

Isoflurane Activates Intestinal Sphingosine Kinase to Protect against Renal Ischemia–Reperfusion-induced Liver and Intestine Injury

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ABSTRACT

Background: Renal ischemia–reperfusion injury (IRI) is a major cause of acute kidney injury and often leads to multiorgan dysfunction and systemic inflammation. Volatile anesthetics have potent antiinflammatory effects. We aimed to determine whether the representative volatile anesthetic isoflurane protects against acute kidney injury–induced liver and intestinal injury and to determine the mechanisms involved in this protection.

Methods: Mice were anesthetized with pentobarbital and subjected to 30 min of left renal ischemia after right nephrectomy, followed by exposure to 4 h of equianesthetic doses of pentobarbital or isoflurane. Five hours after renal IRI, plasma creatinine and alanine aminotransferase concentrations were measured. Liver and intestine tissues were analyzed for proinflammatory messenger RNA (mRNA) concentrations, histologic features, sphingosine kinase-1 (SK1) immunoblotting, SK1 activity, and sphingosine-1-phosphate concentrations.

Results: Renal IRI with pentobarbital led to severe renal, hepatic, and intestinal injury with focused periportal hepatocyte vacuolization; small-intestinal apoptosis; and proinflammatory mRNA up-regulation. Isoflurane protected against renal IRI and reduced hepatic and intestinal injury *via* induction of small-intestinal crypt SK1 mRNA, protein and enzyme activity, and increased sphingosine-1-phosphate. We confirmed the importance of SK1 because mice treated with a selective SK inhibitor or mice deficient in the

What We Already Know about This Topic

- Acute kidney injury results in a systemic inflammatory condition that injures other organs, including the intestine and liver
- Potent volatile anesthetics have antiinflammatory effects and protect against renal ischemia–reperfusion injury

What This Article Tells Us That Is New

- The volatile anesthetic isoflurane protects the intestine and liver after renal ischemia–reperfusion injury by attenuating proinflammatory cytokine up-regulation and intestinal apoptosis through induction of the sphingosine kinase-1/sphingosine-1-phosphate pathway

SK1 enzyme were not protected against hepatic and intestinal dysfunction with isoflurane.

Conclusions: Isoflurane protects against multiorgan injury after renal IRI *via* induction of the SK1/sphingosine-1-phosphate pathway. Our findings may help to unravel the cellular signaling pathways of volatile anesthetic-mediated hepatic and intestinal protection and may lead to new therapeutic applications of volatile anesthetics during the perioperative period.

ACUTE kidney injury (AKI) continues to be a major clinical problem during the perioperative period.¹ The development of AKI often leads to multiorgan dysfunction and systemic inflammation and contributes to significant morbidity and mortality. In particular, hepatic dysfunction occurs frequently in patients with AKI and leads to other complications, such as intestinal barrier dysfunction, respiratory failure, and multiorgan failure (frequently complicated by sepsis).² We recently demonstrated that AKI leads to rapid hepatic and intestinal injury in mice with increased inflammation, apoptosis, and necrosis.³ This hepatic and intestinal injury was mediated by up-regulation of the proinflammatory cytokines tumor necrosis factor (TNF)- α , interleukin (IL)-17A, and IL-6 after AKI.

Volatile anesthetics are an integral part of the perioperative period because they are administered to virtually all patients undergoing general anesthesia in the United States. It

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was previously reported that volatile anesthetics protected against renal ischemia–reperfusion injury (IRI) *in vivo*⁴ and had direct antiinflammatory and antinecrotic effects in cultured human kidney proximal tubule cells.⁵ Most volatile anesthetics are lipophilic molecules⁶ and have increased membrane fluidity and activated sphingomyelin hydrolysis in the renal cortex.⁷ The lysophospholipid sphingosine-1-phosphate (S1P) is a product of sphingomyelin hydrolysis and functions as both an extracellular ligand for specific G protein–coupled receptors and an intracellular second messenger in promoting cell growth and survival and the inhibition of apoptosis.⁸

In this study, we questioned whether volatile anesthetics would provide protection of the liver and intestine after renal IRI. We hypothesized that volatile anesthetics activate the sphingosine kinase-1 (SK1)/S1P pathway in the small intestine to protect against renal IRI-induced hepatic and intestinal injury. We demonstrate rapid hepatic and intestinal injury after renal IRI, with protective effects mediated by the representative volatile anesthetic, isoflurane. Isoflurane reduced hepatic and intestinal proinflammatory cytokine upregulation and intestinal apoptosis *via* induction of SK1 in small-intestinal crypts.

Materials and Methods

Materials

Isoflurane (2-chloro-2-[difluoromethoxy]-1,1,1-trifluoro-ethane) was purchased from Abbott Laboratories, North Chicago, IL; and the selective SK inhibitor (SKI-II; 4-[4-{4-chlorophenyl}-2-thiazolyl]amino phenol) was purchased from Tocris Bioscience, Ellisville, MO. Unless otherwise specified, all other reagents were purchased from Sigma, St Louis, MO.

Murine Model of Renal IRI

All animal protocols were approved by the Institutional Animal Care and Use Committee of Columbia University, New York, NY. We used male C57BL/6 mice (Harlan, Indianapolis, IN) or SK1 knockout mice (provided by R. L. Proia, Ph.D., Chief, Genetics of Development and Disease Branch, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, MD); all mice weighed 20–25 g. The generation and initial characterization of SK1 knockout mice was previously described.⁹ These mice were backcrossed to C57BL/6 mice for more than 10 generations.

