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Mechanism of Hepatic Heme Oxygenase-1 Induction by Isoflurane

Alexander Hoetzel, M.D., Daniel Leitz,† Rene Schmidt, M.D.,* Eva Tritschler,† Inge Bauer, Ph.D.,‡ Torsten Loop, M.D.,* Matjaz Humar, Ph.D.,* Klaus K. Geiger, M.D.,§ Benedikt H. J. Pannen, M.D.‖*

Background: **The heme oxygenase pathway represents a major cell and organ protective system in the liver. The authors recently showed that isoflurane and sevoflurane up-regulate the inducible isoform heme oxygenase 1 (HO-1). Because the activating cascade remained unclear, it was the aim of this study to identify the underlying mechanism of this effect.**

Methods: **Rats were anesthetized with pentobarbital intravenously or with isoflurane per inhalation (2.3 vol%). Kupffer cell function was inhibited by dexamethasone or gadolinium chloride. Nitric oxide synthases were inhibited by either N^{ω}-nitro-L-arginine methyl ester or *S*-methyl thiourea. *N*-Acetyl-cysteine served as an antioxidant, and diethyldithiocarbamate served as an inhibitor of cytochrome P450 2E1. Protein kinase C and phospholipase A_2 were inhibited by chelerythrine or quinacrine, respectively. HO-1 was analyzed in liver tissue by Northern blot, Western blot, immunostaining, and enzymatic activity assay.**

Results: **In contrast to pentobarbital, isoflurane induced HO-1 after 4–6 h in hepatocytes in the pericentral region of the liver. The induction was prevented in the presence of dexamethasone ($P < 0.05$) and gadolinium chloride ($P < 0.05$). Inhibition of nitric oxide synthases or reactive oxygen intermediates did not affect isoflurane-mediated HO-1 up-regulation. In contrast, chelerythrine ($P < 0.05$) and quinacrine ($P < 0.05$) resulted in a blockade of HO-1 induction.**

Conclusion: **The up-regulation of HO-1 by isoflurane in the liver is restricted to parenchymal cells and depends on Kupffer cell function. The induction is independent of nitric oxide or reactive oxygen species but does involve protein kinase C and phospholipase A_2.**

HEPATIC reperfusion injury, *e.g.*, after transplantation, systemic low flow conditions, or disturbances of the hepatic microcirculation, is associated with high morbidity and mortality. To date, no pharmacologic strategy is clinically applicable that effectively prevents hepatic reperfusion injury and improves the outcome.

The heme oxygenase (HO) pathway has been shown to limit reperfusion injury after experimental systemic and regional hepatic ischemia.[1] Heme oxygenase 1 (HO-1) represents the inducible isoform of the microsomal HO family. HO-1 gene expression is up-regulated in response to a variety of stimuli and catalyzes the oxidation of heme to biliverdin-IXa, iron, and carbon monoxide. The HO system exerts major antioxidative, antiinflammatory, antiapoptotic, and vasodilatory characteristics.[1,2] In that regard, preinduction of HO-1 and application of carbon monoxide have been shown to prevent reperfusion injury after liver transplantation or after systemic ischemia in several experimental studies.[3] Therefore, the HO pathway seems to represent a promising candidate for reducing reperfusion injury in patients.

Many efforts have been made to modulate or preinduce HO-1 and to optimize its protective potency against subsequent noxious stimuli. However, most inducers of HO-1 (*e.g.*, oxidative stress, hypoxia, hemin, cobalt chloride, radiation) have numerous side effects and therefore cannot be used in patients. Therefore, it is essential to identify substances or conditions that induce HO-1 without endangering the patient. We recently demonstrated that the volatile anesthetics isoflurane and sevoflurane are able to specifically up-regulate HO-1 gene expression.[4] The mechanism of this inducing action remained unclear. However, characterization of the respective signal transduction pathway could help to establish new concepts aimed at modulating the HO system, furthermore expanding its protective function. In the current study, we investigated potential activating cascades and identified mechanisms of HO-1 regulation by the volatile anesthetic isoflurane.

Materials and Methods

Animals

Male Sprague-Dawley rats (Charles River, Sulzfeld, Germany) weighing 290 ± 21 g were used for all experiments. The experimental protocol was approved by the local animal care and use committee (Regierungspraesidium Freiburg, Germany), and all animals received humane care according to the criteria outlined in the *Guide for the Care and Use of Laboratory Animals*.[5] Rats were fasted for 6 h before the beginning of the experiments but were allowed free access to water. Anesthesia was induced per inhalation of the volatile anesthetic isoflurane for 3 min (Baxter, Unterschleissheim, Germany) and maintained with either pentobarbital sodium (50 mg/kg body weight, intraperitoneal) followed by intravenous bolus injections of 5 mg/kg or by the continuous application of the volatile anesthetic isoflurane (2.3 vol% per inhalation). A tracheostomy was performed, and animals were allowed to breathe spontaneously throughout the experiment. The left carotid artery was cannulated to continuously measure arterial blood pressure.

* Postdoctoral Research Fellow, † Research Assistant and Student, § Professor and Chair of Anesthesiology, ‖ Professor of Anesthesiology, Department of Anesthesiology and Critical Care Medicine, University of Freiburg. ‡ Postdoctoral Research Fellow, Department of Anesthesiology and Critical Care Medicine, University of the Saarland, Homburg, Germany.

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Address reprint requests to Dr. Pannen: Anaesthesiologische Universitaetsklinik, Hugstetter Strasse 55, D-79106 Freiburg, Germany. Address electronic mail to: pannen@ana1.ukl.uni-freiburg.de.

