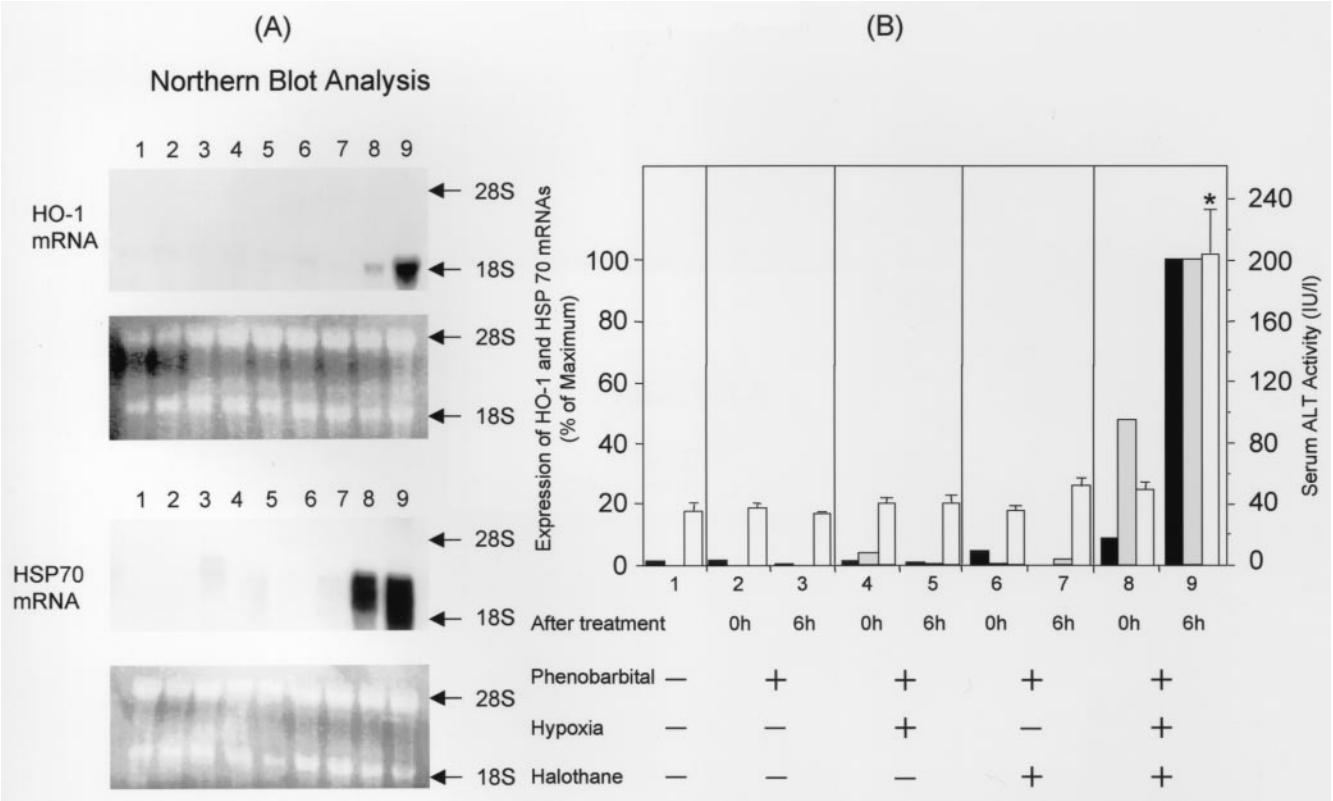


**FIG. 2. Effect of halothane and/or hypoxia on expression of hepatic HO-1 mRNA, HSP70 mRNA, and serum ALT activity in phenobarbital-pretreated rats. Phenobarbital-pretreated rats were exposed for 2 hr to halothane and/or hypoxia. At 0 and 6 hr after each treatment, the liver was removed for northern blot analysis and whole blood was collected for determination of serum ALT activity as described in Materials and Methods. (A) Twenty micrograms of total RNA was subjected to northern blot analysis. Shown are the chemiluminescent signals of the RNA blot hybridized with biotin-labeled riboprobes of rat HO-1 and HSP70 cRNA, respectively. Ethidium bromide staining of the same RNA is shown for loading control. Lane 1, untreated control; lanes 2 and 3, 21% $O_2$; lanes 4 and 5, 14% $O_2$; lanes 6 and 7, 1% halothane under 21% $O_2$; and lanes 8 and 9, 1% halothane under 14% $O_2$. (B) The levels of HO-1 mRNA (solid bars) and HSP70 mRNA (gray bars) are expressed as values relative to the maximal level for each mRNA, respectively. Serum ALT activity (open bars) is expressed as means ± SD (N = 6). Key: (*) $P < 0.01$ vs untreated control.**