In our model of renal IRI, mice were initially anesthetized with intraperitoneal pentobarbital, 50 mg/kg body weight or to effect (Henry Schein Veterinary Co, Indianapolis, IN); and subjected to right nephrectomy and 30 min of left renal ischemia. After closure of the abdomen in two layers, the mice were then exposed to an additional 4 h of equipotent doses of either pentobarbital *via* intermittent intraperitoneal administration or isoflurane (1.2% or minimum alveolar concentration, defined as the concentration of volatile anes-

thetic in the lungs that is needed to prevent movement in 50% of subjects in response to a painful stimulus), as previously described.⁴ Briefly, mice were placed in an airtight 10-l chamber on a warming blanket with inflow and outflow hoses located at the top and bottom of the chamber, respectively. Isoflurane was delivered in room air at 5 l/min using an isoflurane-specific vaporizer (Datex-Ohmeda, Madison, WI). The vaporizer was set to maintain chamber isoflurane concentration at 1.2%, monitored by an infrared analyzer sampling gas at the outflow hose. The mice were placed on a heating pad under a warming light to maintain body temperature at approximately 37°C.

To test the effects of SK inhibition, SKI-II was administered to mice undergoing renal IRI. SKI-II (50 mg/kg) was administered subcutaneously 15 min before ischemia and 4 h after reperfusion. SKI-II is an SK-selective inhibitor with minimal effects on other kinases,¹⁰ and this dose had effective inhibition of activity without significant toxicity.¹¹

For experiments involving a sham operation or renal IRI, all samples (including plasma and tissue) were collected from mice 24 h after a sham operation or renal IRI. For experiments involving sham exposure to pentobarbital or isoflurane (SK messenger RNA [mRNA] and protein expression, SK1 immunofluorescence, SK activity, and S1P measurement), mice were exposed to 4 h of pentobarbital or isoflurane (without a sham operation or renal IRI) and allowed to recover for 1 h. Samples were collected 5 h after initial anesthetic exposure.

Plasma Alanine Aminotransferase Activity, Creatinine Concentration, and Histologic Analysis of Liver and Small-Intestine Injury

Plasma alanine aminotransferase (ALT) activity and creatinine concentrations were measured using an ALT assay kit (Infinity) and an enzymatic creatinine reagent kit, respectively, according to the manufacturer's instructions (Thermo Fisher Scientific, Waltham, MA).

For histologic preparations, liver or small intestine (jejunum and ileum) tissues collected from mice were washed in ice-cold phosphate-buffered saline and fixed overnight in 10% formalin. After automated dehydration through a graded alcohol series, tissues were embedded in paraffin, sectioned at 5 μ m, and stained with hematoxylin-eosin. Liver hematoxylin-eosin sections were graded for renal IRI-induced vacuolization injury (score range, 0–4) by a pathologist (V.D.D.'A.) blinded to the samples.

Detection of Small-Intestinal Apoptosis after Renal IRI, Assessment of Liver and Small-Intestine Inflammation and SK Expression, and Vascular Permeability of Liver and Small Intestine after Renal IRI

We detected small-intestinal apoptosis with terminal deoxynucleotidyl transferase biotin-dUTP nick end-labeling (TUNEL) staining. *In situ* labeling of fragmented DNA was performed with TUNEL staining (green fluorescence) using a commer-

Table 1. Primers Used to Amplify mRNAs Encoding SK1, SK2, GAPDH, and Proinflammatory Cytokines Based on Published Genbank Sequences for Mice

Primers in Mice	Accession No.	Sequence (Sense/Antisense)	RT-PCR Product Size, bp	PCR Cycle No.	Annealing Temperature, °C
SK1	NM_011451 (variant 1)	5'-GATGCATGAGGTGGTGAATG-3'	337	22	64
	NM_025367 (variant 2)	5'-GCCCACTGTGAAACGAATC-3'			
SK2	NM_203280 (variant 1)	5'-ACTGCTCGCTTCTTCTCTGC-3'	437	23	68
	NM_020011 (variant 2)	5'-ACCATTGAGGGACAGGTCAG-3'			
MIP-2	X53798	5'-CCAAGGGTTGACTTCAAGAAC-3'	282	28	60
		5'-AGCGAGGCACATCAGGTACG-3'			
ICAM-1	X52264	5'-TGTTTCCTGCCTCTGAAGC-3'	409	21	60
		5'-CTTCGTTTGTGATCCTCCG-3'			
TNF- α	X02611	5'-TACTGAACTTCGGGGTGATTGGTCC-3'	290	24	65
		5'-CAGCCTTGTCCTTGAAGAGAACC-3'			
MCP-1	NM_011333	5'-ACCTGCTGCTACTCATTAC-3'	312	22	60
IL-6	NM_031168	5'-TTGAGGTGGTTGTGGAAAAG-3'	421	30	62
		5'-CCGAGAGGAGACTTCACAG-3'			
IL-17	NM_010552	5'-GGAAATTGGGGTAGGAAGGA-3'	248	32	66
		5'-TCCAGAAGGCCCTCAGACTA-3'			
GAPDH	M32599	5'-ACACCCACCAGCATCTTCTC-3'	450	15	65
		5'-ACCACAGTCCATGCCATCAC-3'			
		5'-CACCACCCTGTTGCTGTAGCC-3'			

bp = base pair; GAPDH = glyceraldehyde 3-phosphate dehydrogenase; ICAM = intercellular adhesion molecule; IL = interleukin; MCP = monocyte chemoattractant protein; MIP = macrophage inflammatory protein; RT-PCR = reverse transcription-polymerase chain reaction; SK = sphingosine kinase; TNF = tumor necrosis factor.

cially available *in situ* cell death detection kit (Roche, Indianapolis, IN), according to the instructions provided by the manufacturer.