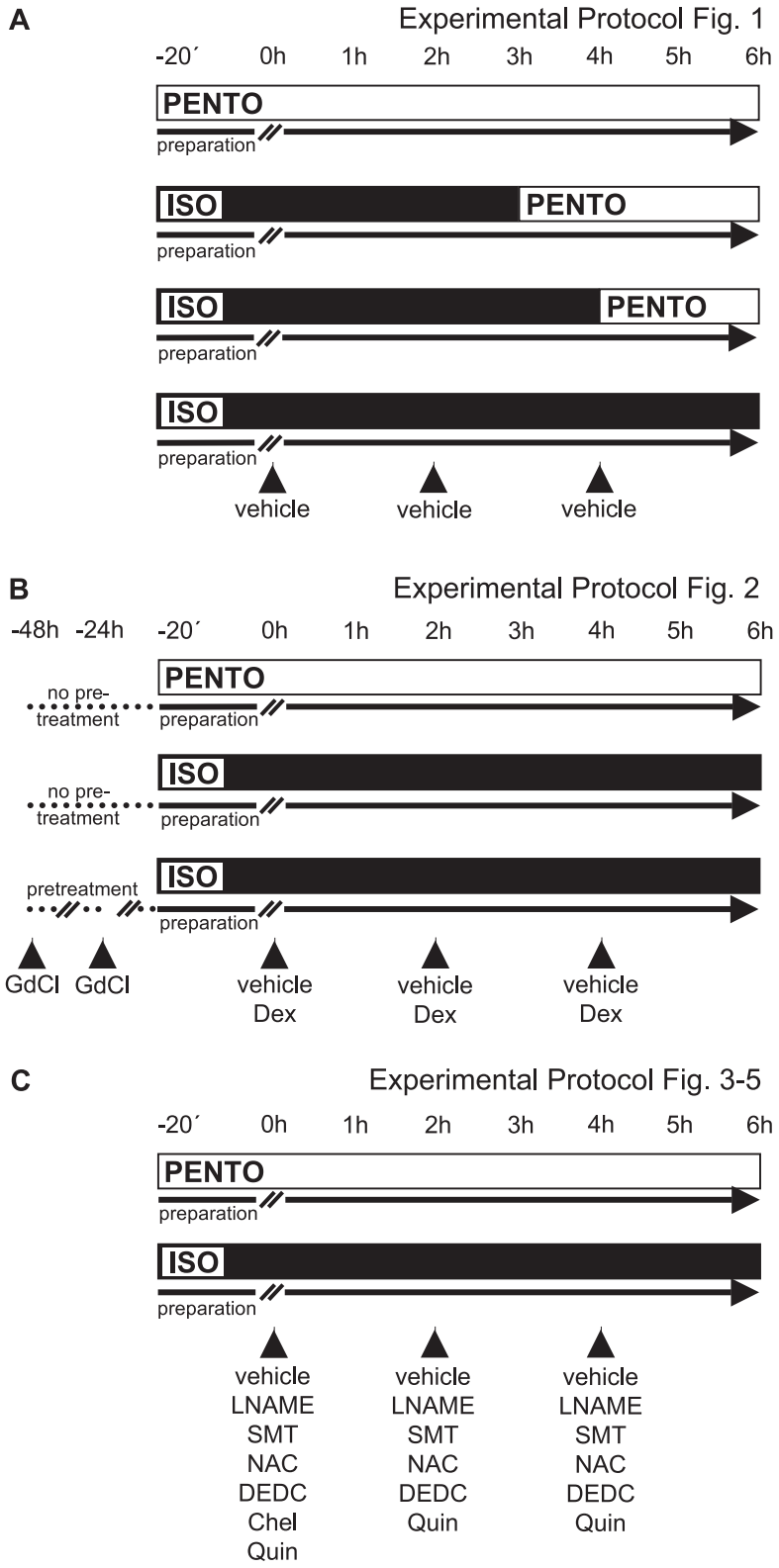

Fig. 1. Experimental protocol. (*A*) Time-dependent experiments. (*B*) Pretreatment experiments. (*C*) All other experiments. Chel = chelerythrine; DEDC = diethyldithiocarbamate; Dex = dexamethasone; GdCl = gadolinium chloride; ISO = isoflurane; L-NAME = N^{ω}-nitro-L-arginine methyl ester; NAC = *N*-acetyl-cysteine; PENTO = pentobarbital; Quin = quinacrine; SMT = *S*-methyl thiourea.

Experimental Protocols

The protocols applied in the different experimental series are summarized in figure 1. The results depicted in figures 2–6 correspond to an independent series of experiments. The pharmacologic agents were administered in the following doses: gadolinium chloride (inhibitor of Kupffer cell function, 10 mg/kg intravenous), dexamethasone (4 mg/kg intravenous), N^{ω}-nitro-L-arginine methyl ester (preferential inhibitor of constitutive nitric oxide synthase, 1 mg/kg intravenous), *S*-methyl thiourea (preferential inhibitor of inducible nitric oxide synthase, 10 mg/kg intravenous), *N*-acetyl-cysteine (anti-

