Title Page:

A Mouse Model of Severe Halothane Hepatitis Based on Human Risk Factors

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Document Statistics:
Number of text pages: 19
Number of tables: 0
Number of figures: 12
Number of references: 42
Number of words in the abstract: 248
Number of words in the introduction: 791
Number of words in the discussion: 1663

List of non-standard abbreviations: ALT, alanine aminotransferase; BSA, bovine serum albumin; FITC, fluorescein isothiocyanate; HRP, horse radish peroxidase; IADRs, idiosyncratic adverse drug reactions; IgG, immunoglobulin G; LPS,
lipopolysaccharide; MOPS, 4-morpholinepropanesulfonic acid; PMN, polymorphonuclear leukocyte; SDS, sodium dodecyl sulfate; TNFα, tumor necrosis factor-alpha; TFA-, trifluoroacetyl-; TRIS, 2-amino-2-hydroxymethylpropane-1,3-diol; TBST, TRIS-buffered saline with 0.01% Tween-20.

Section Assignment: Toxicology
Abstract:
Halothane is an inhaled anesthetic that induces severe, idiosyncratic liver injury, ie, “halothane hepatitis,” in approximately 1 in 20,000 human patients. We employed known human risk factors (female sex, adult age, and genetics) as well as probable risk factors (fasting and inflammatory stress) to develop a murine model with characteristics of human halothane hepatitis. Female and male BALB/cJ mice treated with halothane developed dose-dependent liver injury within 24hrs; however, the liver injury was severe only in females. Livers had extensive centrilobular necrosis, inflammatory cell infiltrate and steatosis. Fasting rendered mice more sensitive to halothane hepatotoxicity, and 8 week-old female mice were more sensitive than males of the same age or than younger (4 week-old) females. C57BL/6 mice were insensitive to halothane, suggesting a strong genetic predisposition. In halothane-treated females, plasma concentration of tumor necrosis factor-alpha was greater than in males, and neutrophils were recruited to liver more rapidly and to a greater extent. AntiCD18 serum attenuated halothane-induced liver injury in female mice, suggesting that neutrophil migration and/or activation are required for injury. Coexposure of halothane-treated male mice to lipopolysaccharide to induce modest inflammatory stress converted their mild hepatotoxic response to a pronounced, female-like response. This is the first animal model of an idiosyncratic adverse drug reaction that is based on human risk factors and produces reproducible, severe hepatitis from halothane exposure with lesions characteristic of human halothane hepatitis. Moreover, these results suggest that a more robust innate immune response underlies the predisposition of female mice to halothane hepatitis.
**Introduction:**

Idiosyncratic adverse drug reactions (IADRs) occur in a minority of patients during drug therapy. They pose a unique public health problem because they cause severe illness and also often result in the withdrawal of otherwise useful drugs from the market. There are currently no predictive preclinical tests to identify drugs that have idiosyncratic potential, and the mechanisms of IADRs are poorly understood. Animal models that share the same sensitivity factors as humans and reproduce the liver lesions seen in people would be useful to study mechanisms of pathogenesis of IADRs and to develop strategies for therapy and prevention. The first step toward this effort must occur with drugs that are known to cause IADRs in people.

Halothane is an inhaled anesthetic that produces a mild and reversible liver injury in 1 in 5 patients (Trowell *et al.*, 1975). A more severe IADR, or “halothane hepatitis,” is observed in 1 in 6,000-22,000 patients who receive the drug (Mushin *et al.*, 1971). Although increasing numbers of reports of halothane-associated liver failure curtailed the use of halothane in the United States, it is still used in other countries, and cases of liver failure from halothane continue to be reported (Eghtesadi-Araghi *et al.*, 2008). The most prominent histologic feature seen in liver biopsies from patients who had halothane hepatitis is centrilobular necrosis (Blackburn *et al.*, 1964). Other findings include fatty degeneration, vacuolation, and inflammatory infiltrate. Risk factors for severe halothane hepatotoxicity are female sex, genetics, age, and multiple halothane exposures (Inman and Mushin, 1974; Cousins *et al.*, 1985). Other susceptibility factors,
such as fasting prior to anesthesia and exposure to inflammasens that accompanies surgery, are possible. For example, it has been reported that the plasma concentration of lipopolysaccharide (LPS) increases in patients at the initiation of several types of surgery (Berger et al., 1997).

Halothane is metabolized by cytochrome P450 2E1 in hepatocytes to form trifluoroacetyl-(TFA-) chloride, which binds covalently to proteins and lipids making TFA-adducts. Direct toxicity from TFA adducts is thought to cause the mild form of injury (Bourdi et al., 2001). The severe form of halothane hepatotoxicity is widely thought to result from an adaptive immune response to TFA-adducted or halothane-modified macromolecules. This is supported by the finding that repeated exposure is a risk factor and by the appearance of antibodies and immune complexes in the sera of some halothane-treated patients (Bird and Williams, 1989).

