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Differential effects of isoflurane and halothane on the induction of heat shock proteins

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Abstract

Isoflurane is considered to be a less hepatotoxic volatile anesthetic than halothane since it not only undergoes quantitatively much less metabolism to form toxic reactive intermediates, but also preserves better hepatic blood flow. However, the biochemical basis for the reduced hepatotoxicity has not been elucidated. In this study, we examined the induction of two heat shock proteins, heat shock protein 70 (HSP70) and heme oxygenase-1 (HO-1), in the livers of rats pretreated with or without phenobarbital, followed by exposure to isoflurane or halothane under hypoxic conditions. In the phenobarbital-pretreated rats, the maximal induction of HSP70 was observed by halothane-hypoxia treatment, followed by a half-maximal induction by isoflurane-hypoxia treatment, and less than 30% induction by hypoxia treatment alone. Serum alanine aminotransferase (ALT) activity, an indicator of hepatic dysfunction, which correlated well with the extent of centrilobular necrosis, showed similar changes with increases in HSP70 mRNA. In contrast, HO-1 mRNA was induced only by treatment with halothane-hypoxia. In addition, changes in the expression of HSP70 and HO-1 mRNAs were correlated with their protein expression in the liver. In non-pretreated rats, neither isoflurane-hypoxia exposure nor halothane-hypoxia exposure caused apparent hepatic injury. There was also no induction of HSP70 or HO-1 mRNA by these treatments in non-pretreated animals. These findings demonstrate that there is a significant difference in hepatic injury, and in the induction of HO-1 and HSP70 between halothane-hypoxia and isoflurane-hypoxia treatments. Isoflurane is known to be safer than halothane, which may, in part, be accounted for by the generation of less oxidative stress in the presence of isoflurane, as assessed by reduced induction of heat shock proteins compared with halothane treatment.

Keywords: Isoflurane; Halothane; Hypoxia; Heat shock protein 70; Heme oxygenase-1

1. Introduction

Isoflurane is considered to be a less hepatotoxic inhalation anesthetic than halothane since it undergoes quantitatively much less metabolism to form toxic reactive intermediates [1], and preserves better hepatic blood flow [2]. Isoflurane is recommended for anesthetic management in major hepatic surgeries such as liver transplantation, in which ischemic insult to the liver is frequently anticipated [3]. It is also well known that during upper abdominal surgery, total hepatic blood flow can be decreased up to 60% by surgery alone [4]. Under these conditions, the mechanism for hepatotoxicity by volatile anesthetics is not understood fully. Thus, we undertook this study to examine and compare the effects of isoflurane and halothane on the expression level of two HSPs and their mRNAs, i.e. hepatic HSP70 [5–7] and HO-1 [8,9], in the presence of 10% oxygen [10–12]. Hepatic injury was assessed by measurements of serum ALT (EC 2.6.1.2) activity and by the analysis of liver histology. Our studies indicate that under hypoxic conditions the effect of isoflurane on the induction of HSPs and on hepatic cell integrity is significantly less than the effect of halothane. Our findings substantiate the fact that isoflurane is a much safer volatile anesthetic than halothane, and provide some biochemical evidence that it induces less oxidative stress than halothane, as suggested

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Abbreviations: ALT, alanine aminotransferase; HO-1, heme oxygenase-1; HSP70, heat shock protein 70; and MAC, minimum alveolar concentration.

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by the induction profile of the two HSPs, which may, in part, account for the observed differences in their effects.

2. Materials and methods

2.1. Animals

All animal experiments were carried out after approval was obtained from the Animal Care Committee of Okayama University Medical School; care and handling of the animals were in accordance with the guidelines of the National Institutes of Health. Male Wistar rats weighing 210–240 g, purchased from Charles River, were used. They were housed in a temperature-controlled [24–26°] room with alternating 12-hr light and dark cycles, and were allowed free access to water and a chow diet until the start of the experiments. Except for untreated control animals, rats were given either 0.1% (w/v) sodium phenobarbital or no additive in their drinking water for 5 days. The rats were fasted on day 5 for 24 hr, before treatment with volatile anesthetics and/or hypoxia (10% oxygen). To achieve equivalent potencies, isoflurane or halothane was used at an MAC of 0.3, which was 0.42% for isoflurane and 0.33% for halothane [10,13]. Animals were exposed to a gas mixture consisting of nitrogen, oxygen, and volatile anesthetics for 2 hr, administered 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 rats between 36.5 and 37.5°. Isoflurane and halothane were delivered by a Forawick (Muraco) or by a Fluotec 3 vaporizer (Cypran Limited), respectively, and the oxygen concentration was controlled by mixing oxygen and nitrogen gases. Isoflurane, halothane, and oxygen concentrations in the anesthetic box were monitored by a multiple gas monitor (Capnomax, Datex), every 15 min. 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), rats were decapitated following anesthesia with ethyl ether. Blood was collected for serum isolation, and livers were excised. Livers were immediately frozen in liquid nitrogen and stored at −80° until used for RNA preparation.

