Background: Methoxyflurane nephrotoxicity results from biotransformation; inorganic fluoride is a toxic metabolite. Concern exists about potential renal toxicity from volatile anesthetic defluorination, but many anesthetics increase fluoride concentrations without consequence. Methoxyflurane is metabolized by both dechlorination to methoxydifluoroacetic acid (MDFA, which may degrade to fluoride) and O-demethylation to fluoride and dichloroacetic acid. The metabolic pathway responsible for methoxyflurane nephrotoxicity has not, however, been identified, which was the aim of this investigation.

Methods: Experiments evaluated methoxyflurane metabolite formation and effects of enzyme induction or inhibition on methoxyflurane metabolism and toxicity. Rats pretreated with phenobarbital, barium sulfate, or nothing were anesthetized with methoxyflurane, and renal function and urine methoxyflurane metabolite excretion were assessed. Phenobarbital effects on MDFA metabolism and toxicity in vivo were also assessed. Metabolism of methoxyflurane and MDFA in microsomes from livers of pretreated rats was determined in vitro.

Results: Phenobarbital pretreatment increased methoxyflurane nephrotoxicity in vivo (increased diuresis and blood urea nitrogen and decreased urine osmolality) and induced in vitro hepatic microsomal methoxyflurane metabolism to inorganic fluoride (2-fold), dichloroacetic acid (1.5-fold), and MDFA (5-fold). In contrast, phenobarbital had no influence on MDFA renal effects in vivo or MDFA metabolism in vitro or in vivo. MDFA was neither metabolized to fluoride nor nephrotoxic. Barium sulfate diminished methoxyflurane metabolism and nephrotoxicity in vivo.

Conclusions: Fluoride from methoxyflurane anesthesia derives from O-demethylation. Phenobarbital increases in methoxyflurane toxicity do not seem attributable to methoxyflurane dechlorination, MDFA toxicity, or MDFA metabolism to another toxic metabolite, suggesting that nephrotoxicity is attributable to methoxyflurane O-demethylation. Fluoride, one of many metabolites from O-demethylation, may be toxic and/or reflect formation of a different toxic metabolite. These results may have implications for interpreting anesthetic defluorination, volatile anesthetic use, and methods to evaluate anesthetic toxicity.

Identification of the Nephrotoxic Metabolic Pathway


FOR nearly half a century, the potential for nephrotoxicity has influenced the pharmaceutical development and clinical use of volatile anesthetics. This concern is grounded in the experience with methoxyflurane, introduced in 1960, which caused dose-related subclinical or overt renal insufficiency in humans and animals. Toxicity was causally related to methoxyflurane metabolism and associated with increased plasma fluoride concentrations. In the intervening decades, every prospective new volatile anesthetic has been experimentally and clinically evaluated for its potential to undergo metabolism, increase plasma fluoride concentrations, and purportedly cause nephrotoxicity. Some candidates might have been rejected simply because they underwent defluorination. Others, such as sevoflurane, were delayed for years.