Despite its popularity, there exists clinical evidence that is incongruent with a strictly adaptive immune-mediated hypothesis. For example, a recent retrospective study demonstrated that 39% of halothane hepatitis patients had no previous history of halothane exposure (Eghtesadi-Araghi et al., 2008). Additionally, some people with antibodies in their serum did not develop liver injury (Walton et al., 1976b; Njoku et al., 2002). Attempts at developing animal models of liver damage using a halothane sensitization and challenge paradigm have resulted in a humoral immune response without associated liver pathology (Hastings et al., 1995). Pohl and Gillette noted in
1982 that after years of intensive investigation the adaptive immunity theory for halothane hepatitis was unproven (Pohl and Gillette, 1982). This remains true today.

It seems possible that halothane hepatitis occurs from a confluence of susceptibility factors, such as proposed by the “multiple determinant hypothesis,” in which an individual’s chance of developing an IADR is a product of several discreet probabilities of specific risk factors particular to the individual and the drug (Li, 2002). This product would be expected to be small, which would explain the rare frequency of IADRs. If the major risk factors could be incorporated into an animal model, it follows that liver injury would occur with high frequency. Animal models of halothane hepatotoxicity have been developed, but most reproduce the mild type of injury (Lind et al., 1990; Bourdi et al., 2001; You et al., 2006). Attempts to develop animal models of severe, halothane-induced hepatotoxicity have focused on using repeated exposures (Hastings et al., 1995), drug metabolizing enzyme inducers, glutathione depletion techniques, and/or hypoxic conditions (McLain et al., 1979; Lind et al., 1992). However, low incidence of responders and/or lack of severe hepatotoxicity limit the usefulness of these models. High incidence of severe halothane-induced liver injury (n=4) was reported in studies using female strain 2 guinea pigs (Lind et al., 1987), but few studies have followed the original report. Here we report a mouse model developed using risk factors seen in humans. The result is the first animal model of halothane hepatitis in which severe liver injury occurs reproducibly without extensive chemical manipulations and which demonstrates histopathologic findings consistent with those observed in humans. The results raise the possibility that human risk factors might be useful in
developing models of hepatotoxic IADRs and suggest that inflammation is a critical factor in the pathogenesis of halothane hepatitis.

Methods:

Materials: Halothane (2-bromo-2-chloro-1,1,1-trifluoro-ethane), highly refined, low acidity olive oil, sodium citrate, oil red O, and lipopolysaccharide (LPS) were purchased from Sigma-Aldrich (St. Louis, MO). Isoflurane (2-chloro-2-(difluoromethoxy)-1,1,1-trifluoro-ethane) was purchased from Abbott Laboratories (Chicago, IL). LPS from Escherichia coli O55:B5 (Lot 075K4038) with an activity of 3.3 x 10^6 endotoxin units (EU/mg) was used in these studies. Rabbit anti-CD18 antiserum against amino acids 89-100 was purchased from New England Peptide (Gardnew, MA). Alanine aminotransferase (ALT) reagent was purchased from Thermo Electron Corp. (Louisville, CO). Anti-TFA-adduct rabbit serum was generously donated by Dr. Lance Pohl. Fluorescein isothiocyanate (FITC) goat anti-rabbit immunoglobulin G (IgG) and horse radish peroxidase (HRP) goat anti-rabbit IgG secondary antibodies were purchased from Invitrogen (Eugene, OR) and Santa Cruz (Santa Cruz, CA), respectively. RIPA buffer and HALT protease inhibitor were purchased from Thermo Scientific (Santa Cruz, MA).

Animals: C57BL/6 or BALB/cJ mice were purchased from Jackson Laboratory (Bar Harbor, ME) and used at an age of 4, 8, or 10-12 weeks, with weights ranging from 10g to 27g. They were housed under conditions of controlled temperature and humidity and 12hr light/dark cycle. They were given continuous access to bottled spring water.
and fed a standard chow (Rodent Chow/Tek 2018, Harlan Teklad, Madison, WI) ad libitum. The mice were allowed to acclimate for 1 week prior to use. Unless otherwise stated, mice were fasted for 15hrs and injected with halothane between 10 AM and noon. Food was returned 30 min after halothane administration. All procedures were carried out according to the humane guidelines of the American Association for Laboratory Animal Science and the University Laboratory Animal Research Unit at Michigan State University.