2.2. Histological study

For histological examination, livers were fixed in 10% neutral buffered formalin, embedded in paraffin, and sectioned at 5 μm thickness. After deparaffinization and rehydration, the sections were stained with hematoxylin and eosin for microscopic examination. When liver sections were subjected to immunohistochemical analysis endogenous peroxidases were blocked with 3% hydrogen peroxide. This was followed by incubation of the sections with mouse monoclonal anti-human HSP70 (Amersham), or rabbit polyclonal anti-rat HO-1 (StressGen Biotechnologies) antibodies at 37° for 3 hr. The antigen-antibody reaction was detected using either biotinylated anti-mouse or anti-rabbit secondary antibodies and an avidin-biotin immunoperoxidase staining kit (DAKO). Positive reactions were visualized by staining with 3,3′-diaminobenzidine. Normal mouse and rabbit sera were used as controls for non-specific staining of HSP 70 and HO-1, respectively. Sections were counterstained with Mayer's hematoxylin solution.

2.3. cRNA probes

Rat HSP70 cDNA was isolated from a fetal rat brain library [14] and cloned into the *Sma*I-*Pst*I sites of the pGEM-4Z vector (Promega). Template cDNA for the HSP70 probe corresponds to bp 163–1230 of the cloned rat HSP70 cDNA. Template cDNA for HO-1 was obtained from rat pRHO-1 [15]. All probes used for northern blot analysis were biotin-CTP labeled anti-sense riboprobes prepared according to the instructions of the manufacturer using a Nonradioactive RNA Labeling System (Life Technologies, Inc.).

2.4. Northern blot analysis

Total RNA was isolated from the liver by the method of Cathala *et al.* [16]. Twenty micrograms of total RNA was analyzed by northern blot analysis as described previously [17]. After blotting onto a sheet of BIODYNE A Nylon Membrane (Pall BioSupport Division, Pall Co.), RNA samples were hybridized with biotin-labeled specific riboprobes. The membranes were then treated with RNase A (1 μg/mL) and washed under stringent conditions. Detection of mRNAs was carried out by using a PHOTOGENE™ Nucleic Acid Detection System, Version 2.0 (Life Technologies, Inc.). Chemiluminescent signals were visualized by exposing the membranes to x-ray film. Expression levels of mRNAs were quantitated by densitometry using a BioImage Analyzer (Millipore Corp.). Briefly, after measuring the integrated optical density (IOD) of each band at each time point up to 24 hr, IOD ratios were calculated for HSP70 and HO-1 at these time points, using the value for the maximum expression of each mRNA (6 hr after exposure to halothane-hypoxia) as the denominator. These ratios were converted to percentages by multiplying each by 100.

2.5. Serum ALT activity

Serum was separated from whole blood by centrifugation at 1600 *g* for 10 min at 4° 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.).

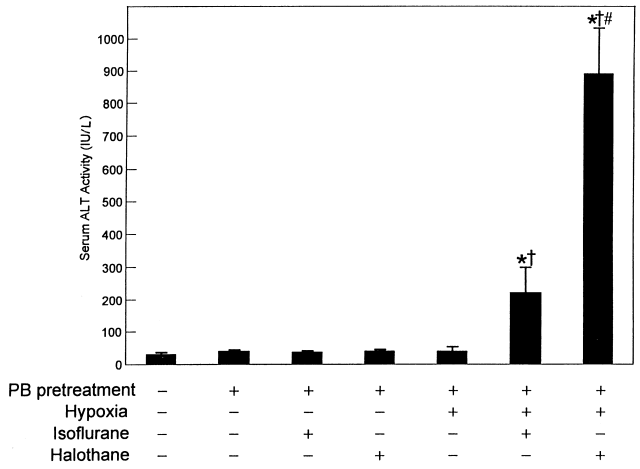

Fig. 1. Effects of isoflurane, halothane, and/or hypoxia on hepatic function. Phenobarbital-pretreated rats were exposed for 2 hr to isoflurane, halothane, and/or hypoxia as described in "Materials and methods." Twenty-four hours after gas treatment, whole blood was collected for the determination of serum ALT activity. Plus or minus denotes with or without treatment. Data are expressed as means ± SD (N = 6). Key: (*) $P < 0.01$ vs untreated control, (†) $P < 0.01$ vs hypoxia, and (#) $P < 0.01$ vs isoflurane-hypoxia.