There is unambiguous evidence, based in part on the seminal contributions of Richard Mazze and others, that methoxyflurane nephrotoxicity is related to its metabolism. Methoxyflurane undergoes oxidative metabolism by cytochrome P450, with liberation of inorganic fluoride ion as a major metabolite. Soon after recognition of methoxyflurane nephrotoxicity, an association with increased plasma fluoride concentrations was noted, and it was suggested that fluoride was the causative nephrotoxin. In animals, increased plasma fluoride concentrations were associated with structural and functional renal abnormalities. Both plasma fluoride concentrations and nephrotoxicity increased as a function of methoxyflurane dose. In animals, induction of P450 enzymes by phenobarbital pretreatment increased methoxyflurane metabolism, accentuated the increase in plasma fluoride concentration, and worsened nephrotoxicity, whereas P450 inhibition decreased metabolism, plasma fluoride, and renal toxicity. Injection of animals with inorganic fluoride, albeit at very large doses, produced changes in renal function and morphology similar to those seen after methoxyflurane. Clinically, there were methoxyflurane dose-related abnormalities in renal function in surgical patients. In addition, there was an association with serum fluoride concentrations. No renal abnormalities were reported at peak fluoride concentrations of less than 40 μM, subclinical toxicity was accompanied by peak fluoride of 50–80 μM, and mild clinical toxicity was associated with peak fluoride of 90–120 μM, and overt nephrotoxicity was associated with peak fluoride concentrations of 80–175 μM.
Based on these associations, it was concluded that methoxyflurane nephrotoxicity was caused by inorganic fluoride. The once classically accepted “fluoride hypothesis,” subsequently generalized to volatile anesthetics in general (albeit without any evidence), was that anesthetics undergo hepatic defluorination, that inorganic fluoride is released and disseminated systemically and acts as a specific renal toxin, and that the threshold for renal toxicity is plasma fluoride concentrations exceeding 50 μM. Nevertheless, it is now clear that hepatic defluorination and systemic translocation of fluoride to the kidney is not the exclusive mechanism of anesthetic toxicity, that a generic plasma fluoride concentration of greater than 50 μM has no causal implications for renal toxicity, and that the 50-μM fluoride threshold for nephrotoxicity has been relegated to historical artifact.15–17 Numerous investigations have demonstrated the absence of renal toxicity with enflurane, isoflurane, and sevoflurane despite plasma fluoride concentrations exceeding 50 μM or even 100 μM, and neither the peak systemic fluoride concentration nor the duration of fluoride increase alone can be applied nonselectively to all anesthetics to explain or predict nephrotoxicity.15 Rather, anesthetic nephrotoxicity seems agent (i.e., methoxyflurane) specific, the role of fluoride in methoxyflurane toxicity is incompletely understood, and the mechanisms of methoxyflurane toxicity remain enigmatic.

Why, then, is methoxyflurane nephrotoxic, how is toxicity related to metabolism, and why was the toxicity associated with a plasma fluoride concentration of greater than 50 μM? And, given that methoxyflurane disappeared from clinical use decades ago, why are these questions important? One potential explanation for methoxyflurane nephrotoxicity is that intrarenal production of fluoride (or other toxins) may be the actual determinant of anesthetic toxicity. A previous investigation showed that human kidney microsomes substantially defluorinated methoxyflurane but not nonnephrotoxic anesthetics.15 Another (not necessarily exclusive) explanation is that a different methoxyflurane metabolite (unique to methoxyflurane) may be the actual toxin. If so, coformed inorganic fluoride would simply be a marker for formation of the actual nephrotoxin—an epiphenomenon. All of the above animal and human experimental and clinical observations could also be explained by a nephrotoxic methoxyflurane metabolite coformed with fluoride.

Methoxyflurane undergoes defluorination by two different routes (fig. 1).6,18 Oxidative dechlorination of the chloromethyl carbon yields 2,2-difluoro-2-methoxyacetic acid (which may degrade to fluoride). Oxidative O-demethylation of the methoxy group yields fluoride and dichloroacetic acid (thought in turn to yield oxalic acid). Both metabolic pathways may form inorganic fluoride. Nevertheless, the metabolic pathway that is responsible for methoxyflurane nephrotoxicity has never been identified.

Materials and Methods

Chemicals
Methoxyflurane was obtained from Abbott Laboratories (Abbott Park, IL). Identity and purity were confirmed by
gas chromatography-mass spectrometry (GC-MS). Dichloroacetic acid (DCAA), barium sulfate, and phenobarbital were purchased from Sigma Chemical Company (St. Louis, MO). Trifluoroacetic acid, Magtrieve, and benzophenone hydrazone were purchased from Aldrich (Milwaukee, WI). Chlorodifluoroacetic acid was purchased from Fluka (Milwaukee, WI). Methylene chloride and hydrochloric acid were purchased from Fisher Scientific (Pittsburgh, PA). Ethyl acetate (GC grade) was obtained from Burdick and Jackson (Muskegon, MI). Anhydrous ether was from J.T. Baker (Phillipsburg, NJ).