**Experimental Protocol:** Unless otherwise stated, experiments were performed using BALB/cJ mice. Halothane was mixed with olive oil in a septum-covered glass vial at concentrations of 0.047M, 0.063M, 0.094M, 0.187M, 0.375M, 0.562M to deliver the respective doses of 3.75, 5, 7.5, 15, 30, 45mmol/kg. Isoflurane mixtures were prepared similarly. Mice were fasted overnight to mimic the duration humans are typically fasted prior to surgery. Mice were given halothane or olive oil, i.p. For polymorphonuclear leukocyte (PMN) functional inhibition, mice were treated with 100 ul of antiCD18 serum (1:1 in saline), normal rabbit serum (1:1 in saline), or saline i.v. 6 hrs after halothane administration. They were anesthetized with isoflurane and euthanized at various times after the administration of halothane. Blood was drawn from the vena cava into a syringe containing sodium citrate (final concentration of 0.76%) for preparation of plasma. The left lateral lobe of the liver was fixed in 10% neutral buffered formalin (Fisher Scientific, NJ) and then blocked in paraffin. The median lobe was snap frozen, and the caudal lobe was fixed in formalin, immersed in 20% sucrose, snap frozen in
Tissue-Tek OCT embedding media from VWR Labshop (Batavia, IL), and cryosectioned.

Male, BALB/cJ mice were treated with 5.0 \times 10^6 \text{ EU/kg LPS} (i.v.) 5.5hrs following halothane administration. The dose chosen for LPS was nonhepatotoxic but consistently produces increased plasma cytokine concentrations and hepatic PMN accumulation.

**Histopathology:** Paraffin-embedded, left lateral liver lobes were sectioned, stained with hematoxylin & eosin and examined by light microscopy. Snap frozen sections were sectioned and stained with Oil Red O (0.5% Oil Red O in isopropranol) according to a previously described method (Lillie and Ashburn, 1943).

**Immunohistochemistry and Microscopy:** For PMN identification, formalin-fixed liver sections were probed with anti-mouse PMN antibodies using the protocol described by Yee et al. (2003). Stained PMNs were counted in 4-5 random, 400X fields for each animal with a Nikon Eclipse E400 light microscope. For evaluation of TFA-adducted proteins, 8 um frozen liver sections from treated animals were probed with TFA-adduct antiserum. Using a fluorescent microscope, optical fields were captured as computer images. Five centrilobular and periportal regions were analyzed, and the percent of positive pixels in the centrilobular area was calculated and divided by the percent in the periportal region, in which fluorescent staining was minimal in all sections examined.
**Western Analysis:** Frozen liver samples were processed for whole cell protein isolation with RIPA buffer supplemented with HALT protease inhibitor according to the manufacturer’s directions. Twenty ug of protein were loaded onto Invitrogen NuPAGE 12%-TRIS gels (Carlsbad, CA) and electrophoresed in Invitrogen NuPAGE MOPS SDS Running Buffer at 200V for 1hr. Protein was transferred onto BioRad Immuno-blot PVDF membranes (Hercules, CA) using Invitrogen NuPAGE transfer buffer at 150 mAmp for 2hrs. The blot was blocked in 5% bovine serum albumin (BSA) for 1hr and hybridized with 1:10,000 TFA antiserum in 5% BSA overnight at 4°C. It was washed in TRIS-buffered saline with 0.01% Tween-20 (TBST) three times and hybridized with 1:5,000 goat anti-rabbit HRP in 5% BSA for 2hrs at room temperature. Following TBST wash, the proteins were detected using Amersham ECL Western Blotting Analysis System and Amersham Hyperfilm MP from GE Health Care (Uppsala, Sweden).

**Tumor necrosis factor-alpha (TNFα) Analysis:** The plasma concentration of TNFα was measured using a BD OpEIA mouse TNF ELISA kit (Cat. No 558534) purchased from BD Biosciences (San Diego, CA).

**Statistical Analysis:** Results are presented as mean +/- standard error of the mean. A Student’s t-test was performed on comparisons of two groups. For comparison of more than two groups, a 1-or 2- way ANOVA was used as appropriate after data normalization. The Student-Newman-Keuls test was performed to compare means in studies in which the ANOVA indicated statistical significance. The criterion for significance was p<0.05 for all studies.
Results:

Dose- and Sex-dependent Liver Injury in Halothane-treated Mice. Male and female BALB/cJ mice were treated with halothane, and hepatocellular injury was assessed 24hrs later from the activity of ALT in plasma and liver histopathology. Doses up to 15mmol/kg halothane proved to be subanesthetic, with mild ataxia evident at the 15mmol/kg dose. The 30mmol/kg dose induced a transient anesthesia for less than 30min. Resulting liver injury was dose-dependent in males and females (Figure 1A). Male mice developed relatively mild liver damage at doses up to 45mmol/kg. The response in females was greater than that in males and corresponded to severe hepatocellular injury, as plasma ALT activities approached 10,000U/L. Livers from female mice treated with 30mmol/kg halothane had severe lesions located primarily in centrilobular regions. Lesions were characterized by hepatocellular necrosis and inflammatory cell infiltrate (Figure 1C). Similarly treated males had milder liver lesions than females (Figure 1B).