Since all values were distributed normally, statistical evaluation was made by analysis of variance with the Tukey test, using Statview software (Abacus Concepts). Differences were designated as significant when $P < 0.05$.

3. Results

3.1. Effects of isoflurane, halothane, and/or hypoxia on hepatic function and histological findings

Serum ALT levels were examined in phenobarbital-pretreated rats 24 hr after a 2-hr exposure to gas anesthesia (Fig. 1). Hypoxia (10% oxygen) alone had no significant effect on ALT activity levels in serum, whereas isoflurane treatment under hypoxia (0.3 MAC isoflurane under 10% oxygen) increased the level to about 8-fold compared with that of the untreated control. In contrast, serum ALT levels were markedly increased to >30-fold following exposure to halothane under hypoxic conditions (0.3 MAC halothane under 10% oxygen). Histological examination of livers following hypoxic gas treatment showed vacuolar degeneration of hepatocytes around the portal tracts without apparent centrilobular necrosis (Fig. 2B) [18]. Isoflurane inhalation under hypoxic conditions resulted in centrilobular necrosis with additional vacuolar degeneration around the portal tracts (Fig. 2C). Hepatocyte degeneration was by far the most severe in the halothane-hypoxia inhalation group, which was accompanied by extensive necrotic changes in the centrilobular region (Fig. 2D) [19].

3.2. Time course for the expression of HSP70 mRNA after isoflurane, halothane, and/or hypoxia treatment

We examined the time courses for the expression of HSP70 mRNA following gas treatment (Fig. 3). In all

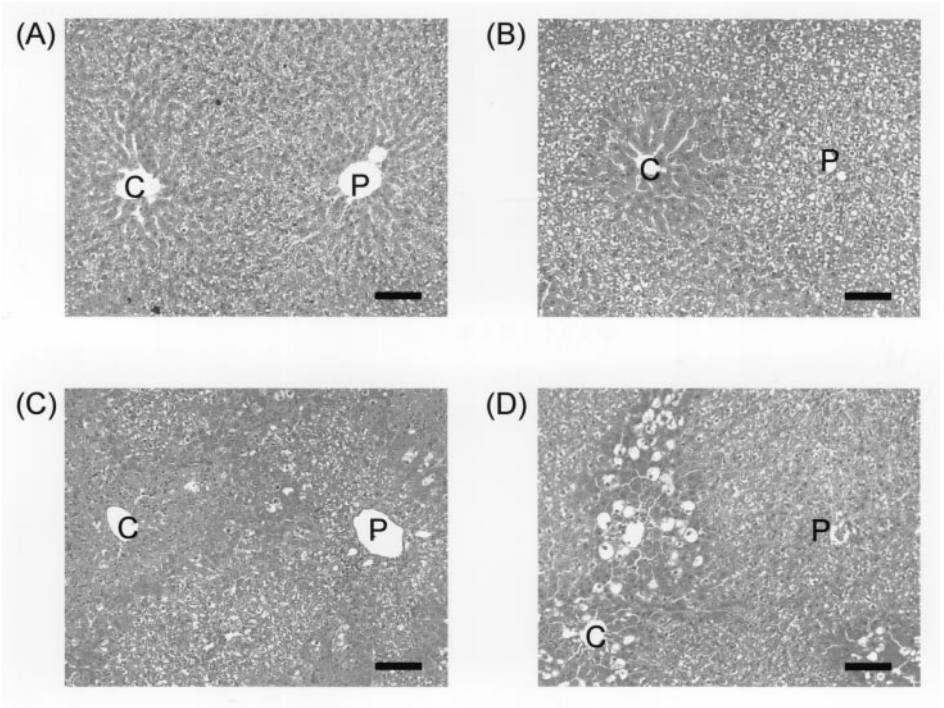

Fig. 2. Histological changes after isoflurane, halothane, and/or hypoxia exposure. Phenobarbital-pretreated rats were exposed for 2 hr to isoflurane, halothane, and/or hypoxia. Twenty-four hours after gas treatment, livers were removed for histological examination using hematoxylin-eosin staining. (A) untreated control; (B) hypoxia; (C) isoflurane-hypoxia; and (D) halothane-hypoxia. The letters C and P within the panels denote central and portal venules, respectively. The bar represents 100 μm.