**Animals and Treatments**

All experiments were approved by the University of Washington Animal Care and Use Committee, Seattle, Washington, and conducted in accordance with American Association for Accreditation of Laboratory Animal Care guidelines. Male Fischer 344 rats (12 weeks old, 250–300 g) were purchased from Harlan (San Diego, CA). Rats were housed in individual metabolic cages, provided food and water ad libitum, and maintained on a 12-h light-dark cycle. They were allowed to acclimate to the metabolism cages for at least 48 h before any experimental treatment. Before any treatment, a baseline 24-h urine collection and blood sample via saphenous vein puncture were obtained (300 μl collected in capillary tubes containing EDTA; Sarstedt, Newton, NC). Blood was centrifuged and plasma stored at −80°C. All urine samples were also stored at −80°C.

To evaluate methoxyflurane metabolism and toxicity, animals were anesthetized for 3 h with 0.5% methoxyflurane (1.3 times the minimum alveolar concentration) in oxygen9,10,20 at 8 l/min via a calibrated methoxyflurane vaporizer, in a 204 inhalation chamber without the use of carbon dioxide absorbent. Animals rested on a warming pad, with constant monitoring to maintain rectal temperature at 37°C. Chamber gas concentrations were monitored constantly to maintain the desired methoxyflurane concentration and minimize any carbon dioxide rebreathing (Capnomac Ultima; Datex, Madison, WI). Two methods were used to monitor methoxyflurane concentrations. Real-time monitoring was via the sevoflurane channel on the Capnomac, which was calibrated using methoxyflurane standards whose concentration was determined by GC-MS, as described in Analytical Methods, below. For example, 0.5% methoxyflurane yielded a Capnomac monitor reading of “0.65% sevoflurane.” In addition, gas samples (100 μl) were removed from the chamber every 30 min using a gastight syringe and injected into a sealed headspace vial, and concentrations were determined by GC-MS. After recovery from anesthesia, animals were returned to the metabolic cages, and blood and 24-h urine samples were obtained daily for 4 days. Blood was obtained from saphenous and tail veins on alternate days. Blood was centrifuged and plasma stored at −20°C until analyzed. Urine was stored at −80°C until analyzed. On the fifth day, rats were killed, the kidneys were excised, a transverse section of the kidney was preserved in 10% formalin for histologic analysis, and the remainder was flash frozen in liquid nitrogen and stored at −80°C.

Experiments were conducted to determine the effect of enzyme induction or inhibition on methoxyflurane metabolism and renal toxicity. To determine the effect of phenobarbital, rats (n = 5) received phenobarbital (80 mg/kg via intraperitoneal injection in 25 mg/ml saline) for 4 days. Controls (n = 4) received saline only (2 ml/kg) for 4 days. One day after the last dose of phenobarbital, all rats were anesthetized with methoxyflurane as described in Animals and Treatments. To test the hypothesis that intrarenal metabolism mediates methoxyflurane nephrotoxicity, an experiment was attempted to manipulate selectively renal P450 activity and metabolism, without altering hepatic metabolism or systemic fluoride concentrations. Barium sulfate was reported to significantly increase renal cytochrome P4502E1 activity (a major P450 isoform responsible for methoxyflurane metabolism21) and decrease total hepatic P450 content.22 Therefore, the effect of barium sulfate on methoxyflurane metabolism and nephrotoxicity was evaluated. Rats received barium sulfate (4 kg/g via intraperitoneal injection in 0.5 g/ml saline; n = 10) or saline (8 ml/kg; n = 8) 48 h before methoxyflurane exposure.22

To determine the effect of enzyme activity on MDFA metabolism and toxicity, rats were randomly assigned to pretreatment with phenobarbital (0.1% in drinking water for 10 days before MDFA dosing and thereafter) or nothing (n = 4). The dose regimen for MDFA was based on preliminary experiments with inhaled methoxyflurane, showing the greatest metabolite excretion on day 1 or 2, an average of half that excretion on subsequent days, and 20–100 μmol MDFA excreted per day (vide infra, and other results not shown). Because the effect of phenobarbital on MDFA metabolism and toxicity was unknown, as was the extent to which MDFA excretion reflected MDFA formation, half of the excreted dose was injected. All animals received MDFA (0.225 mmol/kg via intraperitoneal injection in saline, followed by 0.112 mmol/kg daily for 3 days). Daily blood and urine samples were obtained as described above.