Sensitivity to Halothane-Induced Liver Injury in Fasted Mice. To test whether fasting increases sensitivity to halothane-induced liver injury, female BALB/cJ mice were fasted for 15hrs before halothane administration, and plasma ALT activity was evaluated 24hrs later. In mice fed ad libitum, there was little or no hepatic injury at doses up to 15mmol/kg. In contrast, in fasted mice treated with 7.5 or 15mmol/kg, plasma ALT activity reached 4000U/L. Fasting shifted the dose-response curve to the left so that doses of 7.5mmol/kg or greater were hepatotoxic (Figure 2).
Genetic Background as a Sensitivity Factor for Halothane-induced Liver Injury.

Responses to halothane were compared in two inbred mouse strains, BALB/cJ and C57BL/6. As demonstrated in Figure 2, halothane caused dose-dependent hepatotoxicity in the BALB/cJ mice. In contrast, there were no significant increases in plasma ALT activity at any halothane dose up to 30mmol/kg in the C57BL/6 mice (Figure 3A). The extent of TFA-adduct formation in liver homogenates from halothane-treated C57BL/6 and BALB/cJ female mice was similar (Figure 3B).

In sensitivity to Isoflurane-induced Liver Injury. Isoflurane (1-chloro-2,2,2-trifluoroethyl difluoromethyl ether) is an inhaled anesthetic that is structurally similar to halothane but has less propensity than halothane to cause hepatotoxic IADRs in humans (Hussey et al., 1988). In female BALB/cJ mice, halothane exposure caused dose-dependent hepatotoxicity, whereas ALT activity was not increased at doses of isoflurane up to 30mmol/kg (Figure 4).

Age as a Sensitivity Factor for Halothane-Induced Liver Injury in BALB/cJ Mice.

Responses to halothane were compared in 4- and 8-week old, male and female BALB/cJ mice. After 15mmol/kg halothane exposure, plasma ALT activity was <2,000U/L in 8-week old males and 4-week old mice of either sex (Figure 5). Eight-week old female mice had plasma ALT activity of ~8,000U/L.
Further characterization of Sex Related Differences in Sensitivity in Halothane-induced Hepatotoxicity: Development of Halothane-induced Liver Injury in Male and Female BALB/cJ Mice. Having evaluated the ability of sex, fasting, genetic background and age to modulate the response to halothane, we investigated further the factors involved in the sex-related difference in sensitivity. 10-12 week old male and female mice demonstrated signs of transient (<30min), mild coordination loss following administration of 15mmol/kg halothane. In male mice, halothane treatment caused an increase in plasma ALT activity at 12 and 24hrs after administration, at which times the ALT activity was 124 and 600U/L, respectively (Figure 6). The injury in female mice followed a similar time course but was much more severe, reaching an ALT activity of nearly 7000U/L at these times.

There were no significant lesions in untreated female, mice, whereas halothane-treated females developed pronounced centrilobular necrosis within 12hrs (Figure 7). Immunohistochemical staining for PMNs revealed a greater number in livers of halothane-treated mice compared to control mice. Livers from halothane-treated mice also had greater Oil Red O staining at 6hrs compared to livers from untreated mice at the same time (Figure 7), indicating that halothane administration caused steatosis.

Halothane Bioactivation in Male and Female BALB/cJ Mice. A positive correlation has been reported between the severity of liver injury and the formation of TFA-adducts in livers of halothane-treated guinea pigs (Bourdi et al., 2001). As shown in Figure 8A, TFA-adducted proteins in the livers from halothane-treated male mice had a
centrilobular distribution. A similar distribution was observed in halothane-treated female mice (not shown). There was a similar degree of TFA-adduct formation in livers from halothane-treated male and female mice given the same dose of halothane as determined by immunohistochemistry (Figure 8B) or by western analysis of liver homogenates (Figure 8C). There were no TFA-adducts in the livers of vehicle-treated animals of either sex (Figure 8C).

Plasma TNFα Concentration in Halothane-treated Mice. In vehicle-treated mice or in halothane-treated (15mmol/kg) males, there was no change in plasma TNFα concentration at any time investigated. In contrast, plasma TNFα concentration was significantly increased 12 and 24hrs after halothane exposure in female mice (Figure 9).