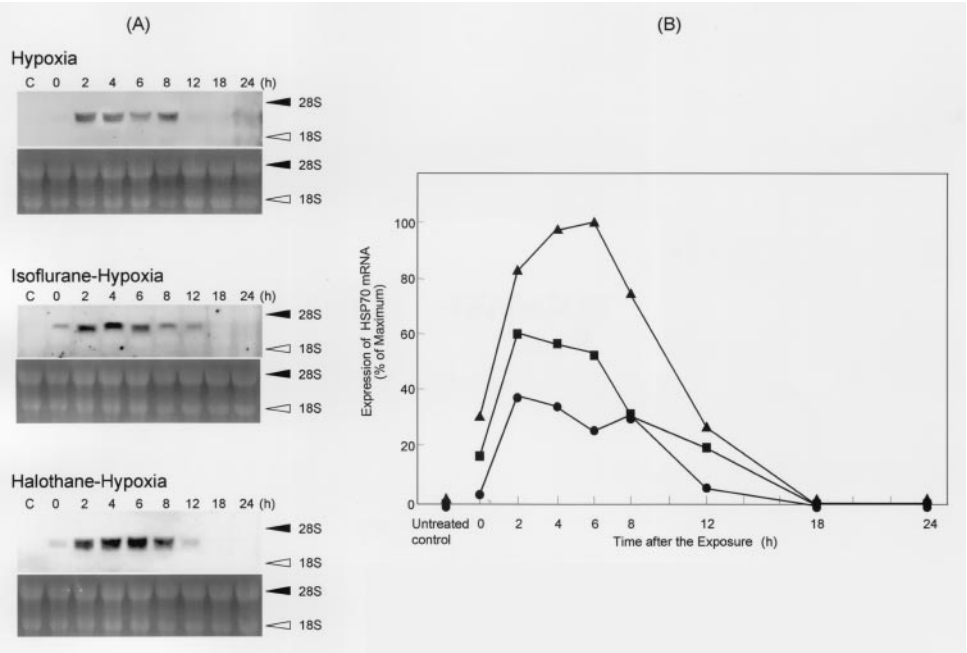

Fig. 3. Time courses for the expression of hepatic HSP70 mRNA after isoflurane, halothane, and/or hypoxia exposure in phenobarbital-pretreated rats. Phenobarbital-pretreated rats were exposed to hypoxia (●), isoflurane-hypoxia (■) or halothane-hypoxia (▲) for 2 hr. After gas exposure, livers were removed at the indicated time points for northern blot analysis. (A) Shown are the chemiluminescent signals of the RNA blot hybridized with a biotin-labeled rat HSP70 cRNA riboprobe. Ethidium bromide staining of the same RNA is shown as a loading control. (B) The levels of HSP70 mRNA at each time point are expressed relative to maximal levels (6 hr after exposure to halothane-hypoxia). Three independent experiments showed similar results, and a typical example is shown in the figure.

three exposure groups, i.e. hypoxia, isoflurane-hypoxia, and halothane-hypoxia, HSP70 mRNA expression started to increase immediately after gas exposure, reached maximal levels within 2 hr, maintained those levels for 8 hr, and returned to control levels by 18 hr. Exposure to 10% oxygen gas by itself resulted in about a 40% induction of HSP70 mRNA starting at 2 hr after treatment and continuing beyond 6 hr. Treatment with isoflurane under 10% oxygen induced HSP70 mRNA in a similar manner, but at significantly higher levels up to 6 hr. Induction of HSP70 mRNA was the highest in halothane-hypoxia treated rats at all time points, compared with controls or isoflurane-hypoxia rats.

3.3. Time course for the expression of HO-1 mRNA after isoflurane, halothane, and/or hypoxia treatment

We also examined the expression of HO-1 mRNA over time in the livers of rats treated with the gas mixtures (Fig. 4). Similar to the changes in the expression levels of HSP70 mRNA, hepatic HO-1 mRNA levels in halothane-hypoxia-treated rats started to increase immediately after gas exposure, reached maximum levels at 2 hr, and remained at those levels for 8 hr before abruptly declining and returning to normal by 18 hr. In contrast, isoflurane-hypoxia treatment caused only a minor increase at 6–8 hr, while exposure to hypoxia alone did not cause any significant induction of HO-1 mRNA at any time.