levels increased markedly following combined treatment with halothane (1%) and hypoxia (14% oxygen). The HO-1 mRNA level was not increased at 0 hr by the combined treatment, but was increased at 6 hr. In contrast, HSP70 mRNA levels were increased by the combined treatment at both 0 and 6 hr. Serum ALT activity was increased by the combined treatment at 6 hr.

### *Time Courses of HO-1 and HSP70 mRNA Levels and Serum ALT Activity Following Halothane–Hypoxia Treatment*

Since the induction of HO-1 mRNA, HSP70 mRNA, and serum ALT activity was observed only in halothane–hypoxia-treated rats at 6 hr, we determined time courses of both mRNAs and serum ALT activity following halothane–hypoxia treatment (Fig. 3). The HO-1 mRNA level started to increase at 4 hr after the gas exposure, rapidly reached a maximum at 6 hr, then declined abruptly, and returned to normal by 18 hr (the top panels in Fig. 3, A and B). In contrast to HO-1 mRNA, HSP70 mRNA showed a very rapid increase within 2 hr after the gas exposure and maintained the high level until 6 hr, but declined thereafter (the bottom panel in Fig. 3A, and the top panel in Fig. 3B). Serum ALT activity showed a significant increase starting at 4 hr, continued to rise during the experimental period, and reached ~20-fold of the untreated value at 24 hr (the bottom panel in Fig. 3B).

### *Change in Hepatic Heme Saturation and ALAS-N mRNA Level after Halothane–Hypoxia Treatment*

Since it is known that HO-1 can be induced by hemin [13], we examined hepatic free heme concentration after halothane–hypoxia treatment (Fig. 4). Although free heme concentration cannot be determined directly because of its extremely low concentration and its status in an extremely dynamic equilibrium, it can be assessed indirectly by the extent of heme saturation of tryptophan pyrrolase activity [20]. Using this assay, free heme concentration was found to be slightly higher than normal (54.3 ± 2.8%) in phenobarbital-pretreated rats (72.2 ± 3.7%), presumably reflecting increased heme synthesis by phenobarbital treatment. When these rats were exposed to halothane–hypoxia for 2