Hepatic PMN Recruitment in Halothane-treated Mice. There was no change in the number of hepatic PMNs in vehicle-treated mice over time. PMN number was slightly elevated by 24hrs in livers of male mice given 15mmol/kg halothane. In contrast, there was a marked increase in hepatic PMNs in female mice at 12 and 24hrs after halothane administration. Hepatic PMN accumulation was significantly greater in female mice compared to male mice at 12 and 24hrs (Figure 10A). Interestingly, PMNs failed to accumulate in halothane-treated, C57BL/6 mice (Figure 10B), which were insensitive to halothane hepatotoxicity (see above).

Halothane-induced Severe Liver Injury in Female Mice Given Anti-CD18 Serum. In a mouse model of mild halothane-induced hepatotoxicity, rabbit antiserum depleted
PMNs and attenuated the increase in plasma ALT activity (You et al., 2006). CD18 is an integrin on leukocyte plasma membranes and is needed for adhesion to vascular endothelium and transmigration of PMNs into the liver parenchyma. Mice given CD18 antiserum in our model of severe halothane hepatitis had less hepatocellular injury compared to those treated with control serum or saline (Figure 11).

**Enhanced Sensitivity of Male Mice to Halothane upon LPS Coexposure.** The results above suggest that the greater sensitivity of female mice might be due to a more robust inflammatory response accompanying halothane exposure. Accordingly, we determined whether LPS-induced inflammation could increase the sensitivity of male mice to halothane hepatotoxicity. Treatment with vehicle or LPS alone was not hepatotoxic (Figure 12A). The animals treated with halothane alone had a small increase in ALT activity, whereas the LPS-cotreated animals had a much larger increase (Figure 12A). Livers from mice treated with LPS and halothane demonstrated severe centrilobular hepatocellular necrosis with marked PMN accumulation (Figures 12B & 12C).

**Discussion:**

Few animal models reproduce severe liver injury caused by drugs with human idiosyncratic potential. Most of the models have involved administration of an inflammagen that prompts the appearance of liver injury from drugs that cause hepatotoxic IADRs in humans (Deng et al., 2009). In other attempts at developing animal models, liver injury has been either modest (You et al., 2006) or nonexistent.
(Ong et al., 2007). For example, nevirapine produces idiosyncratic dermal and liver toxicity in humans; a rat model reproduced the skin lesions but failed to reproduce the liver toxicity (Shenton et al., 2003). Accordingly, it is noteworthy that the present model in mice based on human risk factors resulted in severe liver injury without the coadministration of other agents.

Although an adaptive immune response is commonly thought to be the mode of action of halothane hepatitis, not all human cases support a strictly adaptive-immune mechanism. For example, 4 out of 5 lymphocyte transformation tests and a leukocyte migration inhibition test failed to demonstrate evidence of cellular hypersensitivity for halothane hepatitis patients (Walton et al., 1976a). Antibodies to halothane-modified proteins form in humans (Bird and Williams, 1989); however, evidence linking antibodies to the pathogenesis of hepatitis is lacking (Pohl et al., 1988). Several retrospective clinical studies indicate that halothane hepatitis can occur on the first exposure to halothane (Walton et al., 1976b; Weber et al., 1994), with the the most recent study reporting that 39% of affected patients had no previous exposure (Eghtesadi-Araghi et al., 2008). Furthermore, multiple exposures was only a risk factor for halothane hepatitis when exposures occurred within 28 days (Inman and Mushin, 1974; Inman and Mushin, 1978). This timeframe supports a paradigm in which the first exposure renders the liver more sensitive to a second exposure; however, an adaptive immune response is not the only explanation for the results. For example, TFA-adducted proteins persisted in guinea pigs for at least 21 days after halothane exposure (Chen and Gandolfi, 1997). If the adducts formed in people have similar longevity, then
repeated exposure at intervals that overwhelm the regenerative capacity of the liver could produce cumulative damage. Moreover, the observations that patients who developed clinical signs of liver failure upon first exposure to halothane but not upon subsequent exposure cast doubt on a strictly adaptive immune-mediated mode that explains all instances of idiosyncratic halothane hepatitis (Walton et al., 1976b).

Data presented in Figure 1 demonstrate severe hepatotoxicity resulting from a single exposure of mice to halothane. All of halothane-treated mice developed liver injury at the largest dose administered. The lack of requirement for a priming exposure followed by reexposure indicates a mode of action other than adaptive immune-mediated pathogenesis in this model. Importantly, the histopathological findings in livers from halothane-treated, female mice were consistent with the changes in postmortem specimens from patients who died from halothane-induced liver failure (Blackburn et al., 1964; Kumar et al., 2005). Centrilobular necrosis, inflammatory cell infiltrate and steatosis, all hallmarks of human halothane hepatitis, were observed in livers of halothane-treated mice. Thus, this mouse model mimics to a substantial degree the histopathological features associated with idiosyncratic halothane hepatitis in human patients.