3.4. Distribution of HSP70 and HO-1 proteins in the liver of isoflurane-, halothane-, and/or hypoxia-treated rats

We then examined rat livers immunohistochemically 12 hr after exposure to the gas mixtures. In livers of untreated rats, HSP70 protein was not observed at all (Fig. 5A). After gas treatment, HSP70 protein was expressed by hepatocytes in the centrilobular regions irrespective of the type of gas treatment used. In these regions, HSP70 protein expression was highest following treatment with halothane-hypoxia, intermediate with isoflurane-hypoxia, and lowest after exposure to hypoxia alone (Fig. 5, B-D). In contrast, HO-1 protein was marginally expressed only in Kupffer cells in the livers of untreated (Fig. 6A) [20], hypoxia-treated (Fig. 6B), and isoflurane-hypoxia-treated (Fig. 6C) rats. In the halothane-hypoxia-treated rats, however, positive staining for HO-1 protein was observed not only in Kupffer cells but also in hepatocytes, especially in the centrilobular region (Fig. 6D).

3.5. Effects of isoflurane, halothane, and/or hypoxia on the expression of hepatic HSP 70 and HO-1 mRNAs, and histological findings in non-pretreated rats

To explore the effect of microsomal enzyme induction on hepatotoxicity by the volatile anesthetics, the expression of hepatic HSP70 and HO-1 mRNAs at 6 hr, and hepatic morphological changes at 24 hr were examined in non-

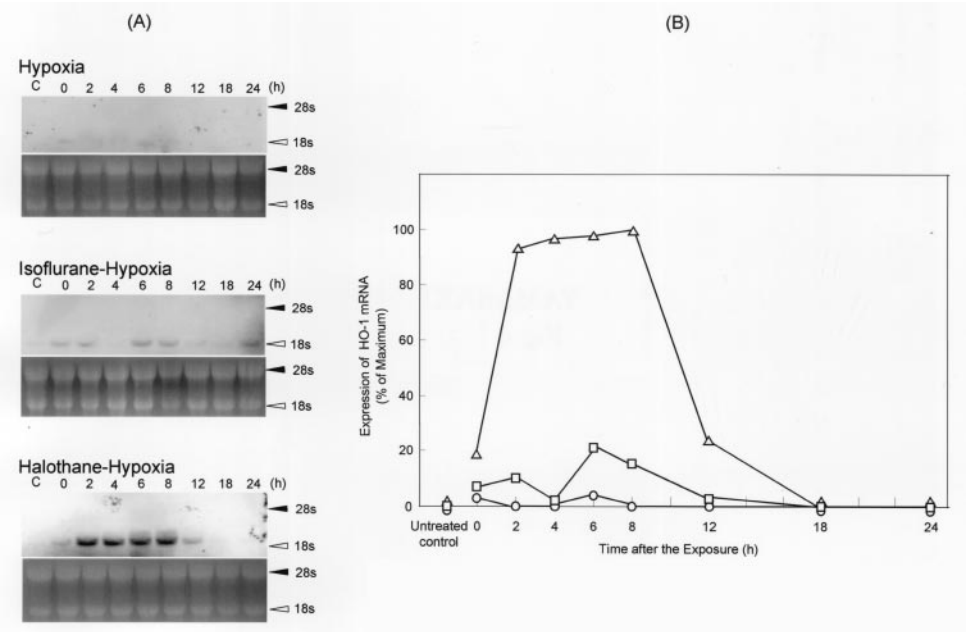

Fig. 4. Time courses for the expression of hepatic HO-1 mRNA after isoflurane, halothane, and/or hypoxia exposure in phenobarbital-pretreated rats. Phenobarbital-pretreated rats were exposed to hypoxia (○), isoflurane-hypoxia (❑), or halothane-hypoxia (Δ) for 2 hr. After each exposure, livers were removed at the indicated time points for northern blot analysis. (A) Shown are the chemiluminescent signals of the RNA blot hybridized with a biotin-labeled rat HO-1 cRNA riboprobe. Ethidium bromide staining of the same RNA is shown as a loading control. (B) The levels of HO-1 mRNA at each time point are expressed relative to maximal levels (6 hr after exposure to halothane-hypoxia). Three independent experiments showed similar results, and a typical example is shown in the figure.