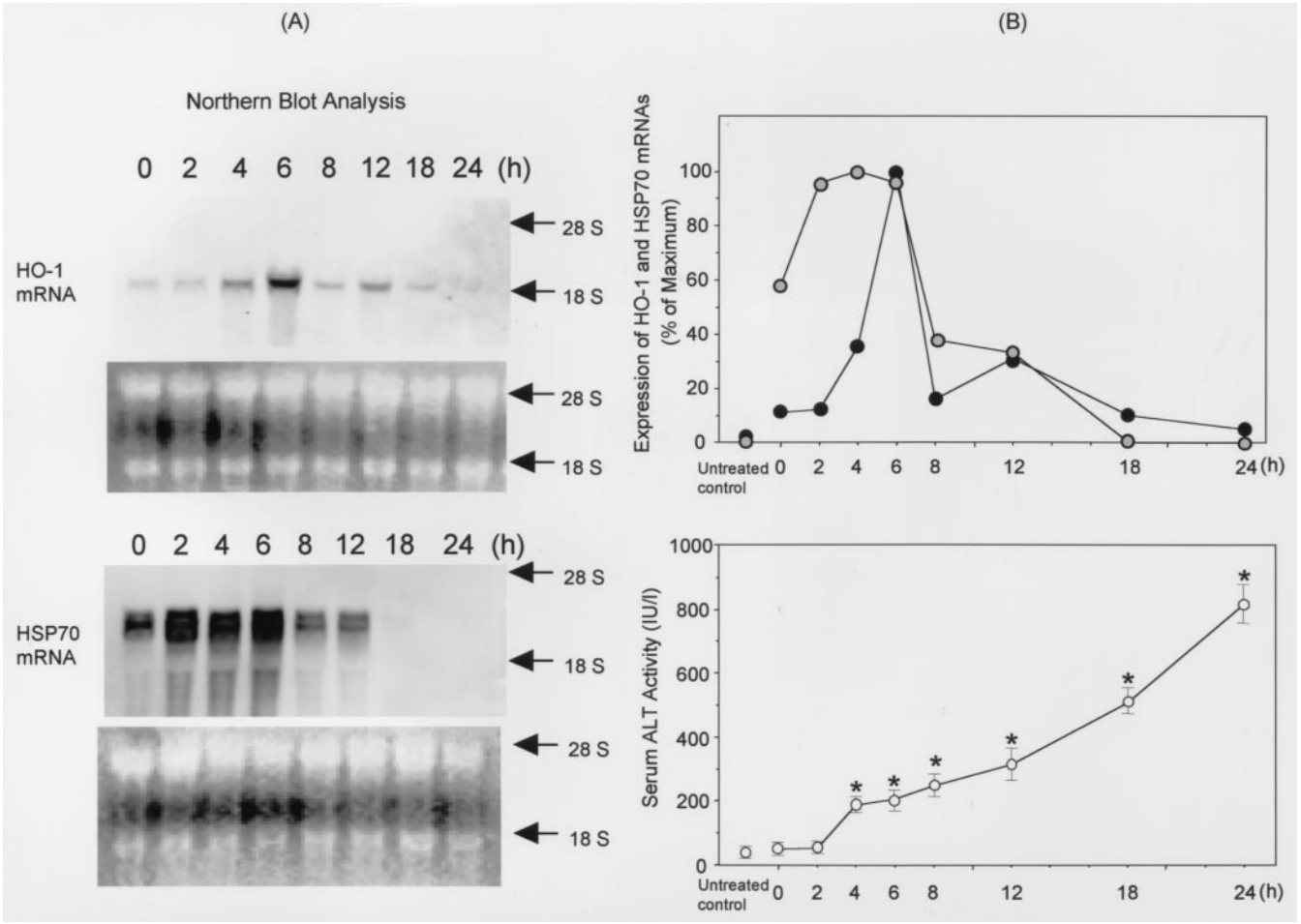


**FIG. 3. Time courses of hepatic HO-1 mRNA, HSP70 mRNA, and serum ALT activity after halothane–hypoxia exposure in phenobarbital-pretreated rats. Phenobarbital-pretreated rats were exposed to 1% halothane under 14% oxygen for 2 hr. After the exposure, the liver was removed for northern blot analysis and whole blood was collected for determination of serum ALT activity as described in Materials and Methods. (A) Twenty micrograms of total RNA was subjected to northern blot analysis. Shown are the chemiluminescent signals of the RNA blot hybridized with biotin-labeled riboprobes of rat HO-1 and HSP70 cRNA, respectively. Ethidium bromide staining of the same RNA is shown for loading control. (B) Top: The levels of HO-1 mRNA (closed circles) and HSP70 mRNA (gray circles) are expressed as values relative to the maximal level for each mRNA, respectively. Bottom: Serum ALT activity at each time point is shown as means ± SD (N = 6). Key: (*) $P < 0.01$ vs untreated control. Time represents hours from the termination of the exposure period to the time when the animals were killed.**

hr, there was a rapid and significant increase in hepatic free heme concentration within 2 hr, which reached a maximum at 4 hr (Fig. 4). The increase in free heme concentration returned to the gas-unexposed level by 12 hr, but remained above the normal level during the experimental period of 24 hr (Fig. 4). To confirm the specificity of the elevation of free heme concentration after halothane–hypoxia treatment, we also examined free heme concentration in normoxia, hypoxia, or halothane-treated rat livers at 4 hr after each treatment. Hepatic free heme concentrations, as determined by the extent of saturation of tryptophan pyrrolase activity with hemin, were 72.2 ± 3.7, 72.3 ± 4.4, 72.6 ± 4.3, and 75.2 ± 6.3%, for pre-exposure, normoxia, hypoxia, and halothane, respectively. These findings indicate that none of these treatments increased free heme concentrations above the pre-exposed level.