Hepatic and intestinal inflammation was determined by measuring mRNA-encoding markers of inflammation, including IL-6, IL-17A, intercellular adhesion molecule (ICAM)-1, monocyte chemoattractant protein (MCP)-1, macrophage inflammatory protein (MIP)-2, and TNF- α (table 1). In addition, SK1 and SK2 mRNA levels were measured. Semiquantitative real-time reverse transcription-polymerase chain reaction was performed as previously described.¹²

Changes in liver and small intestine vascular permeability were assessed by quantitating extravasation of Evans blue dye into the tissue, as previously described.¹³

Immunoblotting Analyses of the Small Intestine

Small-intestinal tissues in mice were collected and homogenized in lysis buffer (20 mM HEPES, pH 7.4; 2 mM EGTA; 1 mM dithiothreitol; 1% Triton X-100; 10% glycerol; and protease inhibitor cocktail) (Calbiochem, La Jolla, CA) on ice with a glass homogenizer. The homogenates were centrifuged for 20 min at 16,000g. The supernatant was collected and used for immunoblotting as previously described.¹⁴ The samples (50–100 μ g protein/lane) were separated on 9 or 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then transferred to membranes (Immobilon; Millipore, Bedford, MA). The membranes were blocked with blocking buffer (5% nonfat dry milk in tris buffered saline (TBS) containing 0.1% Tween 20) and incubated overnight with polyclonal anti-SK1 (3297; 1:1,000 dilution;

Cell Signaling, Beverly, MA), anti-SK2 (ab37977; 1:2,000 dilution; Abcam, Cambridge, MA), or monoclonal anti- β -actin (A5316; 1:5,000 dilution; Sigma) antibodies diluted in blocking buffer at 4°C. After being washed, the membranes were incubated with horseradish peroxidase-conjugated donkey anti-rabbit or sheep antimouse (1:5,000 dilution; ECM Bioscience, Versailles, KY) antibodies for 1 h at room temperature. Finally, the membranes were detected with enhanced chemiluminescence immunoblotting detection reagents (Amersham, Piscataway, NJ) with subsequent exposure to a charge-coupled device camera coupled to an imaging device (UVP Bio-imaging System; UVP, Upland, CA). The band intensities of the immunoblots were within the linear range of exposure for all experiments.

Immunofluorescence Staining for SK1 in the Small Intestine

Immunofluorescence to detect SK1 was performed as previously described.¹⁵ Small-intestinal tissues in mice were collected and embedded in oxytetracycline compound and frozen, and cryosections were incubated with anti-SK1 antibody (AP7237; Abgent, San Diego, CA) overnight at 4°C. After washing with phosphate-buffered saline, the sections were incubated with Texas red-conjugated goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA) for 1 h at room temperature. For nuclear staining, 4',6-diamidino-2-phenylindole (blue) was placed on sections for 1 min. The sections were mounted with antifade reagent (Prolong Gold; Invitrogen, Carlsbad, CA) and observed under a fluorescence microscope (model IX81; Olympus, Center Valley, PA).

In Vivo Intestine Enzyme Preparation from Mice, SK Activity Assay, and High-Pressure Liquid Chromatography Detection of S1P and Protein Determination

Small intestines were collected and homogenized mechanically in cell lysate buffer (100 mM sucrose; 1 mM EGTA; 20 mM 4-morpholinepropanesulfonic acid (MOPS), pH 7.4; 5% Percoll; 0.01% digitonin; and protease [Calbiochem] and phosphatase inhibitors) on ice. After a 1,000 *g* spin for 15 min to pellet cellular debris, protein concentrations were determined. SK activity was measured as described by Vessey *et al.*¹⁶ using 20 μ g protein, with some modifications, as previously described.¹⁷

S1P concentrations were measured in the small intestines of mice using high-performance liquid chromatography, as previously described.¹⁸

Protein content was determined with the bicinchoninic acid protein assay reagent (Thermo Scientific), with bovine serum albumin as a standard.

Statistical Analysis

The data were analyzed with a two-tailed Student *t* test when comparing means between two groups. A one-way ANOVA, plus a Tukey *post hoc* multiple comparison test, was used when comparing multiple groups. The ordinal values of the liver injury scores were analyzed by the Mann–Whitney nonparametric test. In all cases, $P < 0.05$ was considered significant. All data are expressed throughout the text as mean \pm SEM.

Results

Isoflurane Protects against Acute Renal and Hepatic Injury after Renal IRI in Mice

Twenty-four hours after renal IRI, mice exposed to pentobarbital anesthesia developed significant renal dysfunction, as indicated by an increase in plasma creatinine concentration (2.39 ± 0.05 mg/dl, $n = 10$, $P < 0.001$ *vs.* sham) higher than sham values (0.47 ± 0.03 mg/dl, $n = 6$). Isoflurane exposure after renal IRI protected the kidneys, as evidenced by a significant decline in plasma creatinine concentrations (1.61 ± 0.17 mg/dl, $n = 8$, $P < 0.001$ *vs.* pentobarbital renal IRI).

Twenty-four hours after renal IRI, mice developed acute hepatic injury with pentobarbital exposure, as indicated by an increase in plasma ALT concentration higher than sham concentrations (fig. 1A). In contrast, isoflurane exposure after renal IRI protected against liver injury, with a significant reduction in plasma ALT concentrations (fig. 1A).