The present study extends the work of Ju and coworkers (You et al., 2006) by imposing several human risk factors to enable the expression of severe halothane hepatotoxicity. The magnitude of the response to halothane observed in this mouse model was much greater than the mild hepatotoxicity reported in a similar murine
model. For example, the 6000-10,000U/L plasma ALT activity reported here for female, BALB/cJ given 30mmol/kg halothane is much greater than the 1200U/L reported earlier.

The rarity and heterogeneous clinical presentation of many IADRs and the unknown mechanisms of pathogenesis are barriers to the development of adequate animal models. We considered the influence of several risk factors known to be important in people when developing an animal model of severe halothane hepatotoxicity in mice. Female sex is a risk factor for halothane hepatotoxicity with a 2:1 preponderance in women (Cousins et al., 1985). It was recently reported that females were the most at-risk group, representing 81.4% of patients who developed halothane hepatitis (Eghtesadi-Araghi et al., 2008). Female mice developed severe hepatitis at doses of halothane that produced only a mild response in male mice (Figure 1). The disparity in toxicity between males and females was not due to differences in bioactivation of halothane (Figure 8). The greater sensitivity of females has been reported previously for mice and guinea pigs (Lind et al., 1987; You et al., 2006).

Children are thought to be less susceptible to halothane hepatotoxicity, with an estimated incidence of 1-80,000 to -200,000 (Carney and Van Dyke, 1972; Warner et al., 1984). Four-week old female mice had a milder response to halothane than 8-week old, female mice (Figure 5). These results indicate that the human risk factors of female sex and mature age can be recapitulated in a mouse model of severe halothane hepatitis.
The occurrence of rare, halothane hepatitis among closely related family members and an increased frequency of the HLA-DR2 haplotype in patients with severe halothane hepatitis suggest a genetic predisposition (Hoft et al., 1981; Otsuka et al., 1985). The findings that guinea pigs sensitive to halothane produce offspring that are also sensitive to halothane (Lunam et al., 1986) and that there are strain-dependent variations in halothane metabolism in rats (Gourlay et al., 1981) suggest genetic determinants of halothane sensitivity in animals as well. C57BL/6 mice were insensitive to severe halothane hepatitis at all doses tested (Figure 4A), consistent with reports in which sensitivity to more modest liver injury was strain-dependent (Lind et al., 1987; You et al., 2006). The difference in sensitivity between C57BL/6 and BALB/cJ mice was not due to a difference in halothane bioactivation (Figure 4B). We are not aware of specific genetic differences between BALBc/J and C57BL/6 that may account for the difference in sensitivity; however, the results indicate that, as in humans, genetic differences are important in the hepatotoxic response to halothane in animals. Whether the genetic determinants that render BALB/cJ mice sensitive are the same as in susceptible humans is unknown.

Fasting has not been studied as a risk factor for human halothane hepatitis, yet patients are uniformly fasted prior to general anesthesia. Fasting mice before exposure to halothane increased their sensitivity to liver injury (Figure 2). Accordingly, consideration should be given to fasting as a potential contributor to risk for halothane hepatitis in humans. Fasting increases hepatic CYP2E1 expression and decreases hepatic glutathione stores in mice (Walker et al., 1982), and this may reduce protection
against reactive metabolites generated during oxidative metabolism of halothane. Treatment of guinea pigs with an inhibitor of glutathione biosynthesis increased covalent binding of reactive halothane intermediates and enhanced liver injury (Lind et al., 1992).

Halothane treatment resulted in elevated plasma TNFα concentration in female, but not male, mice (Figure 9). Moreover, in the halothane-treated, female mice hepatic PMN accumulation occurred to a greater extent than in males (Figure 10), and liver damage was attenuated by neutralization of CD18, an adhesion molecule required for transmigration of PMNs into the hepatic parenchyma. These results support the hypothesis that PMNs contribute to the pathogenesis of severe halothane hepatitis in mice. The findings also point to a sexually dimorphic inflammatory response to halothane, inasmuch as male mice responded to halothane with only modest liver injury, minimal hepatic accumulation of PMNs, and no increase in serum TNFα. Sexually dimorphic inflammatory responses have been reported in both humans and experimental animals (Wichmann et al., 1996; Marriott and Huet-Hudson, 2006). For example, female sex is a risk factor in alcohol-induced liver injury in people (Becker et al., 1997) as well as animals (Nanji et al., 2001), and alcohol consumption produces greater concentration of inflammatory mediators in female compared to male rats.