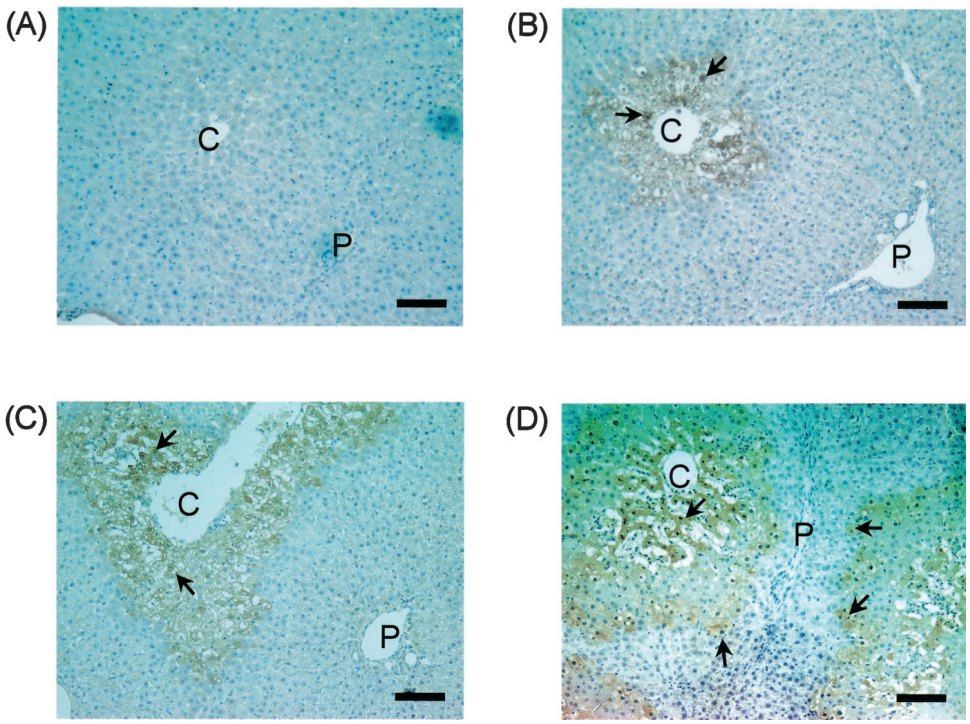

Fig. 5. Immunohistochemical localization of HSP70 in the livers of phenobarbital-treated rats exposed to isoflurane, halothane, and/or hypoxia. Liver sections from phenobarbital-pretreated rats 12 hr after exposure to isoflurane, halothane, and/or hypoxia were used for immunohistochemical detection of HSP70, using mouse monoclonal anti-human HSP70 as a primary antibody. (A) Untreated control; (B) hypoxia; (C) isoflurane-hypoxia; and (D) halothane-hypoxia. The letters C and P within the panels denote central and portal venules, respectively. The bar represents 100 μm. Arrows indicate positively stained hepatocytes.