**In Vitro Metabolism**

Livers were obtained from rats treated with phenobarbital (0.1% in drinking water for 10 days), barium sulfate (4 kg/g via intraperitoneal injection 48 h before death), or saline, perfused with ice-cold phosphate-buffered saline (pH 7.4), and immediately frozen and stored at

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−80°C until used to prepare microsomes, as described previously.15 Incubations (1 ml) contained 3 mg liver or kidney microsomal protein in 50 mM Tris-HCl buffer (pH 7.4), and 2 μl methoxyflurane and nicotinamide adenine dinucleotide phosphate, reduced (final concentration 2 mM), were added to start the reaction, as described previously.15 Reactions were terminated after 30 min by flash freezing with liquid nitrogen. Incubations with MDFA (0.5 mM) were performed in a similar manner. Concentrations of fluoride, MDFA, and DCAA in the quenched reactions were determined as described below, in Analytical Methods.

Analytical Methods

Concentrations of fluoride in urine, blood urea nitrogen (BUN), and urine osmolality were determined as described previously.23 Concentrations of fluoride in microsomal incubations were determined by a fluoride-specific electrode, using standard curves of known fluoride standards and microsomes, as described previously.15 Plasma creatinine concentrations were measured using Vitros Crea slides on a Vitros 250 chemistry system (Ortho-Clinical Diagnostics, Rochester, NY). Kidneys were fixed in 10% neutral-buffered formalin and processed, and histopathologic analysis was performed by a veterinary pathologist who was blinded to animal treatments as described previously.23 The semiquantitative severity score ranged from 0 to 4 (normal, minimal, slight, moderate, and marked, respectively) to reflect the degree and distribution of any tubular necrosis.

Methoxyflurane concentrations in the inhalation chamber were determined by GC-MS using an Agilent (Palo Alto, CA) 6890-5973 with 7694 headspace autosampler containing a DB-VRX column (30 m × 0.32 mm × 1.8 μm film thickness; J&W Scientific, Folsom, CA). The headspace sample oven, sample valve, and headspace transfer line were 50°, 60° and 70°C, respectively. The parameters for the headspace for sampling were as follows: agitation = high; sample equilibration time = 1.0 min; vial pressurization time = 0.1 min; vial pressure = 18 psi; loop fill time = 0.25 min; loop equilibration time = 0.15 min; sample inject time = 0.25 min; and oven stabilization time = 1.0 min. The gas chromatograph was operated in split mode (split ratio 5:1) with the split flow at 10 ml/min and the total flow at 15 ml/min. The inlet and transfer line temperatures were 150° and 250°C, respectively. The helium carrier gas was delivered at a constant flow of 1.3 ml/min. Samples (2 μl) were injected using an Agilent 7983 autosampler. The injector and transfer line temperatures were 250° and 300°C, respectively. The oven was held at 50°C for 1 min, increased at 25°C/min to 250°C, held for 1 min, increased at 35°C/min to 300°C, and held for 6 min. Analytes were detected using selected-ion monitoring for MDFA (m/z 292, 11.2 min), DCAA (m/z 294, 12.5 min), trifluoroacetic acid (m/z 280, 8.7 min), and chlorodifluoroacetic acid (m/z 296, 10.3 min) and quantified using standard curves of peak area ratios (MDFA/trifluoroacetic acid and DCAA/chlorodifluoroacetic acid) versus analyte concentration. Calibration curves were obtained by analyzing blank rat urine containing MDFA and DCAA at 100, 250, 500, 750, 1,000, 2,500, and 5,000 μg/ml. Quality control samples (250, 750, 2,500 μg/ml) of MDFA and DCAA were also analyzed. Coefficients of variation were 6–9% for both analytes.

Statistical Analysis

Data were analyzed using two-way repeated-measures analysis of variance (time and treatment), followed by the Student-Newman-Keuls test for individual comparisons (within group days 1–4 vs. baseline, and between treatments), using SigmaStat (Systat, Point Richmond, CA). All results are reported as mean ± SD. Statistical significance was assigned at P < 0.05.