To evaluate the role of SK in mediating the protective effects of isoflurane after renal IRI, we treated some animals with SKI-II before the induction of renal IRI. SKI-II administration had no detrimental effects on renal function (creatinine concentration, 0.43 ± 0.04 mg/dl; $n = 3$) in sham-operated mice. There were no differences in plasma creatinine values with SKI-II treatment before renal IRI with

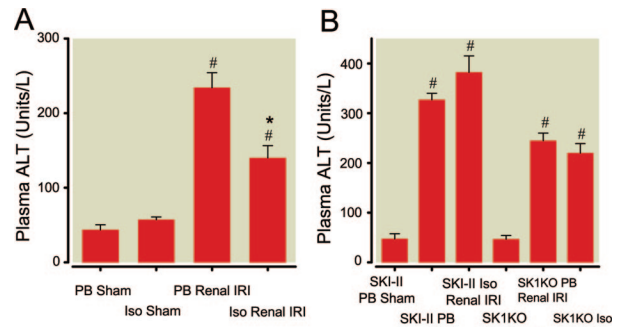


Fig. 1. Plasma alanine aminotransferase (ALT) after renal ischemia–reperfusion injury (IRI). (A) Plasma ALT was measured in C57BL/6 mice exposed to 4 h of pentobarbital (PB) or 1.2% isoflurane (Iso) after sham operation ($n = 7$ for each group) or renal IRI ($n = 8$ for each group). (B) Plasma ALT from C57BL/6 mice treated with the sphingosine kinase (SK) inhibitor SKI-II or from SK1 knockout (SK1KO) mice after sham operation (PB anesthesia; $n = 4$ for each group) or renal IRI ($n = 6$ for each group), followed by exposure to 4 h of PB or Iso. In all cases, plasma ALT was measured 24 h after renal IRI. # $P < 0.05$ versus sham mice. * $P < 0.05$ versus PB renal IRI group. Data are presented as mean \pm SEM.

pentobarbital exposure (creatinine concentration, 2.53 ± 0.18 mg/dl; $n = 6$) compared with isoflurane exposure (creatinine concentration, 2.32 ± 0.09 mg/dl; $n = 5$; $P = 0.47$ *vs.* SKI-II pentobarbital renal IRI). Plasma ALT concentrations increased in SKI-II–treated mice exposed to pentobarbital after renal IRI compared with sham mice (fig. 1B). There was no reduction in plasma ALT concentrations after isoflurane exposure.

In addition, we used a strain of mice deficient in the SK1 enzyme. There were no differences in plasma creatinine values in SK1 knockout mice after renal IRI with pentobarbital exposure (creatinine concentration, 2.33 ± 0.18 mg/dl; $n = 8$) compared with isoflurane exposure (creatinine concentration, 2.25 ± 0.38 mg/dl; $n = 6$; $P = 0.82$ *vs.* SK1 knockout pentobarbital renal IRI). SK1 knockout mice exposed to pentobarbital after renal IRI had increased plasma ALT concentrations compared with sham–operated mice with no reduction with isoflurane exposure (fig. 1B).

Isoflurane Exposure Reduces Hepatic Vacuolization and Small-Intestinal Apoptosis

In figure 2 and figure 3, the protective effects of isoflurane anesthesia are supported further by representative histologic slides. Compared with sham-operated mice (figs. 2A, 3A, and 3B), pentobarbital exposure after renal IRI led to marked hepatocyte vacuolization (fig. 2B) and profound epithelial villous swelling and apoptosis in the small intestine (fig. 3, C and D). Isoflurane exposure after renal IRI dramatically attenuated these injuries in the liver (fig. 2C) and small intestine (fig. 3, E and F).

After renal IRI, the predominant component of hepatic injury was vacuolization. Pentobarbital exposure after renal IRI resulted in severe hepatic vacuolization, as demonstrated

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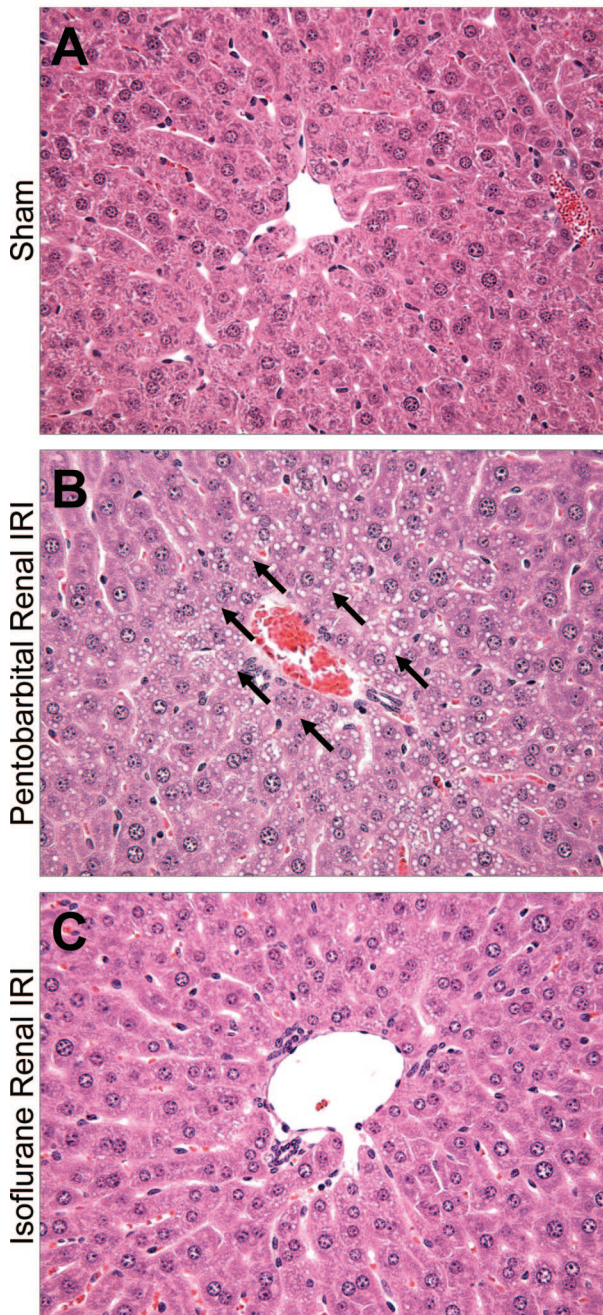