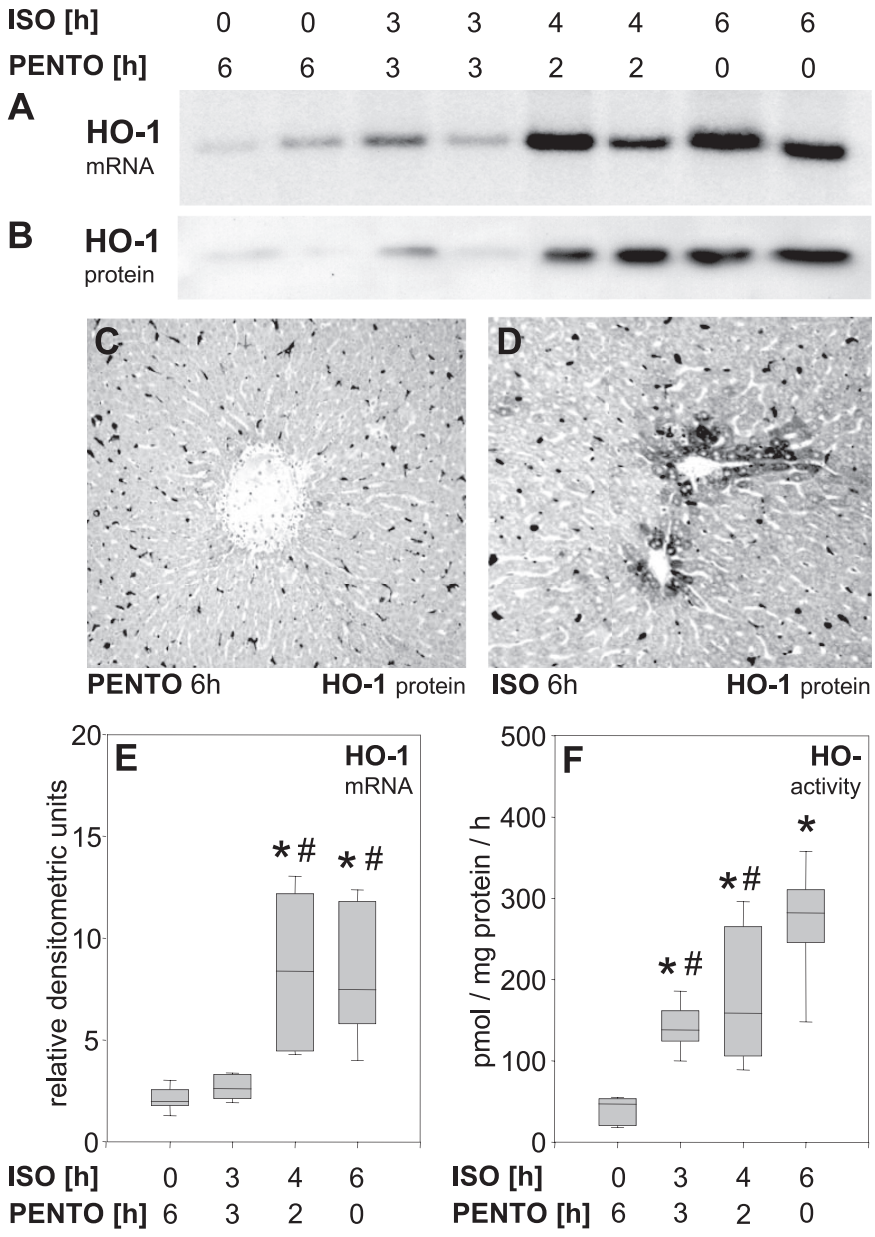

Fig. 2. Expression of heme oxygenase 1 (HO-1) and heme oxygenase (HO) activity in the liver after different time periods of anesthesia with either pentobarbital (PENTO) or isoflurane (ISO). (*A*) Northern blot. (*B*) Western blot. (*A* and *B*) The *two lanes* corresponding to each intervention show the results of two representative experiments. (*C* and *D*) Immunostaining with HO-1 antibody. (*E*) Densitometric analysis of HO-1 messenger RNA (mRNA) and (*F*) HO activity of all individual experiments (n = 5/group). * *P* < 0.05 *versus* pentobarbital. # *P* < 0.05 *versus* isoflurane 3 h (*E*) or 6 h (*F*).

oxidant, 60 mg/kg intravenous), diethyldithiocarbamate (cytochrome P450 2E1 inhibitor, 5 mg/kg intravenous), chelerythrine (protein kinase C [PKC] inhibitor, 1 × 5 mg/kg intravenous), quinacrine (phospholipase A_2 [PLA_2] inhibitor, 5 mg/kg intravenous). All these intravenous injections were performed during inhalational anesthesia with isoflurane. Additional control experiments with pentobarbital anesthesia and administration of dexamethasone, gadolinium chloride, chelerythrine, or quinacrine did not provide any evidence for an intrinsic effect of these agents on the expression of HO-1 (data not shown). Blood gas analyses were performed at the beginning, after 3 h, and at the end of each experiment. Partial pressure of oxygen and carbon dioxide did not differ between groups (data not shown). At the end of each experiment, plasma enzyme activities of alanine amino transferase, aspartate amino transferase, and lactate dehydrogenase did not differ between groups (data not shown). After completion of the experimental protocol, animals were killed, and tissue samples were stored at −80°C for subsequent analysis.

RNA Extraction and Northern Blot Analysis

Total RNA was isolated from liver tissue as previously described.[6,7] The HO-1 probe consisted of an HO-1 complementary DNA (cDNA) restriction fragment (0.8 kb; kindly provided by Shigeki Shibahara, M.D., Ph.D., Department of Applied Physiology and Molecular Biology, Tohoku University School of Medicine, Sendai, Japan).[8] All blots were stripped and reprobed with 18S cDNA. Results were quantified by densitometric analysis and expressed as relative densitometric units calculated as