Since it is known that ALAS-N is increased by various chemical treatments including phenobarbital, and is subject to suppression by hemin treatment [31], we determined the changes in the ALAS-N mRNA level after halothane–hypoxia treatment. The ALAS-N mRNA level was increased markedly by pretreatment with phenobarbital for 5 days (Fig. 4). Following exposure to the gas mixture, there was a rapid and significant decrease in ALAS-N mRNA level within 4 hr, which continued to decrease for 24 hr (Fig. 4). The time course of ALAS-N mRNA was almost a reverse image of that of free heme concentration, suggesting that the increased free heme concentrations down-regulated the level of ALAS-N gene expression.

### *Distribution of HO-1 Protein in the Liver of Halothane–Hypoxia-Treated Rats*

Although northern blot analysis indicated that HO-1 mRNA was expressed in the livers of halothane–hypoxia

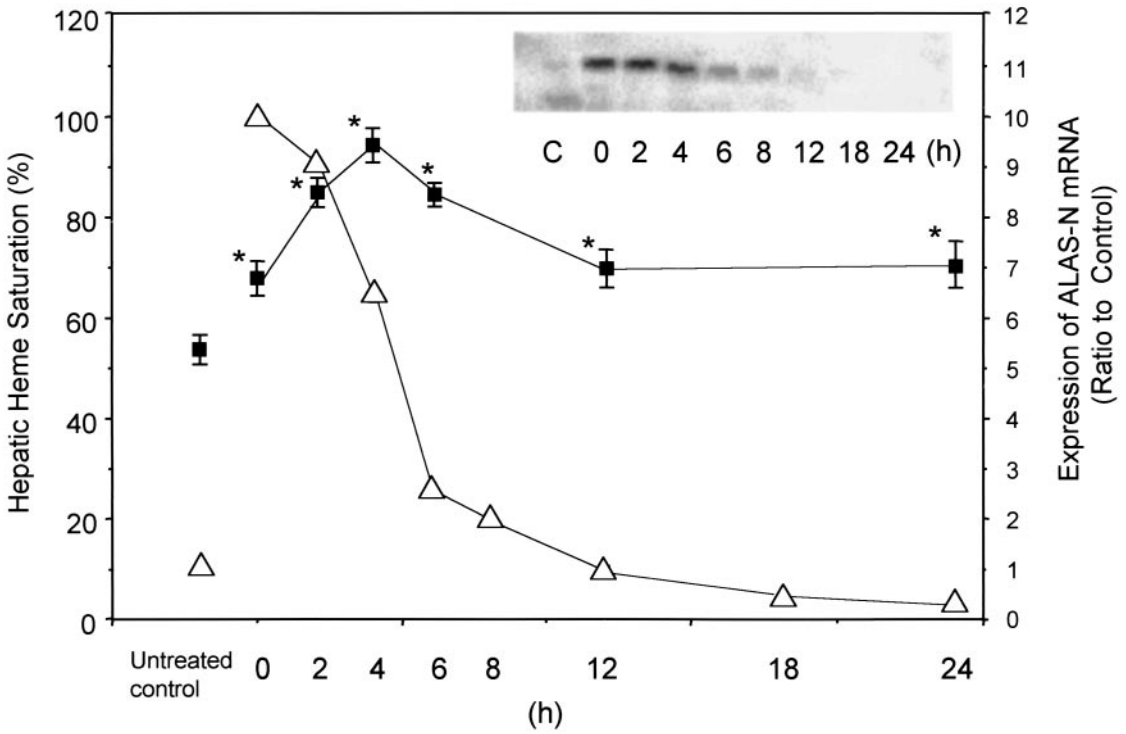


**FIG. 4. Effect of halothane and hypoxia on hepatic heme saturation and expression of ALAS-N mRNA in phenobarbital-pretreated rats. Phenobarbital-pretreated rats were exposed to 1% halothane under 14% oxygen. After the exposure, the liver was removed for northern blot analysis and for measurement of hepatic heme saturation as described in Materials and Methods. The levels of hepatic heme saturation (closed squares) are expressed as means ± SD (N = 6). Key: (*) $P < 0.05$ vs untreated control. Twenty micrograms of total RNA was subjected to northern blot analysis. Shown are the chemiluminescent signals of the RNA blot hybridized with biotin-labeled riboprobes of rat ALAS-N cRNA (inset). The levels of ALAS-N mRNA (open triangles) are expressed as values relative to the maximal level. Time represents hours from the termination of the exposure period to the time when the animals were killed.**

model rats, it was not clear whether the transcript was translated into its protein or not. Furthermore, it was also unclear what types of cell(s) in the liver expressed HO-1. Therefore, we carried out a immunohistochemical study of the livers from rats 8 hr after halothane–hypoxia exposure. In the untreated liver, HO-1 was expressed exclusively in the Kupffer cells (Fig. 5A) [32]. In the halothane–hypoxia-treated liver, positive staining for HO-1 protein was observed not only in the Kupffer cells but also in the hepatocytes, especially around the central vein (Fig. 5B). The section treated with non-immune rabbit serum showed no signals in treated liver (Fig. 5C).