Results

Experiments were conducted to determine the mechanism by which pretreatment with phenobarbital, an inducer of multiple hepatic and renal cytochrome P450s,24 increased methoxyflurane defluorination and nephrotoxicity in animals in previous investigations.11–13 Specifically tested was the hypothesis that MDFA and DCAA concentrations in urine and microsomal incubations were determined by GC-MS. Rat urine (unknown, calibration, or quality control samples, 20 μl) was added to 16 × 125-mm glass tubes, followed by the internal standards trifluoroacetic acid and chlorodifluoroacetic acid (100 μl of 4 mg/ml; 400 μg of each standard), and 250 μl of 0.1N HCl, and vortexed. Samples were twice extracted with 1 ml diethyl ether and vortexing, followed by centrifugation at 700g for 10 min. The organic layers were removed and combined, and the diphenyldiazomethane derivitizing agent (75 μl, prepared as described previously23) was added and vortexed. After 45 min, 20 μl methanol was added to quench the reaction. Samples were transferred into autosampler vials and sealed for GC-MS analysis, using the same instrument as for methoxyflurane analysis above, but equipped with a DB-5 fused-silica capillary column (30 m × 0.32 mm × 0.5 μm; J&W Scientific). The helium carrier gas was delivered at a constant flow of 1.3 ml/min. Samples (2 μl) were injected using an Agilent 7983 autosampler. The injector and transfer line temperatures were 250° and 300°C, respectively. The oven was held at 50°C for 1 min, increased at 25°C/min to 250°C, held for 1 min, increased at 35°C/min to 300°C, and held for 6 min. Analytes were detected using selected-ion monitoring for MDFA (m/z 292, 11.2 min), DCAA (m/z 294, 12.5 min), trifluoroacetic acid (m/z 280, 8.7 min), and chlorodifluoroacetic acid (m/z 296, 10.3 min) and quantified using standard curves of peak area ratios (MDFA/trifluoroacetic acid and DCAA/chlorodifluoroacetic acid) versus analyte concentration. Calibration curves were obtained by analyzing blank rat urine containing MDFA and DCAA at 100, 250, 500, 750, 1,000, 2,500, and 5,000 μg/ml. Quality control samples (250, 750, 2,500 μg/ml) of MDFA and DCAA were also analyzed. Coefficients of variation were 6–9% for both analytes.

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Phenobarbital would induce methoxyflurane toxicity and metabolism to MDFA and fluoride. Methoxyflurane alone caused weight loss (7% at 24 h vs. baseline, not shown), decreased urine osmolality, and minimal diuresis or change in BUN (fig. 2). There were also only minor changes in renal histology, with occasional mild swelling and vacuolization of focal clusters of proximal tubules noted in some animals. Phenobarbital induction enhanced methoxyflurane effects on renal function, including increased diuresis, BUN, and decreased urine osmolality (fig. 2). Baseline urine volumes in phenobarbital-treated rats were higher than in controls, as observed previously.\textsuperscript{11,12,20} Phenobarbital pretreatment further increased total 4-day urine output after methoxyflurane, from 38 \pm 7 to 55 \pm 5 ml (P < 0.05; fig. 2). Two of five phenobarbital-pretreated rats had modestly greater histologic injury than those treated with methoxyflurane alone (which showed little effect). This injury included widespread tubular swelling and vacuolation, tubular dilation, and rare, focal, tubular necrosis. There was, however, no statistical difference between groups (median score 0.2 vs. 0.5 in controls and phenobarbital-treated rats).

After methoxyflurane anesthesia, inorganic fluoride and MDFA were both readily excreted in urine, although no DCAA was detectable (fig. 3). Phenobarbital induction decreased urine MDFA excretion and had minimal effect on daily urine fluoride excretion. Total 0- to 96-h urine MDFA excretion was 130 \pm 17 versus 204 \pm 45 \mu mol in controls (P < 0.05), and total 0- to 96-h urine fluoride excretion was 122 \pm 7 versus 112 \pm 20 \mu mol in controls (not significant). No DCAA was detectable in the urine of control or phenobarbital-induced rats anesthetized with methoxyflurane.