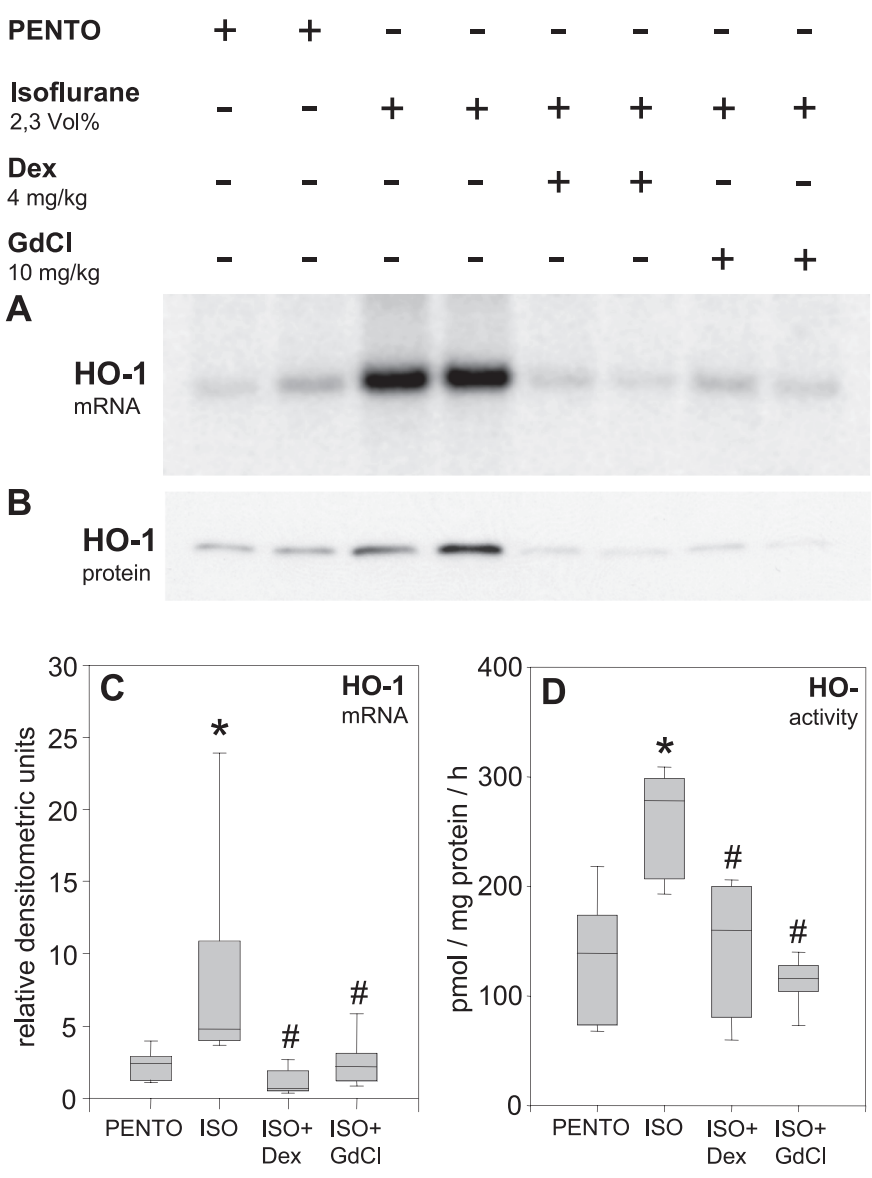

Fig. 3. Expression of heme oxygenase 1 (HO-1) and heme oxygenase (HO) activity in the liver after pentobarbital (PENTO) or isoflurane (ISO) anesthesia, with or without dexamethasone (ISO + Dex), or gadolinium chloride (ISO + GdCl). (*A*) Northern blot. (*B*) Western blot. (*A* and *B*) The *two lanes* corresponding to each intervention show the results of two representative experiments. (*C*) Densitometric analysis of HO-1 messenger RNA (mRNA) and (*D*) HO activity of all individual experiments (n = 5–7/group). * $P < 0.05$ *versus* pentobarbital. # $P < 0.05$ *versus* isoflurane.

dividends of the background-corrected densitometric values of HO-1/18S.

Immunohistochemical Staining

Frozen liver sections were used to assess the effect of isoflurane on the cell-type–specific expression pattern of HO-1 by immunohistochemical staining, as we previously described.[6] Slides were incubated with anti–HO-1 antibody (dilution 1:200, StressGen; Biotechnologies, Victoria, Canada) as a primary antibody. A biotinylated goat anti-rabbit antibody was used as a secondary antibody for streptavidin–biotin complex peroxidase staining.

Western Blot Analysis

Western blot analysis was performed with total protein isolated from frozen liver tissue as previously described.[6,7] A rabbit polyclonal anti–HO-1 antibody (1:1,000 dilution, SPA 895, StressGen; Biotechnologies) was used as a primary antibody, and an anti-rabbit antibody (1:5,000 dilution, ECL-detection kit; Amersham Pharmacia, Freiburg, Germany) was used as a secondary antibody.

HO Activity Assay

Heme oxygenase enzymatic activity was measured in liver tissue as we have previously reported,[6,7] and results were expressed as the formation of bilirubin in pmol · mg protein^{-1} · h^{-1}.

Data Analysis

Data are presented as medians with 25th and 75th percentiles (boxes) and 10th and 90th percentiles (error bars). Statistical differences between experimental groups were determined by one-way analysis of variance on ranks followed by the nonparametric Student-Newman-Keuls *post hoc* test for multiple comparisons. A P