### *Effect of Hemin Pretreatment on Hepatic HO Activity, Serum ALT Activity, and Hepatic Injury in Halothane–Hypoxia-Treated Rats*

To examine the functional role of HO-1 in this model, we administered hemin, a strong and specific inducer of HO-1, prior to the halothane–hypoxia treatment, and examined its effect on hepatic HO activity and hepatic dysfunction. Hepatic HO activity is known to be decreased in rats treated with phenobarbital [33]. Pretreatment of rats with hemin caused a significant increase in HO activity compared with saline-treated control animals (Fig. 6). Twelve hours after halothane–hypoxia treatment, hepatic HO activity was markedly high in both the saline-treated group and the hemin-treated group, but after 24 hr, HO activity in the saline-treated group returned to the untreated control level, while it still remained at a high level in the hemin-treated group (Fig. 6). Serum ALT activity was markedly high in the saline-administered group after 24 hr of halothane–hypoxia treatment, while it returned to the control level in the hemin-administered group (the top panel in Fig. 7). Histological examination of liver in halothane–hypoxia model rats revealed severe hepatocyte necrosis associated with infiltration of inflammatory cells around the central vein in the saline-treated group. In contrast, only a few necrotic cells were observed around the central vein in the hemin-treated group, with only minor vacuolization of the hepatocytes around the portal tracts (the bottom panels in Fig. 7).

## DISCUSSION

The present study demonstrated that HO-1 mRNA was induced in the rat liver after halothane–hypoxia exposure in phenobarbital-pretreated rats. A rapid and significant induction of HO-1 mRNA takes place in this model, which may be partially mediated by a free radical mechanism as judged by induction of HSP70 mRNA. More importantly,