Experiments were conducted to determine the effect of MDFA on renal function, and the influence of phenobarbital on MDFA metabolism and renal function. Specifically tested was the hypothesis that MDFA is nephrotoxic, or that phenobarbital would increase MDFA toxicity (and possibly metabolism). Administration of 0.225 mmol/kg MDFA followed by 0.112 mmol/kg on the next 3 days had no effect on animal weight (not shown), nor on BUN, urine volume, or urine osmolality (fig. 4). Higher doses of MDFA (as high as 1.9 mmol/kg, then 0.9 mmol/kg on the next 3 days) also had no significant effect on urine volume, urine osmolality, or BUN concentrations, and there was no histologic evidence of renal toxicity.\textsuperscript{25} Phenobarbital pretreatment did not alter the response to MDFA (fig. 4), and no renal histologic abnormalities were observed in rats treated with phenobarbital and MDFA. Total 4-day urine output after MDFA was 23 \pm 7 ml in the phenobarbital-treated group versus 23 \pm 7 in those receiving MDFA alone (not significant).

The pattern of metabolite excretion after MDFA administration is shown in figure 5. MDFA was excreted in urine, in small amounts. Fluoride was also detected in urine, albeit also in low amounts. DCAA was not detected in urine. After administration of approximately
140 μmol MDFA over 4 days, cumulative metabolite recovery in controls was 49 ± 6 μmol MDFA and 22 ± 2 μmol fluoride. Phenobarbital had negligible effect on MDFA metabolism or excretion (fig. 5). Cumulative metabolite recovery after phenobarbital pretreatment was 44 ± 6 μmol MDFA and 17 ± 3 μmol fluoride, which was not different from that in rats receiving MDFA alone.

To test the hypothesis that intrarenal metabolism mediates methoxyflurane nephrotoxicity, the effect of barium sulfate on methoxyflurane metabolism and nephrotoxicity was evaluated. Pretreatment with barium sulfate diminished the diuresis, decrease in urine osmolality, and increase in BUN caused by methoxyflurane (fig. 6). Total 4-day urine output was 20 ± 9 ml in the pretreated group versus 35 ± 9 ml in rats receiving methoxyflurane alone (P < 0.05). The pattern of normal to mild histologic change seen with methoxyflurane was not altered...
by pretreatment with barium sulfate. Barium sulfate pretreatment decreased methoxyflurane metabolism, evidenced by diminished urine MDFA and fluoride excretion (fig. 7). Total 0- to 96-h urine MDFA excretion was 114 ± 24 versus 252 ± 40 μmol in controls (P < 0.05), and total 0- to 96-h urine fluoride excretion was 60 ± 15 versus 98 ± 27 μmol in controls (P < 0.05). No DCAA was detectable in the urine of either control or barium sulfate-pretreated rats anesthetized with methoxyflurane.

Experiments were conducted to determine the effect of phenobarbital and barium sulfate pretreatment on the hepatic and renal metabolism of methoxyflurane and MDFA in vitro (table 1). Phenobarbital induction significantly increased hepatic microsomal methoxyflurane metabolism. In contrast, there was no evidence for...
MDFA metabolism by liver microsomes from control rats and no effect of phenobarbital induction (i.e., no metabolism), based on the formation of MDFA and fluoride. No DCAA formation from MDFA was observed. Barium sulfate pretreatment had no effect on renal microsomal methoxyflurane metabolism, and decreases in hepatic microsomal methoxyflurane metabolism were not statistically significant ($P < 0.08$ for MDFA).

**Discussion**

Methoxyflurane metabolism clearly results in nephrotoxicity. The two major routes of methoxyflurane metabolism, O-demethylation and dechlorination, yield inorganic fluoride and DCAA (further metabolized to glyoxylate and oxalic acid), and MDFA, respectively. MDFA can also yield inorganic fluoride, thought to result from either decomposition or further metabolism. Thus, both metabolic pathways may theoretically generate fluoride, whose formation is positively associated with nephrotoxicity. Nevertheless, the metabolic pathway responsible for methoxyflurane nephrotoxicity has never been identified. This investigation attempted to identify which pathway of methoxyflurane metabolism mediates nephrotoxicity in rats.

