Enflurane Metabolism Produces Covalently Bound Liver Adducts Recognized by Antibodies from Patients with Halothane Hepatitis

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The existence of a rare syndrome of "enflurane hepatitis" similar to that described for halothane and of a cross-sensitization between halothane and enflurane has been controversial, largely due to equivocal clinical case reports and a lack of a plausible molecular mechanism for the hepatotoxicity. The present study suggests a possible hypersensitivity basis for enflurane hepatitis and the apparent cross-sensitization between halothane and enflurane involving covalently bound liver microsomal adducts. Immunoblotting studies have revealed that antibodies in the sera of patients with halothane hepatitis recognize liver microsomal antigens of Mr = 100,000, or both 100,000 and 76,000, formed in rats treated with enflurane or halothane. These antigens were not detected in microsomes from isoflurane- or sesame oil-treated rats. The recognition of these antigens could be abolished by preincubation of the sera with microsomes from halothane-treated rats. These data suggest that the difluoromethoxydifluoracetyl halide metabolite of enflurane, as well as the trifluoroacetyl halide metabolite of halothane, covalently bind to similar hepatic proteins, and may become immunogens in susceptible patients. This mechanism may also account for the apparent cross-sensitization between halothane and enflurane anesthesia, and the development of hepatic necrosis. (Key words: Anesthetics, volatile: enflurane. Liver, hepatitis: enflurane. Immune response: immunotoxicity.)

There is considerable debate in the current literature regarding the potential hepatotoxicity associated with enflurane anesthesia.¹-³ Several clinical case reports have suggested a possible syndrome of idiosyncratic hepatic necrosis resulting from enflurane anesthesia,¹,³,9-12 analogous to that produced by halothane. Further clinical evidence for an "enflurane hepatitis" is presented by reports of a possible cross-sensitization between enflurane and halothane; that is, enflurane-induced hepatic necrosis in patients previously exposed to halothane.⁵,6,13-15 These reports led Lewis et al. to conclude that a syndrome of enflurane-associated hepatic necrosis, although rare, does exist.¹

This view has recently been challenged. Eger et al. reevaluated the data examined by Lewis et al. and additional reports, and concluded that the evidence does not support the existence of a syndrome of "enflurane hepatitis."⁹ One of the key arguments used by Eger et al. is that a molecular mechanism by which enflurane might produce hepatotoxicity analogous to that produced by halothane is difficult to envision and support experimentally.

Current evidence strongly suggests that halothane hepatitis may be an immune-mediated drug toxicity characterized by the presence of specific serum antibodies recognizing several liver microsomal proteins altered covalently by trifluoroacetyl halide, the oxidative metabolite of halothane.¹⁶-²¹ These antibodies are thought to play a role in the pathogenesis of the idiosyncratic hepatic necrosis produced by halothane, because they have been shown to mediate destruction of halothane-altered hepatocytes by normal lymphocytes in vitro,²² and are not found in patients experiencing the milder form of hepatic dysfunction after halothane,²³ in patients with hepatic necrosis resulting from viral hepatitis or direct hepatotoxins such as acetaminophen,²²,²⁴ or in patients anesthetized with halothane who developed liver damage attributable to other causes.²⁵

The metabolism of enflurane and isoflurane produces reactive intermediates which form covalently altered, acylated hepatic protein adducts similar to those formed by the metabolism of halothane.²⁶,²⁷ Although the hapten produced by enflurane is chemically distinct from the

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Hapten produced by halothane (fig. 1), the enflurane-induced adducts cross-react with specific hapten antibodies that recognize the covalently bound trifluoracetyl halide metabolite of halothane.

The present study demonstrates that antibodies found in the serum of patients with halothane-induced hepatic necrosis also recognize liver microsomal proteins covalently altered by the reactive acyl metabolite of enflurane, thus establishing a possible molecular mechanism by which enflurane might produce a syndrome of idiosyncratic hepatic necrosis in susceptible patients.