Interestingly, although male, BALB/cJ mice responded to halothane with only modest liver injury and inflammation, cotreatment with a nontoxic dose of LPS led to pronounced hepatotoxicity. Similar results have been observed by others in a hypoxia-halothane model in rats (Lind et al., 1984). LPS effects biological responses through
Toll-like receptor 4. A viral mimetic (PolyI:C) that stimulates Toll-like receptor 3 potentiated halothane hepatotoxicity in female mice (Cheng et al., 2009). These results suggest that inflammation might contribute to halothane hepatotoxicity and that the contribution of inflammatory stimuli might be mediated through at least Toll-like receptors 3 or 4. It is tempting to speculate that the modest hepatotoxic response to halothane alone in male mice is tantamount to the mild liver injury seen in 20% of patients, whereas the pronounced response seen in combination with LPS relates to the rarer, severe halothane hepatitis seen in humans.

In summary, to our knowledge this is the first animal model of idiosyncratic hepatotoxicity based on human risk factors for an IADR. The animal model of severe halothane hepatitis shares the human risk factors of female sex, mature age, and genetic predisposition and a possible contribution of fasting, which is imposed on all individuals prior to general anesthesia. The model in female, BALB/cJ mice captured important aspects of the pathology of human halothane hepatitis, including severe centrilobular necrosis, inflammatory infiltrate and steatosis. Importantly, the results suggest that an adaptive immune response requiring sensitization and challenge exposures to halothane is not needed to precipitate severe halothane hepatitis, since injury was produced in mice upon a single exposure to the drug. The greater sensitivity of female mice was associated with a more robust inflammatory response than occurred in males, and the importance of inflammatory stress in the hepatotoxicity was suggested by the ameliorative effect of CD18 neutralization. The ability of a nontoxic dose of LPS to convert a modestly hepatotoxic response in males to a robust, female-like response
further testifies to the importance of an inflammatory response in the expression of halothane hepatitis in this model. This animal model should inform our thinking about the mode(s) of action of human halothane hepatitis and may prove to be useful in the future study of mechanisms underlying IADRs from halothane and other drugs.
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Footnotes:

This work was supported by National Institute of Health [Grant GM075865]. CMD was supported by National Institute of Environmental Health Sciences [Grant T32 ES07255].
Legends for Figures:

Figure 1. Halothane-induced Hepatotoxicity in Male and Female Mice. A. Plasma ALT activity evaluated 24 hrs after halothane treatment (ip) of mice fed ad libitum (n = 3-6 per group). *significantly different from males given the same dose. # significantly different from 7.5 mmol/kg-treated sex-matched animals. B and C. H&E-stained liver sections from 30 mmol/kg halothane-treated male and female mice, respectively. Liver section from the male mouse shows minimal necrosis, whereas the lesion is more severe in the female mouse. Labeled in picture are central vein (CV) and portal triad (PT).

Figure 2. Fasting Enhances Sensitivity to Halothane-induced Liver Injury. Female mice were either fasted overnight or not and then given halothane at the doses indicated (n = 3-5 per group). Blood was collected 24 hrs later for the determination serum ALT activity. *significantly different from fed mice given the same dose. #significantly different from 3.75 mmol/kg group.

Figure 3. Genetic Background is a Sensitivity Factor for Severe Halothane Hepatotoxicity in Mice. Fasted female C57BL/6 and BALB/cJ mice were given halothane at the doses indicated (n = 3-5 per group), and blood was collected 24 hrs later. A. ALT activity in plasma. B. Immunoblot detection of TFA-protein adducts in liver homogenates from mice treated with 15mmol/kg halothane. *significantly different from BALB/cJ mice.
Figure 4. Lack of Isoflurane-induced Hepatotoxicity in Female Mice. Fasted, female BALB/cJ mice were given halothane or isoflurane at the doses indicated (n = 3-5 per group), and blood was collected at 12hrs for plasma ALT activity. All isoflurane-treated mice had plasma ALT activities < 50 U/L. *significantly different from the respective halothane-treated group.

Figure 5. Age is a Sensitivity Factor for Severe Halothane Hepatotoxicity in BALB/cJ Mice. Fasted 4- and 8-week old, male and female BALBc/J mice (n = 5 per group) were given 15 mmol/kg halothane, i.p., and blood was collected 24 hrs later. *significantly different from age-matched males. #significantly different from 4-week old females.

Figure 6. Development of Halothane-induced Liver Injury. Fasted female and male BALB/cJ mice were given vehicle or 15 mmol/kg halothane, i.p. (n = 4-6 per group). Blood was collected at various times for the determination of plasma ALT activity. There was no time- or sex-related difference in ALT activity in the vehicle-treated mice, so the results were combined and represented as zero-time. *significantly different from male mice at the same time. #significantly different from vehicle-treated animals (zero-time).