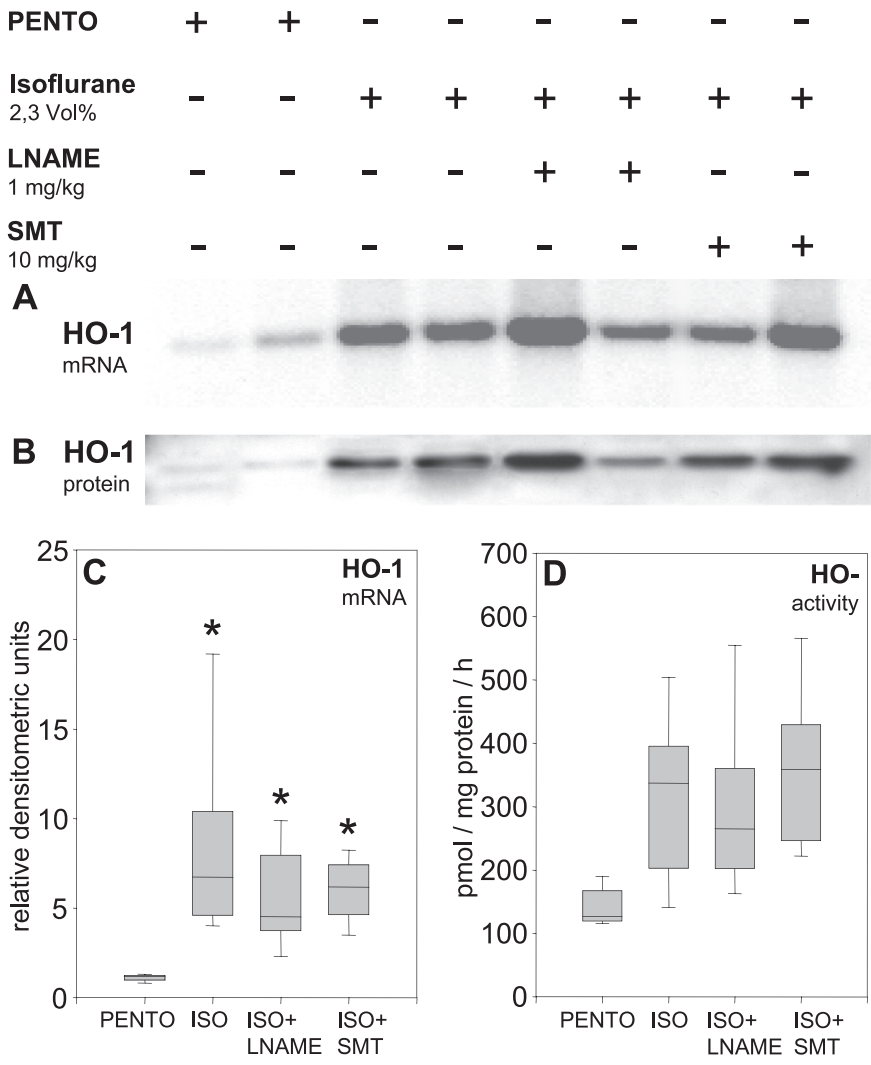

Fig. 4. Expression of heme oxygenase 1 (HO-1) and heme oxygenase (HO) activity in the liver after pentobarbital (PENTO) or isoflurane (ISO) anesthesia, with or without N^{ω}-nitro-L-arginine methyl ester (ISO + L-NAME) or *S*-methyl thiourea (ISO + SMT). (*A*) Northern blot. (*B*) Western blot. (*A* and *B*) The *two lanes* corresponding to each intervention show the results of two representative experiments. (*C*) Densitometric analysis of HO-1 messenger RNA (mRNA) and (*D*) HO activity of all individual experiments (n = 5/group). * $P < 0.05$ *versus* pentobarbital.

value less than 0.05 was considered to indicate a significant difference.

Results

Time-dependent Effect of Isoflurane on HO-1 Expression and HO Activity

In liver tissue of pentobarbital-anesthetized animals, HO-1 mRNA was barely detectable (fig. 2A). The administration of isoflurane for up to 3 h did not lead to a significant up-regulation of HO-1 mRNA (figs. 2A and E). In contrast, 4 or more hours of isoflurane treatment markedly induced HO-1 gene expression (figs. 2A and E). To study the cell-type–specific expression of HO-1 protein, immunohistochemical staining was performed. In pentobarbital control animals, HO-1 could only be detected in Kupffer cells (fig. 2C). In contrast, isoflurane-treated animals showed *de novo* HO-1 accumulation in parenchymal cells of the liver, mainly in the pericentral region (fig. 2D). HO enzymatic activity was increased after 3 and 4 h of isoflurane treatment and increased even further after 6 h (fig. 2F).

Effect of Dexamethasone and Gadolinium Chloride on Isoflurane-mediated HO-1 Expression and HO Activity

As depicted in figures 3A and C, isoflurane induced HO-1 mRNA. In sharp contrast, the additional administration of dexamethasone or gadolinium chloride resulted in a significantly lower HO-1 mRNA accumulation than that of isoflurane alone (figs. 3A and C). The inhibitory effect of dexamethasone and gadolinium chloride on isoflurane-induced HO-1 mRNA expression was paralleled on the translational level. Western blot experiments as well as the measurement of HO activity showed an increase in HO-1 protein accumulation (fig. 3B) and enzymatic activity (fig. 3D) after administering isoflurane alone. This was no longer existent when dexamethasone or gadolinium chloride was applied in addition.