Materials and Methods

SERUM ANTIBODY SAMPLES

The sera used throughout these studies were collected by JGK while at the Liver Unit, Kings College Hospital, London. Sera from patients with halothane hepatitis contain antibodies recognizing specific halothane-induced liver antigens, as determined by immunoblotting and enzyme-linked immunosorbent assay techniques. Serum from a patient anesthetized with halothane but without hepatic dysfunction and serum from a patient with acetaminophen-induced hepatic necrosis were used as controls.

MICROSOMAL ANTIGEN PRODUCTION

To produce the anesthetic-induced antigens, pairs of male Sprague Dawley rats (100–200 g, Taconic Farms, Germantown, NY) were injected intraperitoneally with halothane (10 mmole/kg, Halocarbon Lab. Inc., Hackensack, NJ, distilled before use), enflurane (Ethane, Anaquest, Madison, WI, 21 mmole/kg) or isoflurane (Forane, Anaquest, 21 mmole/kg) dissolved in sesame oil. Five hours later, a second dose of enflurane (21 mmole/kg) or isoflurane (21 mmole/kg) was given, followed 7 h later by a third dose of enflurane or isoflurane (10 mmole/kg). Control animals received equivalent volumes of sesame oil. The rats were killed 3 h after the final dose (15 h after the initial dose), the livers in each group pooled, and hepatic microsomes prepared by differential centrifugation. Total microsomal protein was determined according to Lowry et al., using crystalline bovine serum albumin as the standard. All microsomal preparations were stored at −80°C until analyzed.

To investigate the possibility that clinical anesthesia with enflurane or halothane can produce covalently bound liver adducts, liver biopsy samples were obtained from three patients anesthetized with enflurane (2 men, 1 woman), and two patients anesthetized with halothane (2 women) undergoing staging exploratory laparotomies. This study was approved by the Institutional Clinical Research Committee of the National Heart, Lung, and Blood Institute, NIH, and all patients gave informed consent to participate. No patient had a history of drug hypersensitivity. All patients were given an opiate and sedative preoperatively. Intraoperatively, anesthesia was induced with sodium thiopental and paralysis was obtained with pancuronium bromide and succinylcholine. An initial liver wedge biopsy was obtained, while the anesthesia was maintained with a mixture of oxygen and nitrous oxide supplemented with an opiate and pancuronium bromide. After this sample was removed, enflurane or halothane was added to the maintenance anesthetic for an average of 1.26 MAC hours (1.26, 1.13, and 1.38) or 2.40 MAC hours (2.31 and 2.48), respectively. At the end of this period, a second wedge biopsy was removed. Microsomal fractions were prepared from each biopsy as described previously.

IMMUNOBLOTTING PROCEDURES

Proteins were separated according to their molecular weights from the complete microsomal fractions by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), carried out essentially according to Laemmli, as described previously. The separated proteins were then transferred electrophoretically to the surface of nitrocellulose paper, prior to incubation with the serum samples.

Immunoblotting procedures were identical to those reported previously. This technique utilizes a three-step detection process for visualizing specific antigen-antibody

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Fig. 2. Recognition of enflurane and halothane-induced antigens by serum antibodies from patients with halothane hepatitis. Immunoperoxidase staining with serum (1:50 dilution) from three patients with halothane hepatitis or serum from a patient anesthetized with halothane but without developing hepatic necrosis, of a SDS-PAGE blot (75 µg protein per lane) of pooled (N = 9) rat liver microsomes (prepared from rats treated ip with three doses of enflurane (E) or isoflurane (I) or sesame oil (S), or a single dose of halothane (H)). Three additional sera from patients with halothane hepatitis were tested and gave comparable results.

complexes. First, an appropriate serum (antibody 1) solution is allowed to react with the antigens bound to the surface of the nitrocellulose paper. After washing, a second antibody (antibody 2) that is covalently linked to an enzyme (i.e., horseradish peroxidase) and that will bind to the first antibody is added and allowed to react. Finally a chromogenic substrate for the antibody-bound enzyme is added, and a colored area will develop wherever the specific antigen-antibody(1)-antibody(2) bound-enzyme complex is found.