Phenobarbital pretreatment induced hepatic methoxyflurane metabolism in vitro to both inorganic fluoride and MDFA and increased methoxyflurane nephrotoxicity in vivo. In contrast, phenobarbital had no effect on MDFA metabolism in vitro, no effect on MDFA metabolism in vivo, and no influence on the renal effects of MDFA in vivo. Indeed, MDFA seemed not to undergo metabolism in vitro or in vivo, and had no effect on renal function under any condition. Together, these results suggest that phenobarbital effects on methoxyflurane nephrotoxicity are not attributable to induction of the dechlorination pathway, an increase in MDFA formation, toxicity of MDFA, or induction of MDFA metabolism to another toxic metabolite. Rather, these results support the hypothesis that phenobarbital effects on methoxyflurane nephrotoxicity are due to induction of the O-demethylation pathway.

What is the source and significance of increased inorganic fluoride after methoxyflurane? The relative lack of MDFA metabolism and defluorination in vitro and in vivo is consistent with previous observations showing only small amounts of fluoride formation after MDFA administration, which was attributed to nonenzymatic degradation of MDFA after excretion in urine. Thus, inorganic fluoride formation from methoxyflurane seems to arise primarily from the O-demethylation pathway, rather than the dechlorination pathway.

These results imply that well-described associations in vivo between increased (or decreased) methoxyflurane metabolism, increased (or decreased) plasma and urine fluoride concentrations, and increased (or decreased) methoxyflurane nephrotoxicity, in animals and humans, are attributable to an underlying association between renal toxicity and methoxyflurane O-demethylation. Although dechlorination was quantitatively greater than O-demethylation, in agreement with previous results, O-demethylation seems to be the toxic pathway.

Several metabolites arise from methoxyflurane O-demethylation, including the putative unstable compounds dichlorodifluoroethanol and dichloroacetyl fluoride (which may acylate tissue macromolecules) and the stable metabolites inorganic fluoride, DCAA, and its subsequent metabolites glyoxylate and oxalic acid. One or more of these metabolites may be implicated in me-
thoxyflurane nephrotoxicity. Inorganic fluoride can cause nephrotoxicity, albeit at high doses.\textsuperscript{9,10,12} For example, renal changes similar to those after methoxyflurane were replicated by injecting 100 µmol sodium fluoride, but the accompanying serum fluoride concentrations (400 µM) were relatively high.\textsuperscript{10} DCAA and other haloacids are renal mitochondrial toxins and can cause nephrotoxicity.\textsuperscript{27,28} Oxalic acid was previously discounted as the causative metabolite in methoxyflurane nephrotoxicity.\textsuperscript{8,12} It is clear, however, that coformation of inorganic fluoride along with, or from, these other metabolites implies that measured fluoride concentrations may reflect the formation of one or more of these other, potentially toxic, metabolites. The identity, however, remains unknown. The effects of other methoxyflurane metabolites, alone or in combination, is unknown. Identification of the nephrotoxic methoxyflurane metabolite(s) requires further investigation.

In addition to metabolite-specific considerations, there may be tissue-specific considerations. A previous investigation showed that human kidney microsomes substantially defluorinated methoxyflurane but not the non-nephrotoxic anesthetic sevoflurane.\textsuperscript{15} This suggested that an intrarenally generated toxic metabolite (fluoride or others) may contribute to methoxyflurane nephrotoxicity, and the relative paucity of renal defluorination of other volatile anesthetics may explain the absence of their nephrotoxicity, despite plasma fluoride concentrations which may exceed 50 µM.\textsuperscript{15}

The ideal experiment to test the hypothesis that intrarenal metabolism mediates methoxyflurane nephrotoxicity would selectively manipulate renal P450 activity and metabolism, without altering hepatic metabolism or systemic fluoride concentrations. Based on reports of selective induction of renal P450 and suppression of hepatic P450 by fibrates and particulate irritants,\textsuperscript{22,29,30} such an experiment was attempted. Preliminary studies with hepatic and renal microsomes from rats treated with ciprofibrate, clofibrate, celeste, or barium sulfate characterized their effects on methoxyflurane metabolism. Unfortunately, no regimen resulted in both renal induction and hepatic inhibition of methoxyflurane metabolism (not shown), although barium sulfate did result in mild down-regulation of hepatic microsomal methoxyflurane metabolism without a significant alteration in renal microsomal methoxyflurane metabolism. Therefore, barium sulfate effects on methoxyflurane metabolism and toxicity were evaluated \textit{in vivo}. Both dechlorination and O-demethylation were inhibited, and methoxyflurane toxicity was prevented. These results suggest that hepatic methoxyflurane metabolism does impact methoxyflurane nephrotoxicity, although they do not inform on the hypothesis regarding renal metabolism. Nevertheless, it is interesting to note that in a previous investigation, phenobarbital induction (and enzyme inhibition) markedly increased (and decreased) the concentration of fluoride, and presumably other methoxyflurane metabolites in kidneys, said to be consistent with enhanced (and inhibited) renal methoxyflurane O-demethylation.\textsuperscript{21}