Effect of N^{ω}-Nitro-L-arginine Methyl Ester and S-Methyl Thiourea on Isoflurane-mediated HO-1 Expression and HO Activity

The induction of HO-1 mRNA by isoflurane is shown in figures 4A and C. Inhibition of nitric oxide synthases by

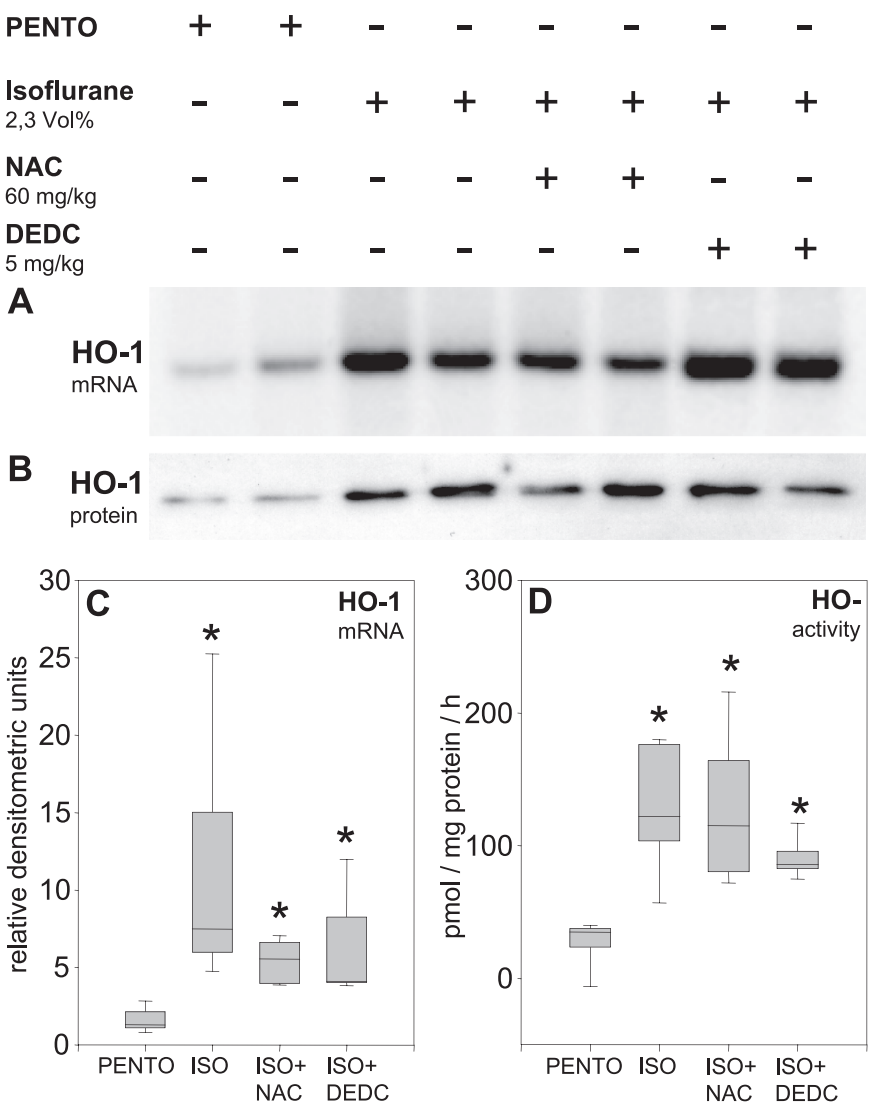

Fig. 5. Expression of heme oxygenase 1 (HO-1) and heme oxygenase (HO) activity in the liver after pentobarbital (PENTO) or isoflurane (ISO) anesthesia, with or without *N*-acetyl-cysteine (ISO + NAC) or diethyldithiocarbamate (ISO + DEDC). (*A*) Northern blot. (*B*) Western blot. (*A* and *B*) The *two lanes* corresponding to each intervention show the results of two representative experiments. (*C*) Densitometric analysis of HO-1 messenger RNA (mRNA) and (*D*) HO activity of all individual experiments (n = 5/group). **P* < 0.05 *versus* pentobarbital.

either the preferential inhibitor of its constitutive isoform (N^{ω}-nitro-L-arginine methyl ester) or a selective inhibitor of the inducible nitric oxide synthases (*S*-methyl thiourea) did not affect the isoflurane-mediated HO-1 mRNA up-regulation (figs. 4A and C). Similarly, analysis of HO-1 protein and HO activity revealed no significant differences when isoflurane was administered alone or in the presence of the nitric oxide synthase inhibitors (figs. 4B and D).

Effect of N-Acetyl-cysteine and Diethyldithiocarbamate on Isoflurane-mediated HO-1 Expression and HO Activity

Figure 5 demonstrates that the HO-1 mRNA induction by isoflurane was not altered in the presence of the antioxidant *N*-acetyl-cysteine or the cytochrome P450 2E1 inhibitor diethyldithiocarbamate (figs. 5A and C). The expression pattern of HO-1 mRNA was paralleled by an up-regulation of HO-1 protein (fig. 5B) and an increase in HO activity (fig. 5D) that was also not affected by *N*-acetyl-cysteine or diethyldithiocarbamate.

Effect of Chelerythrine and Quinacrine on Isoflurane-mediated HO-1 Expression and HO Activity

As described above (figs. 2–5), isoflurane alone induced HO-1 mRNA compared with pentobarbital control conditions (figs. 6A and C). The PKC inhibitor (chelerythrine) as well as the inhibitor of PLA_2 (quinacrine) prevented the HO-1 mRNA accumulation that could otherwise be observed in response to isoflurane (figs. 6A and C). Moreover, the isoflurane-induced accumulation of HO-1 protein and the increase in HO enzymatic activity were also abolished by chelerythrine and quinacrine (figs. 6B and D).