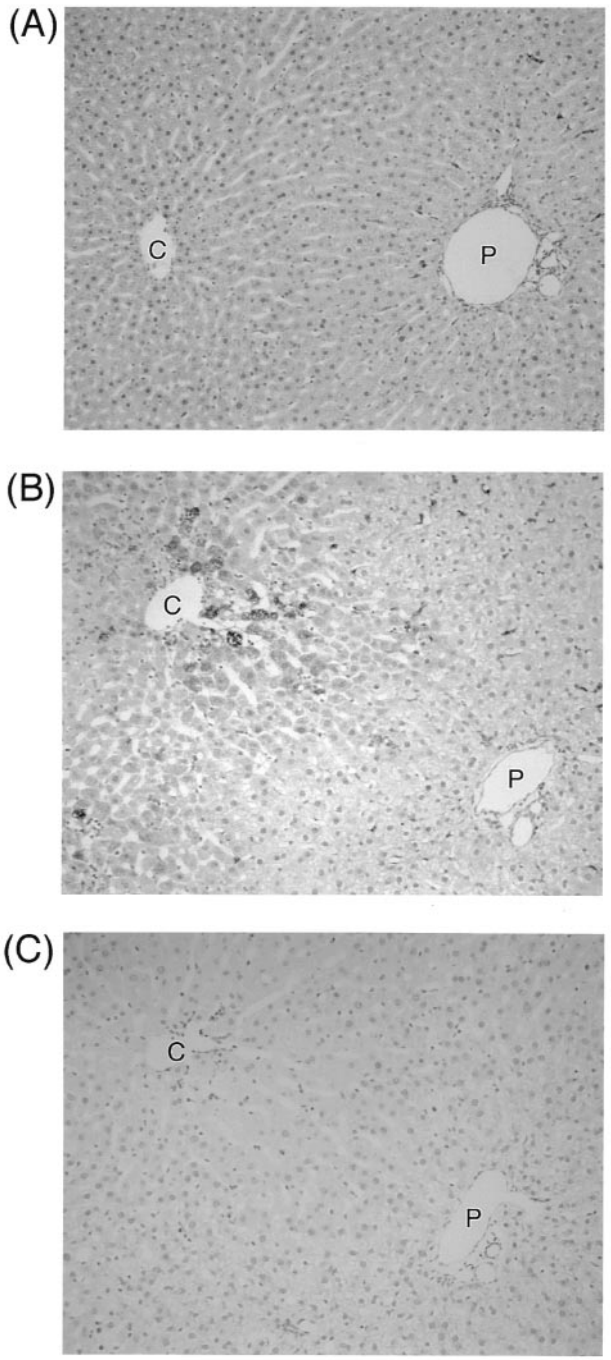


**FIG. 5. Immunohistochemistry of HO-1 in the livers of halothane–hypoxia-treated rats. Hepatic sections from rats exposed to halothane–hypoxia after phenobarbital pretreatment were used for immunohistochemical detection of HO-1. P and C denote portal and central venules, respectively. (A) Untreated control; rabbit polyclonal anti-rat HO-1 was used as a primary antibody. (B) Halothane–hypoxia-treated rat (8 hr after the exposure); rabbit polyclonal anti-rat HO-1 was used as a primary antibody. (C) Halothane–hypoxia-treated rat; normal rabbit serum was used as a primary antibody. Immunohistochemical staining was carried out as described in Materials and Methods (original magnification x100).**

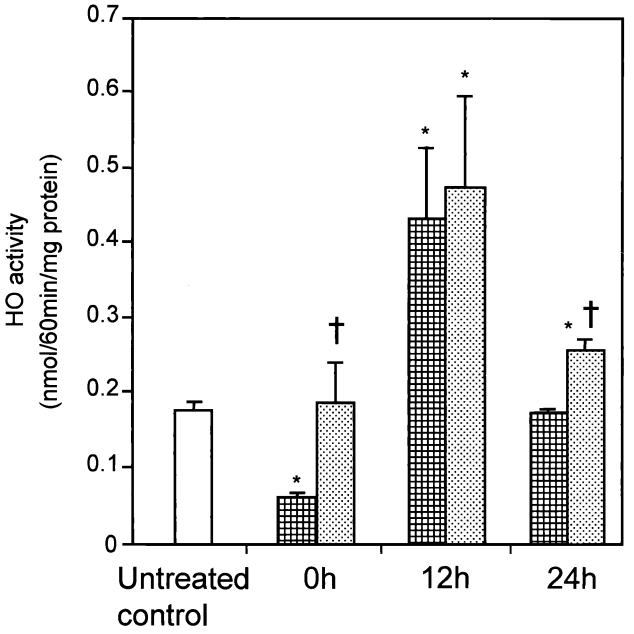


**FIG. 6. Effect of hemin administration on hepatic HO activity. Rats were administered hemin (30 μmol/kg), or physiological saline s.c. 24 hr before halothane–hypoxia treatment. Before (0 hr) and 12 and 24 hr after halothane–hypoxia treatment, livers were removed and HO activity was measured as described in Materials and Methods. White bars, untreated; checked bars, saline-administered; dotted bars, hemin-administered. Data are expressed as means ± SD (N = 6). Key: (*) $P < 0.01$ vs untreated, and (†) $P < 0.01$ vs saline-administered.**