Figure 7. Histopathology of Livers from Halothane-treated Mice. Female BALB/cJ mice were fasted overnight and then given vehicle or halothane (15 mmol/kg, i.p.). Representative liver sections from mice treated 12 hrs earlier were examined after hematoxylin and eosin (H&E) staining or after immunohistochemical staining for PMNs,
in which PMNS appear as pink cells. Liver sections taken from mice 6hrs after halothane administration were stained with Oil Red O, in which lipid. Labeled in the picture are central vein (CV) and portal triad (PT). Photomicrographs were taken at 200X magnification.

**Figure 8. Halothane Metabolism is Similar in Male and Female Mice.** Fasted male and female BALB/c mice were treated with vehicle or 15 mmol/kg halothane, and liver samples were collected 12hrs later. A. Representative liver section from a halothane-treated, male mouse stained immunohistochemically for TFA-adducts and visualized with green color. DAPI nuclear stain appears blue. B. Ratio of positive pixels in the centrilobular and periportal regions (CV/PT)(n= 3 per group). C. Immunoblot detection of TFA-protein adducts in liver homogenates.

**Figure 9. Sex-specific Difference in Plasma TNFα Concentration after Halothane Exposure.** Female and male BALB/cJ mice were given vehicle or 15 mmol/kg halothane (n = 4-6 per group), and blood was collected at various times. Plasma TNFα concentration was determined using a BD optEIA Mouse ELISA. The average plasma concentration of TNFα in vehicle-treated animals was less than 40pg/ml.*significantly different from males at the same time. # significantly different from vehicle controls (zero-time).

**Figure 10. Hepatic PMN Recruitment is Sex- and Strain-Dependent.** Female and male BALB/cJ mice were given vehicle or 15 mmol/kg halothane (n = 4-5 per group)
and female BALB/cJ and C57BL/6 mice were given 30 mmol/kg halothane (n=4). The number of PMNs in liver sections was determined by counting PMNs in immunostained tissue. A. Hepatic PMNs in male and female BALB/cJ mice. The number of PMNs was < 8 for vehicle-treated mice and did not change with time, so those values were combined and represented as zero-time. *significantly different from halothane-treated males. #significantly different from vehicle controls (zero time). B. Hepatic PMNs in female, BALB/cJ and C57BL/6 mice. *significantly different from halothane-treated BALB/cJ mice.

Figure 11. CD18 Neutralization Attenuates Severe Halothane Hepatotoxicity. Fasted, female BALB/cJ mice were treated with 15 mmol/kg halothane (i.p.) and either saline, control rabbit serum (NRS) or anti-CD18 rabbit serum (CD18 RS) as described in Methods (i.v.). Plasma was collected 12 hrs after halothane administration and evaluated for ALT activity (n=6 per group). * significantly different from all other groups.

Figure 12. Inflammation Enhances Sensitivity to Halothane Hepatotoxicity in Male BALB/cJ Mice. Mice were treated with vehicle or halothane and then either 5x10^6 EU/kg LPS or saline vehicle 6 hrs later (n = 3-8 per group). Blood and liver samples were collected 24hrs after halothane administration. A. Plasma ALT activity. *significantly different from halothane-treated and LPS-treated animals. B. H&E section of liver from a representative halothane/LPS-cotreated mouse. C. Immunohistochemical staining for PMNs in a cotreated mouse; PMNs stain bright pink and nuclei stain blue. Both photomicrographs were taken at 200X magnification. Labeled in the pictures are central vein (CV) and portal triad (PT).
Figure 1
Figure 2
Figure 3
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Figure 9

A line graph showing the time course of TNFα (pg/mL) in males and females. The x-axis represents time in hours (0, 6, 12, 24), and the y-axis represents the concentration of TNFα in pg/mL. Males are represented by a dashed line, and females by a solid line. Significant differences are indicated by asterisks (*) and hashtags (#).
Figure 10
Figure 11
Figure 12
A Mouse Model of Severe Halothane Hepatitis Based on Human Risk factors

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Figure A. Mild halothane-induced liver injury. Female mice fed *ad libitum* were treated with halothane (15 mmol/kg; ip), and 24 hrs later liver injury was assessed. H&E-stained section from a mouse with plasma ALT activity of 1,038 U/L demonstrating mild liver damage with centrilobular loss of glycogen and few, scattered necrotic cells (arrows). Panel on left was taken at 100X; panel on right is a portion of the same section taken at 200X. CV, central vein; PT, portal triad.
Figure B. Severe halothane-induced liver injury. Female mice fed *ad libitum* were treated with halothane (30 mmol/kg; ip), and 24 hrs later liver injury was assessed. H&E-stained section from a mouse with plasma ALT activity of 11,663 U/L demonstrating large areas of bridging necrosis. Panel on left was taken at 100X; panel on right is a portion of the same section taken at 200X. CV, central vein; PT, portal triad.