Fig. 2. Isoflurane protects against liver injury after renal ischemia–reperfusion injury (IRI). Representative photomicrographs of liver from 4 experiments (hematoxylin–eosin staining; magnification, $\times 400$) of mice subjected to a sham operation (A) or renal IRI followed by 4 h of pentobarbital (B) or 1.2% isoflurane (C). Tissues were collected 24 h after renal IRI. Arrows point to areas of significant hepatocyte vacuolization.

by the vacuolization score (3.4 ± 0.2 ; $n = 5$; score range, 0–4). In contrast, hepatic vacuolization was less severe with isoflurane exposure after renal IRI (1.6 ± 0.4 , $n = 4$, $P < 0.05$ vs. pentobarbital renal IRI). Sham mice had normal hepatic histologic features, as demonstrated by a vacuolization score of 0.0 ± 0.0 ($n = 4$).

We failed to detect significant TUNEL-positive cells in small-intestinal sections from sham–operated mice exposed to pentobarbital (fig. 4A) or isoflurane (fig. 4B). Mice exposed to pentobarbital after renal IRI (fig. 4C) showed many TUNEL-positive cells in the small intestine (representative of 4 experiments). Mice exposed to isoflurane after renal IRI (fig. 4D) had a reduction in TUNEL-positive cells in the small intestine.

Mice Exposed to Isoflurane after Renal IRI Show Reduced Proinflammatory Gene Expression in the Liver and Small Intestine

We found increased mRNA expression of TNF- α , ICAM-1, IL-17A, MCP-1, MIP-2, and IL-6 in the livers and small intestines of mice exposed to pentobarbital after renal IRI compared with sham mice.³ When mice were exposed to isoflurane after renal IRI, there were significantly reduced expressions of most proinflammatory mRNAs (TNF- α , IL-17A, MCP-1, ICAM-1, and MIP-2) in both the liver and intestine compared with pentobarbital exposure (fig. 5). Isoflurane exposure decreased IL-6 expression in the liver, but not intestine, after renal IRI.

Isoflurane Exposure Decreases Hepatic and Small-Intestinal Vascular Permeability after Renal IRI

We measured liver and small-intestinal vascular permeability using Evans blue dye, which binds to plasma proteins; its appearance in extravascular tissues reflects an increase in vascular permeability.¹⁹ Renal IRI caused significant increases in vascular permeability, as measured by increased Evans blue dye content compared with sham mice in the liver, jejunum, and ileum (fig. 6). Vascular permeability was significantly decreased with isoflurane exposure after renal IRI in the liver, jejunum, and ileum.

Isoflurane Increases Small-Intestinal SK1, but not SK2, Expression, Increases Small-Intestinal Crypt SK1 Expression, and Increases Small-Intestinal SK Activity and S1P Concentrations

Isoflurane anesthesia increased small-intestinal SK1 mRNA expression (fig. 7) and protein expression (fig. 8) in sham mice compared with mice anesthetized with pentobarbital. However, there were no changes in SK2 mRNA or protein expression in the small intestine after isoflurane exposure (figs. 7 and 8).

Compared with pentobarbital anesthesia (fig. 9A), isoflurane anesthesia increased staining for SK1 in the small intestine, specifically in intestinal crypts (red fluorescence, fig. 9B, representative of 4 experiments).

The small intestines of sham mice anesthetized with isoflurane demonstrated higher SK activity compared with mice anesthetized with pentobarbital (fig. 10A). Correspondingly, small-intestinal S1P concentrations were higher in mice after isoflurane anesthesia than after pentobarbital anesthesia (fig. 10B).