however, HO-1 induction appears to be mediated by an increase in hepatic free heme concentration, which was clearly supported not only by the increase in heme saturation of tryptophan pyrrolase but also by the reduction in ALAS-N mRNA expression. Most importantly, HO-1 mRNA induction followed the rapid increase in free heme concentration in the liver. Immunohistochemical study demonstrated that HO-1 protein induction occurred exclusively in the hepatocytes at the perivenular zone. Our findings that hemin pretreatment significantly prevented hepatic cell injury in this model also suggest that HO-1 induction prior to halothane–hypoxia exposure may represent a critically important protective response to the halothane-induced radical reactions, which can be additionally amplified by an increase in hepatic free heme concentration.

HO-1 mRNA and HSP70 mRNA are induced only in halothane–hypoxia treated rats. These inductions were associated with the elevation of serum ALT activity, which indicates hepatic dysfunction. These findings indicate that halothane–hypoxia treatment is necessary for the induction of both mRNAs and hepatic injury in phenobarbital-pretreated rats. Our findings suggest that, whereas both HO-1 and HSP70 are heat-shock proteins, HO-1 and HSP70 genes are regulated separately, and their expressions undergo distinct time courses in the halothane–hypoxia model.

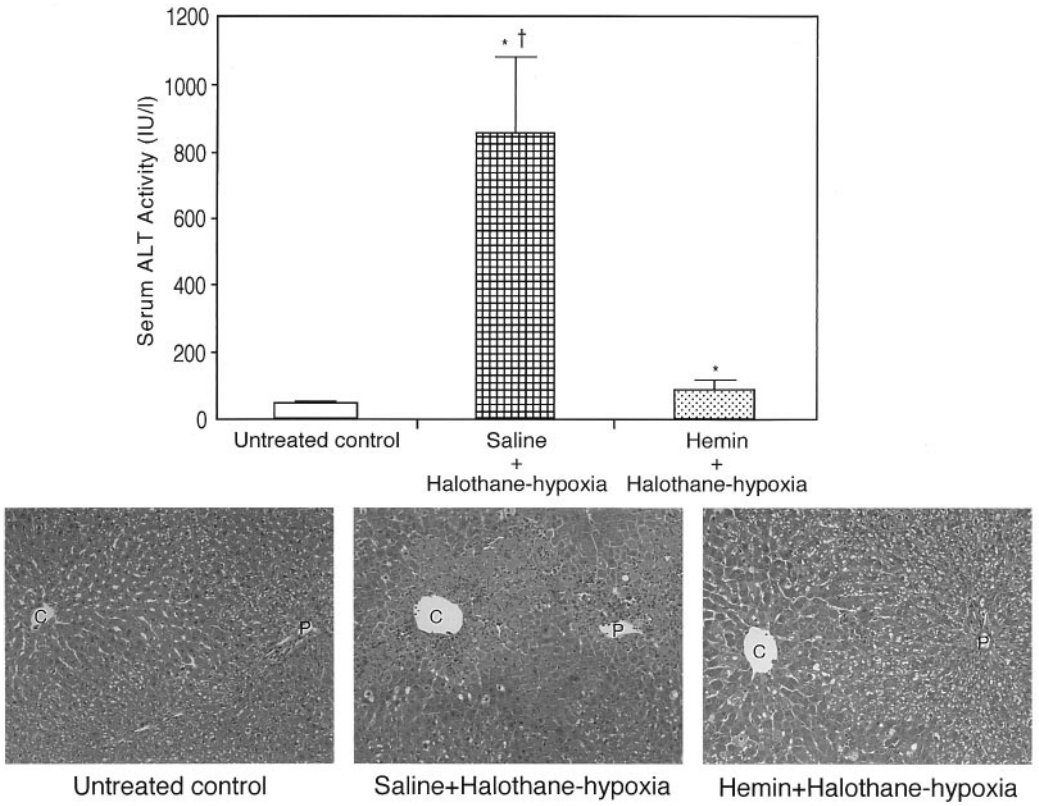


**FIG. 7. Effect of hemin administration on serum ALT activity and histological changes in the halothane-hypoxia model. Rats were administered hemin (30 μmol/kg) or physiological saline s.c. 24 hr before halothane–hypoxia treatment. Twenty-four hours after the treatment, the liver was removed for histological study, and the serum ALT activities were determined as described in Materials and Methods. Top: Serum ALT activity. Data are expressed as means ± SD (N = 6). Key: (*) $P < 0.01$ vs untreated control, and (†) $P < 0.01$ vs hemin-administered rats. Bottom: Hematoxylin–eosin staining was carried out as described in Materials and Methods (original magnification x100). P and C denote portal and central venules, respectively.**