Briefly, after transfer, the nitrocellulose strip containing molecular weight standards was cut off and stained with amido black. The remainder of the blot was immersed in a 2.5% buffered casein solution for 3 h at room temperature, then 24-48 h at 4°C to block any non-specific reactive sites on the nitrocellulose. The 2.5% casein solution was decanted from the blots and replaced with a 1:50 dilution of human serum in 0.5% buffered casein. The blots were shaken slowly at room temperature for 4 h, the serum solution was decanted, and the blots washed to remove non-specifically bound antibodies as described previously. Goat anti-human, horseradish peroxidase conjugated IgG (Tago Immunodiagnostics, Burlingame, CA) diluted 1:100 in buffered 0.5% casein was added to the washed blots and the blots were incubated for 4 h at room temperature. The blots were again washed, and the antibody reactivity was visualized by the addition of the horseradish peroxidase substrate 4-chloro-1-naphthol.

The cross reactivity of the antibodies from patients with halothane hepatitis with enflurane-induced liver antigens was confirmed by pre-incubating the sera with liver microsomes from halothane- or sesame oil-treated rats prior to use in the immunoblotting assay. Serum samples were diluted 1:100 with buffered 0.5% casein containing the protease inhibitors 1 mM phenylmethylsulfonyl fluoride and 2 mM e-amino caproic acid. To first remove non-specific reactivity, hepatic microsomes from rats treated with sesame oil were added to the serum samples to give a final protein concentration of 1 mg/ml, and the suspension was mixed and allowed to stand at room temperature for 3-5 h. The mixture was centrifuged at 100,000 g for 1 h, the supernatant was decanted and microsomes from rats treated with either halothane or sesame oil were added to give a final protein concentration of 1 mg/ml, and the mixture was allowed to stand overnight at 4°C. The suspension was centrifuged again to pellet the microsomes and the supernatant was incubated with the transblotted microsomes.

**Results**

Antibodies in the serum of patients with halothane hepatitis that previously were found to recognize halothane-induced liver antigens recognized similar rat liver antigens produced by enflurane metabolism (Fig. 2). No antibody reactivity was detected when liver microsomes from isoflurane- or sesame oil-treated rats were tested. Control sera, from a patient anesthetized with halothane but without sustaining liver damage (Fig. 2) or from a patient with acetaminophen-induced hepatic necrosis (data not shown), did not contain antibodies recognizing anesthetic-induced liver microsomal adducts. Previous studies have demonstrated that identical patterns of antigen formation are produced in rats or rabbits given halothane by intraperitoneal injection (10 mmole/kg) or inhalation (1% in oxygen for 45 min), therefore establishing the validity of administering the inhalation anesthetics by intraperitoneal injection.

It was previously shown that halothane and enflurane metabolism produces a spectrum of haptenically labelled microsomal proteins ranging from Mr = 245,000 to less than 45,000. The six human sera tested in the present study recognized antigens of apparent Mr = 100,000, or both Mr = 100,000 and 76,000. Antibodies recognizing these antigens are most commonly found in patients with
The recognition of enfurane- and halothane-induced liver microsomal adducts by the serum antibodies could be abolished by preincubation of the serum with liver microsomes from halothane-treated rats prior to the immunoblotting (Fig. 3). In contrast, preincubation of the serum with liver microsomes from saline oil-treated rats had virtually no effect on the antibody recognition of the enfurane- and halothane-induced adducts (Fig. 3).

The formation of microsomal enfurane-or halothane-induced antigens in patients anesthetized with enfurane or halothane for approximately 2 h could not be demonstrated by immunoblotting 75 μg of microsomal protein and incubation with the serum from patients with halothane hepatitis used to probe the rat liver microsomes (data not shown). Other experiments utilizing immunoprecipitation techniques and more stringent antibody incubation conditions were also unsuccessful.

**Discussion**

Although the acyl hapten produced by the oxidative metabolism of enfurane and halothane are chemically distinct (Fig. 1), the liver protein adducts containing these hapten are similar enough to be recognized by serum antibodies from patients with halothane hepatitis (Fig. 2), and specific anti-trifluoroacetyl hapten antibodies.