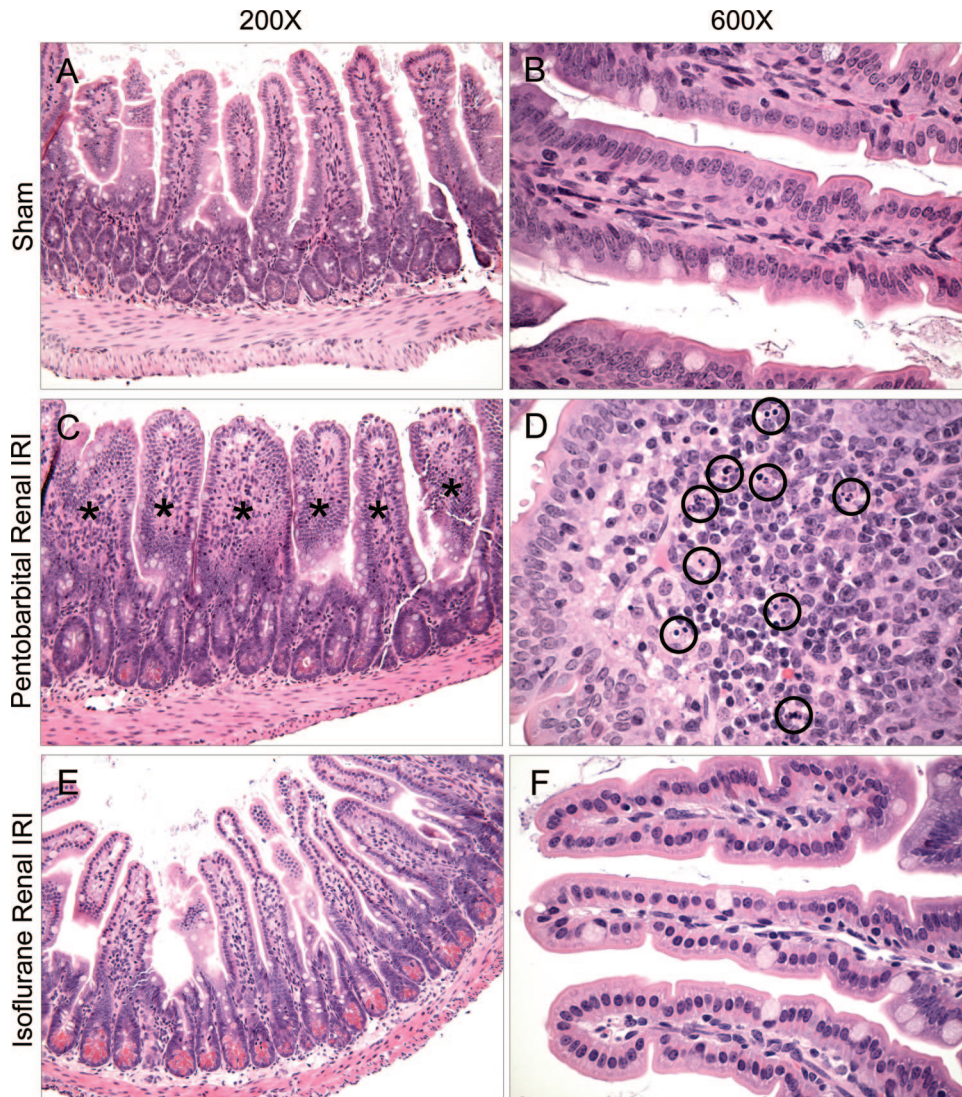


Fig. 3. Isoflurane protects against small-intestinal injury after renal ischemia–reperfusion injury (IRI). Representative photomicrographs of small intestine from 4 experiments (hematoxylin–eosin staining; magnifications, $\times 200$ and $\times 600$). (A, B) Sham-operated mice show normal intestinal morphologic features. The intestines of mice exposed to 4 h of pentobarbital after renal IRI demonstrate marked epithelial villous swelling: (C) swollen villi highlighted by an asterisk, and (D) an enlarged image of a single swollen villous showing numerous apoptotic bodies (circled). (E, F) In contrast, the intestines of mice exposed to 4 h of 1.2% isoflurane after renal IRI were protected from severe injury. Tissues were collected 24 h after renal IRI.

Discussion

The major finding of this study is that a clinically relevant concentration of isoflurane (1 minimum alveolar concentration) administered after renal IRI reduced the degree of renal and hepatic dysfunction and hepatic and intestinal injury by reducing inflammation and apoptosis while improving vascular permeability. The protective effects of isoflurane were mediated by the SK1/S1P pathway because isoflurane failed to protect mice treated with an inhibitor of SK activity (SKI-II) or in mice lacking the SK1 enzyme. Moreover, isoflurane induced small-intestinal SK1 mRNA, protein expression, and enzymatic activity, leading to higher S1P concentrations in the small intestine.

AKI continues to be a significant clinical problem in the perioperative period, and the development of postoperative

AKI requiring renal replacement therapy has a 60% mortality rate.²⁰ Extrarenal dysfunction associated with AKI, including liver and respiratory failure, was predictive of in-hospital mortality.²¹ AKI is an inflammatory process involving multiple cellular and systemic responses, including activation of proinflammatory cytokines and chemokines²² and infiltration by leukocytes, such as neutrophils, macrophages, and T cells.²³ Indeed, modulation of the inflammatory cascade (*e.g.*, with adenosine generation and signaling modulation *via* ecto-5'-nucleotidase²⁴ and the A2B-adenosine receptor²⁵) provides powerful protection against AKI after renal IRI. There is growing interest in the extrarenal manifestations of AKI because it is becoming clear that AKI leads to a systemic inflammatory state affecting distant organs.