Results of the current investigation can be compared with those reported previously. The identity of DCAA as the organic acid methoxyflurane metabolite in urine, established here using mass spectrometry, confirms the identification by nuclear magnetic resonance spectroscopy.\textsuperscript{16,19} Effects of methoxyflurane anesthesia, including weight loss, diuresis, and decrease in urine osmolality, were similar to those seen by Mazze \textit{et al.}\textsuperscript{9,10,12,20,32,33} The absence of necrosis under light microscopy with 0.5% methoxyflurane for 3 h is similar to that reported previously.\textsuperscript{20,34} The extent of methoxyflurane metabolism and toxicity (diuresis, osmolality) was, however, less than that reported previously. This seems related to age. We used male Fischer 344 rats (3 months old, 250–300 g), whereas Mazze \textit{et al.}\textsuperscript{9–12,20} used much older (6- to 12-month-old) rats. Older rats (12 months vs. 6 weeks) had greater methoxyflurane blood concentrations, greater methoxyflurane defluorination as evidenced by greater serum inorganic fluoride concentrations (37 ± 5 vs. 126 ± 25 µM) and urine fluoride excretion (0.1 vs. 0.3

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<th>Table 1. Effect of Phenobarbital and Barium Sulfate on the Hepatic Microsomal Metabolism of Methoxyflurane and MDFA</th>
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* Significantly different from untreated control (P < 0.05). † Residual methoxydifluoroacetic acid (MDFA) concentrations were not different between phenobarbital-treated and control animals. DCAA = dichloroacetic acid; ND = not determined.
Because methoxyflurane disappeared from clinical use decades ago, why are these findings important? They may have mechanistic and clinical implications. Methoxyflurane causes nephrotoxicity as a result of metabolism, and methoxyflurane toxicity is associated with plasma fluoride concentrations of greater than 50 μM, but other volatile anesthetics have little or no effect on renal function under conditions in which plasma fluoride concentrations exceed 50–100 μM. This remained previously unexplained. Methoxyflurane O-demethylation, the apparent pathway of toxification, generates inorganic fluoride as well as many other metabolites. If one of these other metabolites that are unique to methoxyflurane, alone or in concert with fluoride, mediates methoxyflurane toxicity, this may provide an apparent explanation for the selective nephrotoxicity of methoxyflurane but not other volatile anesthetics. If so, exclusive focus on plasma fluoride concentrations as a harbinger of renal toxicity would seem unfounded. Furthermore, with regard to anesthetic development, the use of in vitro assays that evaluate defluorination as the primary screen for potential in vivo toxicity merits reevaluation.

In summary, these results suggest that neither MDFA nor methoxyflurane metabolism to MDFA is nephrotoxic, and that phenobarbital exacerbatation of methoxyflurane nephrotoxicity in rats is not attributable to enhanced methoxyflurane dechlorination or increased MDFA formation. Results suggest that inorganic fluoride arises primarily from methoxyflurane O-demethylation, that phenobarbital exacerbatation of methoxyflurane nephrotoxicity is attributable to enhanced methoxyflurane O-demethylation, and that inhibition of methoxyflurane O-demethylation decreases nephrotoxicity. Together, these results suggest that O-demethylation is the mechanism of methoxyflurane nephrotoxicity. Because inorganic fluoride is one of many metabolites arising from O-demethylation, it may be responsible for methoxyflurane toxicity and/or simply reflecting the formation of a different toxic metabolite. These results may have implications for the interpretation of clinical anesthetic defluorination, use of volatile anesthetics, and the laboratory methods used to evaluate potential anesthetic toxicity.

References