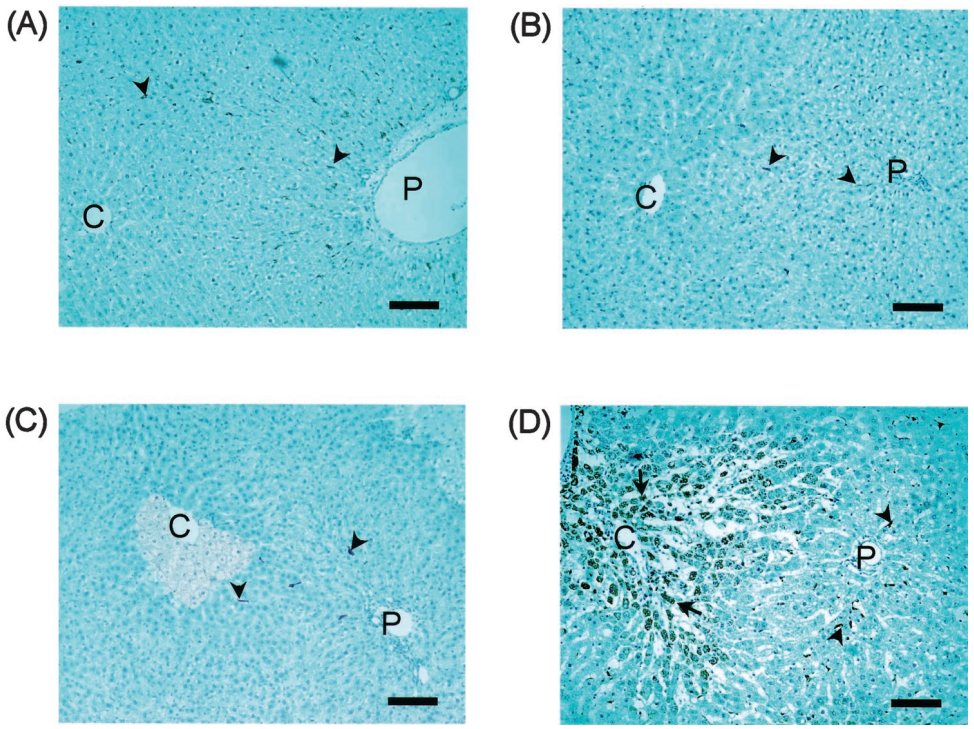

Fig. 6. Immunohistochemical detection of HO-1 in the livers of phenobarbital-pretreated rats exposed to isoflurane, halothane, and/or hypoxia. Liver sections from phenobarbital-pretreated rats 12 hr after exposure to isoflurane, halothane, and/or hypoxia were used for immunohistochemical detection of HO-1, using rabbit polyclonal anti-rat HO-1 as a primary antibody. (A) Untreated control; (B) hypoxia; (C) isoflurane-hypoxia; and (D) halothane-hypoxia. The letters C and P within the panels denote central and portal venules, respectively. The bar represents 100 μm. Arrows and arrowheads indicate positively stained hepatocytes and Kupffer cells, respectively.

pretreated rats after each gas treatment (Fig. 7). HSP70 mRNA was not detectable in non-pretreated control animals exposed to either hypoxia or isoflurane-hypoxia (Fig. 7; top). In contrast, following halothane-hypoxia treatment, expression of hepatic HSP70 mRNA markedly increased to levels found in phenobarbital-pretreated rats exposed to halothane-hypoxia (Fig. 7; top). HO-1 mRNA was only barely detectable following exposure to each gas mixture (Fig. 7; top left). Although histological examination of livers from non-pretreated rats exposed to the gas mixtures showed vacuolar degeneration and congestion around the portal tracts, particularly following halothane-hypoxia exposure, there was no centrilobular necrosis following exposure to these gas mixtures (Fig. 7; bottom).

4. Discussion

The present study demonstrates that in phenobarbital-pretreated rats exposure to isoflurane-hypoxia caused a smaller induction of HSP70 mRNA and protein than did exposure to halothane-hypoxia (Figs. 3 and 5). HSP70 mRNA levels were already somewhat increased when animals were exposed to hypoxia alone (Fig. 3). In contrast, induction of HO-1 mRNA was insignificant in rats treated with hypoxia alone, or with the isoflurane-hypoxia mixture, whereas HO-1 mRNA was clearly induced following exposure to halothane-hypoxia in phenobarbital-pretreated rats (Figs. 4 and 6). Our findings indicate differential effects of isoflurane and halothane on the expression of HSPs under hypoxic conditions. Histological examination demonstrated that injured hepatocytes were observed predominantly in the centrilobular region of the liver in phenobarbital-pretreated rats exposed to isoflurane-hypoxia or halothane-hypoxia (Fig. 2). In contrast there was no centrilobular necrosis following either isoflurane-hypoxia or halothane-hypoxia treatment in non-pretreated rats (Fig. 7; bottom). There was also no induction of HSP70 or HO-1 mRNA in non-pretreated rats following any gas exposure (Fig. 7; top). These results suggest that not only halothane but also isoflurane has some potential to cause hepatic injury under hypoxic conditions.

In phenobarbital-pretreated rats, induction of HSP70 mRNA and protein was observed irrespective of the type of gas treatment used. The best induction was achieved by exposure to halothane-hypoxia followed by isoflurane-hypoxia (intermediate) and hypoxia alone (lowest) (Figs. 3 and 5). This is consistent with the degree of hepatic injury, serum ALT levels, and the extent of centrilobular necrosis observed (Figs. 1 and 2). Induction of HO-1 mRNA and protein in the livers of phenobarbital-pretreated rats was observed only following exposure to halothane-hypoxia (Figs. 4 and 6). It is well known that oxidative stress increases the expression of HSP70 [21]. We have demon-