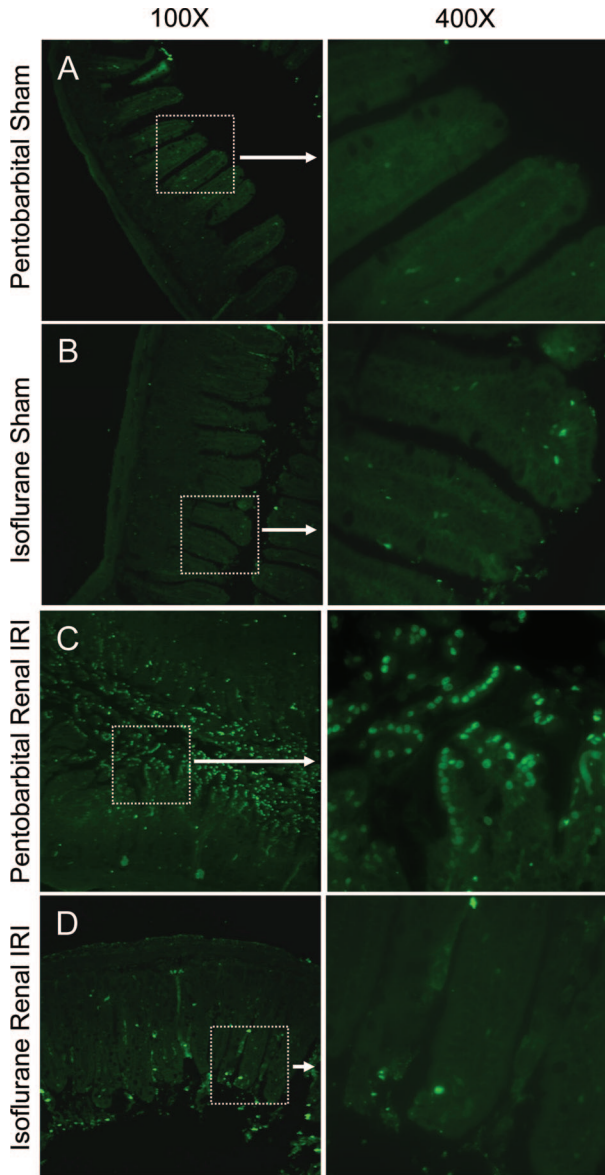


Fig. 4. Isoflurane protects against intestinal apoptosis after renal ischemia–reperfusion injury (IRI). Representative fluorescence photomicrographs (of 4 experiments) illustrating apoptotic nuclei (terminal deoxynucleotidyl transferase biotin-dUTP nick end-labeling [TUNEL] fluorescence staining, green) in the small intestine. The left side of each panel depicts a $\times 100$ fluorescence photomicrograph with a highlighted area (white box) enlarged to $\times 400$ on the right side. Mice were exposed to 4 h of pentobarbital after a sham operation (A) or renal IRI (C) or to 4 h of 1.2% isoflurane after a sham operation (B) or renal IRI (D). Tissues were collected 24 h after renal IRI.

AKI caused increases in pulmonary capillary leak and interstitial neutrophil infiltration *via* IL-6,²⁶ led to worsening of cardiac function and induced cardiomyocyte apoptosis,²⁷ increased neuronal pyknosis and microgliosis in the brain, and increased the proinflammatory cytokines keratinocyte-derived chemokine (KC) and granulocyte-colony stimulating factor (G-CFS).²⁸

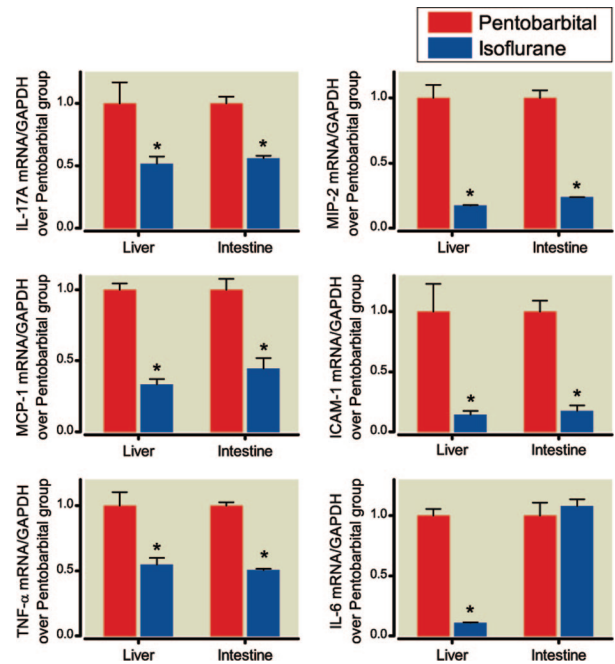


Fig. 5. Isoflurane protects against renal ischemia–reperfusion injury (IRI)–mediated hepatic and intestinal proinflammatory messenger RNA (mRNA) up-regulation. Mice were subjected to renal IRI, followed by exposure to 4 h of pentobarbital (red) or 1.2% isoflurane (blue). Liver and small-intestine tissues were collected 24 h after renal IRI. Densitometric quantifications of band intensities relative to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) from reverse transcription–polymerase chain reactions. $n = 4$ per group. * $P < 0.05$ versus the appropriate pentobarbital group. Data are presented as the mean \pm SEM. ICAM = intercellular adhesion molecule; IL = interleukin; MCP = monocyte chemoattractant protein; MIP = macrophage inflammatory protein; TNF = tumor necrosis factor.

Previous studies²⁹ have shown that the gut plays an important role in mediating the hyperdynamic response early in sepsis. Hepatic dysfunction after AKI has been described³⁰; recently, Paneth cells, located in small-intestinal crypts, were

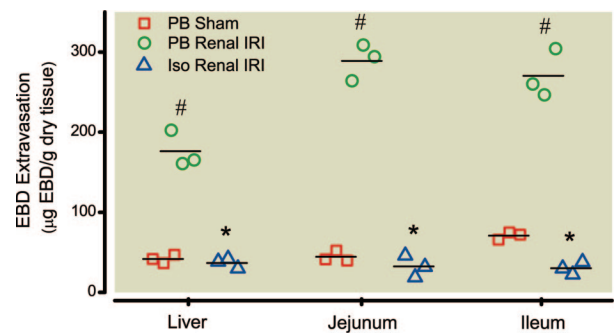


Fig. 6. Isoflurane reduces vascular permeability after renal ischemia–reperfusion injury (IRI). Quantification of Evans blue dye extravasation (EBD) as an index of vascular permeability of liver, jejunum, and ileum tissues in mice 24 h after a sham operation (sham, pentobarbital [PB] anesthesia) or renal IRI followed by exposure to 4 h of PB or 1.2% isoflurane (Iso). $n = 3$ per group. Data are presented as the mean \pm SEM. # $P < 0.05$ versus the PB sham group. * $P < 0.05$ versus the PB renal IRI group.