Our results also demonstrated that, prior to induction of HO-1, there was a rapid and significant increase in heme saturation of tryptophan pyrrolase, suggesting that an increase in intracellular free heme concentration may contribute to the induction of HO-1 mRNA [13]. Coincident with an increase in free heme concentration, ALAS-N mRNA levels also were decreased rapidly after halothane–hypoxia treatment. This finding is consistent with the well-known heme-mediated repression of ALAS-N gene expression [31]. The heme of microsomal cytochrome P450 may likely be the source of "free heme," since induced cytochrome P450 in phenobarbital-pretreated rat liver was decreased markedly after halothane–hypoxia treatment (Fig. 1) [34]. It also has been reported that cytochrome P450 undergoes suicidal inactivation, releasing heme, in the reductive metabolism of halothane under hypoxic conditions [35]. Thus, it is likely that halothane–hypoxia treatment may destabilize heme from cytochrome P450 and increase the hepatic free heme concentration, leading to the induction of HO-1 and the suppression of ALAS-N.

There may be several reasons for the decrease in cytochrome P450 content in the livers of halothane–hypoxia-treated rats. First, the heme moiety of cytochrome P450 is known to be released by the reductive metabolism of halothane under hypoxia [35]. Second, the resultant increase in hepatic free heme concentration suppressed ALAS-N expression (Fig. 4), the well-known heme-mediated repression of this rate-limiting enzyme in heme biosynthesis [36]. Third, increased free heme concentration induces HO-1 expression [36]. All of these mechanisms may participate in the halothane–hypoxia-induced decrease in cytochrome P450, and may contribute to hepatotoxicity, unless deranged hepatic heme synthesis is restored in a short period of time.

While heme is required as the prosthetic group for hemoproteins that are necessary for cellular viability [37], an excess amount of free heme is deleterious, since it acts as a potent pro-oxidant, leading to the generation of oxygen radicals [38, 39]. In the halothane–hypoxia rat model, halothane can be metabolized reductively to free radical intermediates that may initiate lipid peroxidation. Thus, an increase in intracellular free heme (Fig. 4) may contribute further to free radical production, leading to hepatocellular injury. HO-1 is known to function, not only as a heme-cleaving enzyme [28], but also as a heat shock protein [12] or an acute phase reactant [40]. Thus, induced HO-1 may protect hepatic cells from additional injury by free heme.

This interpretation is consistent with the result that HO-1 was induced dominantly around the central vein, where the most severe cell damage was observed. Besides, HO-1 induction by hemin administration prior to the halothane–hypoxia treatment resulted in the prevention of hepatic injury in this model, as judged not only by a lack of increase in serum ALT activity, but also by the normal histology of the liver (Fig. 7).

Halothane is known to be metabolized via two main pathways, both of which are catalyzed by microsomal cytochrome P450 [29, 30]. In a sufficient concentration of oxygen, halothane is mainly metabolized oxidatively to form a stable end product, trifluoroacetic acid [41]. On the other hand, when oxygen tension is below normal, it is metabolized by a reductive pathway to produce a free radical intermediate(s) [42]. It generally has been thought that severe hepatitis and mild hepatic injury are related to the oxidative metabolism and the reductive metabolism of halothane, respectively [2]. However, it is also important to note that not all patients exposed to halothane necessarily exhibit hepatotoxicity, suggesting that genetic polymorphism in halothane metabolism may influence variable expression of hepatotoxicity. Such differences may involve individual differences in cytochrome P450s responsible for the reductive metabolism of halothane, but also differences in the regulation of hepatic free heme concentration, induction responses of HO-1, and repression of ALAS-N in the liver.

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