These data suggest that enfurane-induced adducts might be potentially immunogenic, and thereby participate in the pathogenesis of enfurane-induced hepatic necrosis. The presence of serum antibodies against enfurane-induced liver adducts in patients with "enfurane hepatitis" remains to be determined.

The present study also suggests that a molecular mechanism involving covalently acylated liver proteins may be responsible for the apparent cross-sensitization to enfurane in patients previously given halothane, and the subsequent development of hepatic necrosis. A similar mechanism may explain the anecdotal reports of a cross-sensitization between halothane and methoxyflurane, because the oxidative metabolism of methoxyflurane is theoretically capable of forming a covalently bound hapten very similar to that formed by halothane and enfurane (Fig. 1).

The serum antibodies from patients with halothane hepatitis did not recognize isoflurane-induced liver adducts, although the metabolism of isoflurane does produce very small amounts of covalently bound liver adducts in rats. These data would support the cautious speculation that the immunogenic potential of isoflurane should be relatively low. As reviewed by Stoelting et al., the current literature does not indicate a clinical syndrome of isoflurane-induced hepatic dysfunction. It is interesting to note, however, that the recent report of hepatic necrosis in a patient anesthetized with isoflurane who had been anesthetized previously with halothane and enfurane.

The lack of detectable antigen formation in human liver after approximately 2 h of anesthesia with enfurane is not totally unexpected for two major reasons. First, the rate of enfurane metabolism is relatively slow, and appreciable adduct accumulation would likely occur after discontinuation of the anesthetic, as the drug is being eliminated. Hitt et al. estimated that most of the oxidative metabolism of enfurane occurs after the anesthetic is terminated, while White et al. demonstrated that, in the rat, metabolism accounted for a greater proportion of the elimination of subanesthetic levels of enfurane, a situation encountered during recovery from anesthesia. This time dependency is emphasized by animal studies demonstrating greater trifluoroacetylated liver adduct formation in rats sacrificed 12 h after a single dose of halothane, versus rats killed 4 h after the dose.

Secondly, although not measured directly, the estimated total dose of enfurane administered to the patients in this study was approximately 6 mmole/kg. This value agrees reasonably well with the total uptake of enfurane determined by Chase et al. for patients undergoing a similar clinical exposure. While the total enfurane dose administered to the rats was 52 mmole/kg, divided into three doses, patients could theoretically receive significantly higher total doses than 6 mmole/kg during clinical exposures longer than the 2 h used in the present study.
The levels of hepatic antigens might also be increased in patients exposed to agents that increase the oxidative metabolism of enflurane by cytochromes P450. Isoniazid and ethanol induce a specific isozyme of cytochromes P450,\textsuperscript{43,44} and have been shown to increase the rate of enflurane metabolism in rats.\textsuperscript{45-47} Indeed, Mazze et al. found increased rates of oxidative defluorination of enflurane in a subset of patients receiving isoniazid therapy.\textsuperscript{48} Experimentally produced diabetes is also associated with an increased rate of oxidative defluorination of enflurane,\textsuperscript{49} and induces the same cytochromes P450 isozyme as ethanol and isoniazid, possibly through the ketone body acetone.\textsuperscript{50} Whether this isozyme is found in human liver and is susceptible to the same inducers remains to be demonstrated.

As emphasized in the reviews of Lewis et al.,\textsuperscript{1} and Eger et al.,\textsuperscript{3} the evaluation of anecdotal clinical case reports of hepatic dysfunction after enflurane anesthesia is fraught with ambiguity. The provisional diagnosis of "enflurane hepatitis" or other anesthetic-induced hepatitis is a diagnosis of exclusion confounded by many clinical variables, such as the possibility of non-A non-B viral hepatitis, intraoperative portal hypotension and hypoxia, and the presence of multiple drugs.\textsuperscript{3,6} Data presented in this study, however, establish a plausible mechanism by which enflurane-induced hepatotoxicity could occur, and suggest a potential screening procedure for serum which could be used to confirm clinical diagnoses of "enflurane hepatitis."

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