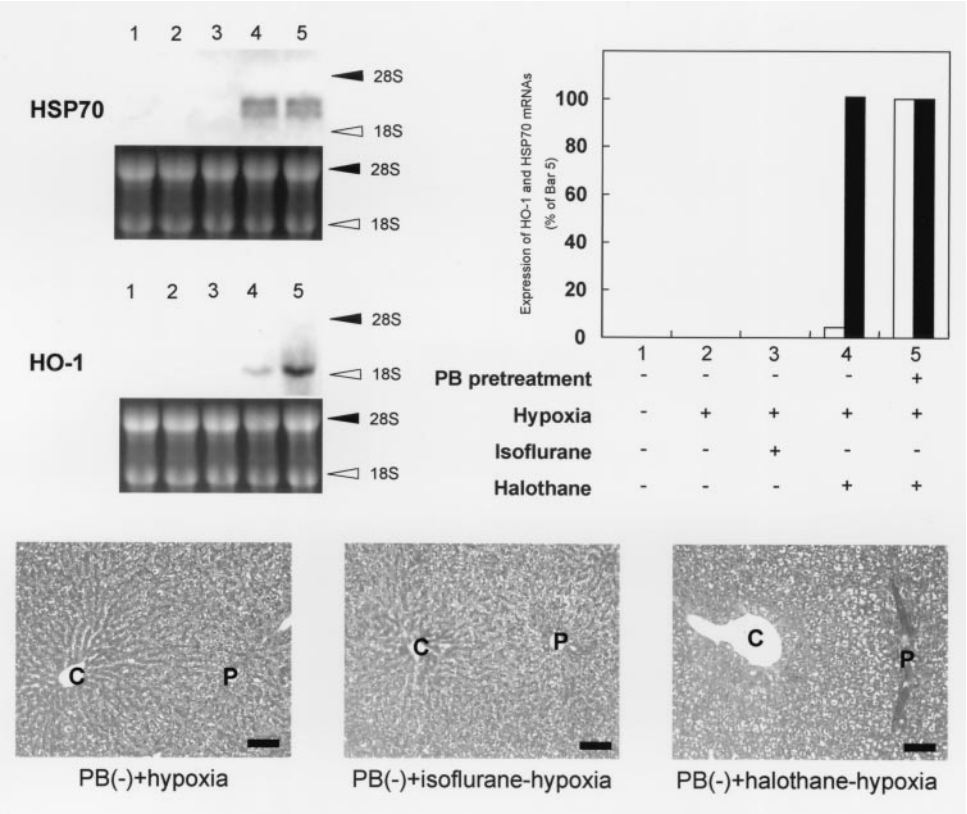

Fig. 7. Effect of isoflurane, halothane, and/or hypoxia on the expression of hepatic HSP 70 mRNA and HO-1 mRNA, and histological findings in non-pretreated rats. Non-pretreated rats were exposed to hypoxia, isoflurane-hypoxia, or halothane-hypoxia for 2 hr. Livers were removed for northern blot analysis at 6 hr (top left panels) and for histological study at 24 hr (bottom panels) after each gas exposure. Top left: Northern blot analysis. Shown are the chemiluminescent signals of the RNA blot hybridized with either biotin-labeled rat HSP70 or HO-1 cRNA riboprobes. Ethidium bromide staining of the same RNA is shown as a loading control. Top right: Quantitation of northern blots. HSP70 (■) and HO-1 (❑) mRNA levels are expressed relative to maximal levels (6 hr after exposure to halothane-hypoxia in phenobarbital-pretreated rats). Three independent experiments showed similar results, and a typical example is shown in the figure. Bottom: Hematoxylin-eosin staining was carried out as described in "Materials and methods." The bar represents 100 μm. The letters C and P within the panels denote central and portal venules, respectively.

strated previously that halothane-hypoxia treatment of phenobarbital-pretreated rats results in a rapid increase in hepatic intracellular free heme, which is presumably derived from cytochrome P450, and results in the induction of HO-1 [22]. While we have not determined the amount of free heme released from cytochrome P450 in the livers of isoflurane-hypoxia-treated rats, it is likely to be less than that produced from the halothane-hypoxia-treated livers as suggested by the reduced induction of HO-1 by isoflurane-hypoxia treatment. Consistent with our hypothesis, it has been shown recently that hydroxyl radical formation in the reperfused working rat heart was decreased more significantly by isoflurane treatment than by halothane treatment [23]. Thus, while an immune mechanism is postulated in the genesis of halothane-mediated hepatotoxicity [24], our findings suggest that there is additionally a toxic reactive intermediate-based injury of hepatocytes by halothane, and the well-known safety aspects of isoflurane [25,26] may be, in part, related to the formation of fewer toxic intermediates with isoflurane than with halothane.

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