Discussion

In a recent issue of ANESTHESIOLOGY, we demonstrated that the volatile anesthetics isoflurane and sevoflurane induce the expression of the hepatoprotective enzyme HO-1 in the liver.[4] Nevertheless, the signal transduction pathway in-

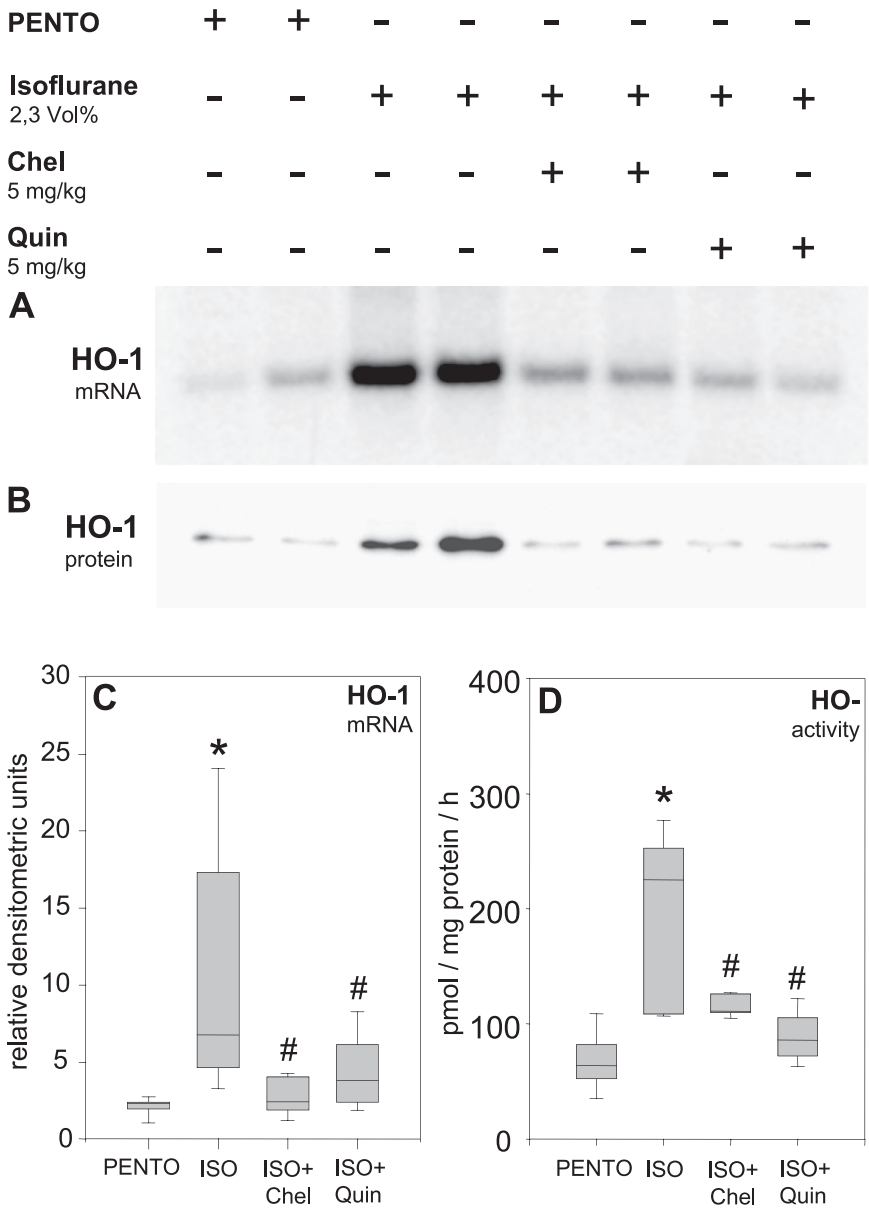

Fig. 6. Expression of heme oxygenase 1 (HO-1) and heme oxygenase (HO) activity in the liver after pentobarbital (PENTO) or isoflurane (ISO) anesthesia, with or without chelerythrine (ISO + Chel) or quinacrine (ISO + Quin). The *two lanes* corresponding to each intervention show the results of two representative experiments. (*C*) Densitometric analysis of HO-1 messenger RNA (mRNA) and (*D*) HO activity of all individual experiments (n = 5–7/group). * $P < 0.05$ *versus* pentobarbital. # $P < 0.05$ *versus* isoflurane.

volved in this process was still unknown. Therefore, it was the aim of the current study to identify the mechanism of HO-1 regulation by volatile anesthetics.

Several inducers of HO-1 gene regulation in the liver are promoted by Kupffer cells.[9,10] Therefore, we hypothesized that Kupffer cell function might also be essential for the hepatocellular expression of HO-1 in response to isoflurane. In fact, our results demonstrate that dexamethasone, a potent inhibitor of the above-mentioned signaling pathways in Kupffer cells,[11,12] completely abolishes the isoflurane-mediated induction of HO-1. This provides indirect evidence that Kupffer cells could be involved in the up-regulation of HO-1 by isoflurane in hepatocytes. However, these data do not allow for exclusion of a direct hepatocellular effect of isoflurane on HO-1 expression, because dexamethasone is able to inhibit other activating pathways (*e.g.*, transcriptional activation, transcription, posttranscriptional modulation). Its action is therefore not restricted to Kupffer cells. A specific approach to investigate the role of Kupffer cells in gene regulation is the inhibition of their function by gadolinium chloride. This agent alters the acinar distribution of Kupffer cells and provokes a switch in their phenotype so that these cells no longer express Kupffer cell gene products.[13] In our experiments, the application of gadolinium chloride prevented the induction of HO-1. This finding is in accord with the results of a previous study showing that gadolinium chloride diminishes the expression of HO-1 in hepatocytes in response to lipopolyssacharides,[9] and it proves the hypothesis that isoflurane induces hepatic HO-1 *via* Kupffer cells.

Consequently, the question remained as to which signal transduction pathway might account for Kupffer cell–mediated HO-1 regulation. Several lines of evidence

suggested that nitric oxide could be involved. First, nitric oxide can induce HO-1 in a variety of cells.[14] Second, nitric oxide is responsible for the up-regulation of hepatocellular HO-1 by lipopolysaccharide, which requires Kupffer cell function.[15] To elucidate the role of nitric oxide in isoflurane-mediated HO-1 regulation, the constitutive as well as the inducible isoforms of the nitric oxide synthase were inhibited by N^{ω}-nitro-L-arginine methyl ester or *S*-methyl thiourea, respectively. However, none of the nitric oxide synthase inhibitors affected the expression pattern of HO-1 by isoflurane. These findings exclude a major role of nitric oxide during this inducing process.

Reactive oxygen intermediates (ROIs) are another important class of signaling molecules regulating HO-1. Because Kupffer cells are a major source of ROIs in the liver, their essential role for the induction of HO-1 by isoflurane in our experiments could serve as an indirect hint toward an involvement of ROIs in this process. Furthermore, dexamethasone is known to attenuate HO-1 up-regulation in response to ROIs.[16] Another argument in favor of the participation of ROIs is based on the fact that volatile anesthetics can cause free radical release as a result of their interaction with tissues[17] and of their metabolization through cytochrome P450 2E1.[18] To investigate whether ROIs are essential for HO-1 induction by isoflurane, we administered *N*-acetyl-cysteine, a potent antioxidant. Despite the fact that the inhibitor was administered in a variety of dosages[19] that have been previously shown to inhibit HO-1 in response to other stimuli,[20] HO-1 up-regulation by isoflurane remained unaffected. Furthermore, activator protein 1 and nuclear factor κB, two redox-sensitive transcription factors,[16] did not show increased DNA binding in response to isoflurane treatment (data not shown). In a second step, cytochrome P450 2E1 was specifically inhibited by diethyldithiocarbamate.[21] This enzyme is predominantly responsible for the microsomal defluorination of volatile anesthetics.[21] Furthermore, this isoform is not only involved in increased ROI production, but, most interestingly, cytochrome P450 2E1 activity has previously been shown to induce HO-1.[22] However, our data demonstrate that diethyldithiocarbamate does not inhibit HO-1 up-regulation under these experimental conditions. Summed up, these data argue against a significant role of ROI production in the gene regulation of HO-1 by isoflurane.

A third major signal transduction pathway regulating the HO-1 gene involves PKC and PLA_2.[20,23] Both enzymes exist in Kupffer cells as well as in hepatocytes.[24–27] Although only a few studies provided evidence for an interaction between volatile anesthetics and PLA_2,[28,29] it was reported previously that volatile anesthetics exert some of their actions *via* PKC.[30–33] Therefore, we tested the hypothesis that isoflurane induces HO-1 *via* PKC, PLA_2, or both. The blockade of each enzyme prevented the isoflurane-mediated up-regulation of HO-1. These results are of particular interest.

First, it is well known that PKC is a key enzyme in late preconditioning and that chelerythrine, in the dosage we used, blocks this effect.[34–36] Therefore, one might speculate as to whether the induction of the protective enzyme HO-1 by isoflurane is part of a preconditioning effect of volatile anesthetics. In fact, it has already been postulated that heat stress–evoked HO-1 is involved in preconditioning, because a specific inhibitor of HO activity abrogated the protecting effect.[37]

Second, we report that both enzymes, PKC and PLA_2, are required for the up-regulation of HO-1. This might be due to the fact that they interact strongly with each other, *e.g.*, PKC mediates phosphorylation of PLA_2 and enhances its activity,[38] and PLA_2 and its products are substrates of PKC.[39] Furthermore, PLA_2 catalyzes the production of prostaglandins and arachidonic acid, and these products have been shown to up-regulate HO-1.[22,40] These data support the assumption that PKC and PLA_2 act together and are essential for the signal transduction leading to HO-1 induction in hepatocytes by isoflurane.

Third, these observations raise the question of whether the activation of PKC and PLA_2 that is responsible for the induction of HO-1 occurs in Kupffer cells or in hepatocytes. The following observations support the hypothesis that isoflurane does not directly act on PKC/PLA_2 in parenchymal cells. First, when Kupffer cell function was inhibited by gadolinium chloride, isoflurane did not induce HO-1 in hepatocytes. Second, we incubated HepG2 and Hepa 1–6 cells with isoflurane in various concentrations *in vitro*. However, none of the cell lines studied showed an up-regulation of HO-1 under these conditions (data not shown). Therefore, the sum of these results argues against a direct stimulation of PKC/PLA_2 by isoflurane in hepatocytes.

Fourth, to the best of our knowledge, it has never been reported that volatile anesthetics induce PKC/PLA_2 dependent pathways in the liver. Hence, this is the first demonstration that PKC and PLA_2 mediate biologic effects of volatile anesthetics in the liver. In contrast to the heart, where it has been shown that these enzymes mediate major protective effects,[32] our data provide evidence for the existence of a similar pathway in another major organ, *i.e.*, the liver. This may have implications for the development of new strategies for anesthetic preconditioning.

It is important to note that the pharmacologic agents used to inhibit PKC and PLA_2 may also affect the activity and function of other enzymes, such as cyclic nucleotide phosphodiesterases or flavoproteins.[41,42] Therefore, because of this potential limitation in specificity, we cannot exclude the possibility that, in addition to PKC and PLA_2, other signal transduction pathways may be involved in the induction of HO-1 by isoflurane.

In summary, our data demonstrate that the volatile

anesthetic isoflurane induces the HO-1 gene in the liver in a time-dependent fashion. This hepatic induction is restricted to hepatocytes in the pericentral region. Kupffer cell function is essential to mediate the HO-1 gene expression in parenchymal cells in response to this stimulus. Although neither nitric oxide nor ROIs seem to play a major role within the signal transduction pathway, PKC and PLA_2 are mandatory operators for the hepatic induction of the protective enzyme HO-1 by isoflurane.

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