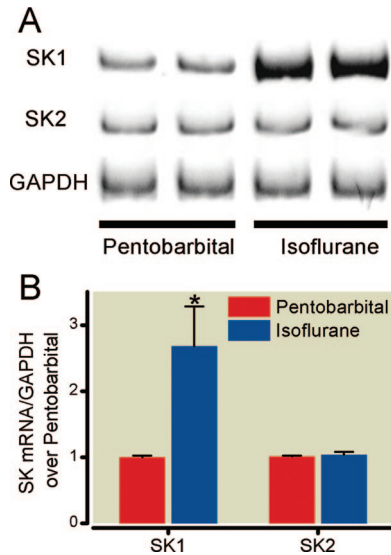


Fig. 7. Isoflurane activates intestinal sphingosine kinase (SK) 1, but not SK2, messenger RNA (mRNA) expression. (A) Representative gel images of reverse transcription–polymerase chain reaction (RT-PCR) (of 4 experiments) of SK1, SK2, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) from the small intestines of sham mice exposed to 4 h of pentobarbital or 1.2% isoflurane. (B) Densitometric quantifications of band intensities relative to GAPDH from RT-PCRs. * $P < 0.05$ versus the pentobarbital sham group. Data are presented as the mean \pm SEM.

identified as the source of the inflammatory mediator, IL-17A, seen in mice after AKI.³¹ The release of IL-17A from Paneth cells led to hepatic dysfunction and a cascade of inflammation, including generation of TNF- α and IL-6. Fur-

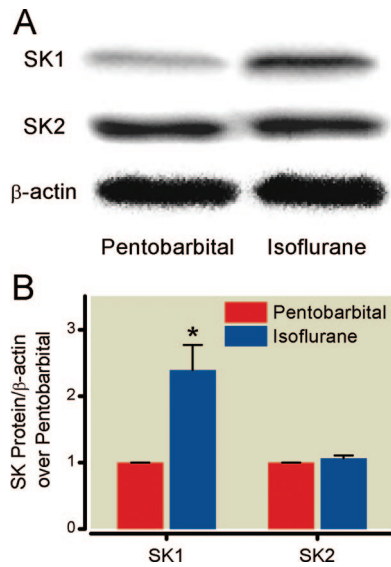


Fig. 8. Isoflurane increases intestinal sphingosine kinase (SK) 1, but not SK2, protein expression. (A) Representative immunoblot images (of 4 experiments) of SK1, SK2, and β -actin from the small intestines of sham mice exposed to 4 h of pentobarbital or 1.2% isoflurane. (B) Densitometric quantifications of band intensities relative to β -actin from immunoblot images. * $P < 0.05$ versus the pentobarbital sham group. Data are presented as the mean \pm SEM.

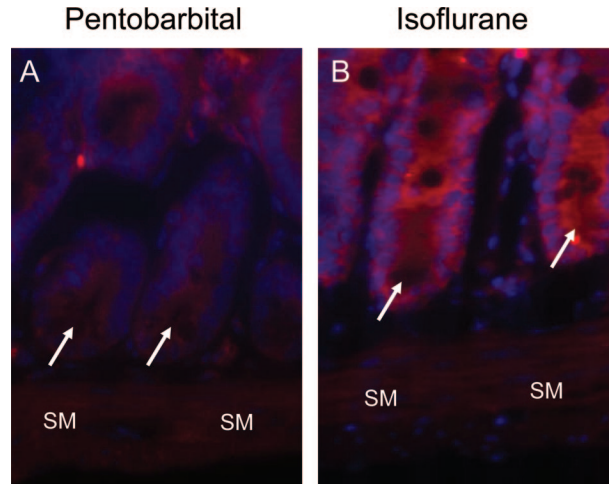


Fig. 9. Isoflurane increases sphingosine kinase (SK) 1 in small-intestinal crypts. Representative immunofluorescence images (of 4 experiments) for SK1 (red) and nuclear (blue) staining from sham mice exposed to 4 h of pentobarbital (A) or 1.2% isoflurane (B). SM designates the smooth muscle layer, and the arrows point to small-intestinal crypts.

thermore, mice deficient in TNF- α , IL-17A, or IL-6 or mice treated with antibodies to TNF- α , IL-17A, or IL-6 had attenuation of hepatic and small-intestinal inflammation.³

In our model, isoflurane, decreased the expression of IL-17A, TNF- α , MIP-2, MCP-1, and ICAM-1 in the liver and small intestine after renal IRI, reflecting the direct antiinflammatory effects of isoflurane. The antiinflammatory effects of volatile anesthetics are well described because they decreased TNF- α -mediated release of heme oxygenase-1 and IL-8 in human monocytic THP-1 cells³² and reduced KC and MIP-2 in a model of endotoxin-induced lung injury.³³

IL-17A is involved in innate immune defense and inflammation and is mainly produced by a subset of T cells known as T helper (Th) 17 cells.³⁴ Th17 cells can be found in the intestinal lamina propria of healthy mice³⁵ but rarely in the spleen, mesenteric lymph nodes, or Peyer patches.³⁶ IL-17A

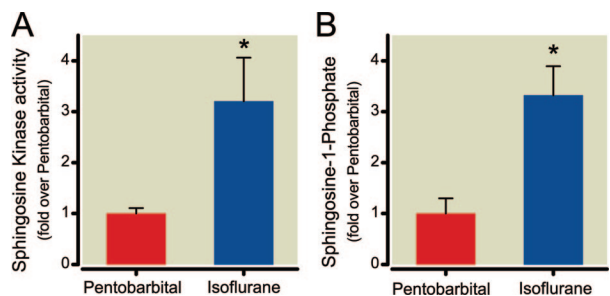


Fig. 10. Isoflurane exposure increases sphingosine kinase (SK) 1 activity and sphingosine-1-phosphate (S1P) formation. (A) Relative SK1 activity (fold over pentobarbital group) from intestines of sham mice exposed to 4 h of pentobarbital or isoflurane (n = 6 per group). (B) Formation of S1P (fold over pentobarbital group) from the intestines of sham mice exposed to 4 h of pentobarbital or isoflurane (n = 4 per group). * $P < 0.05$ versus the pentobarbital group. Data are presented as the mean \pm SEM.

derived from intestinal Paneth cells mediated shock induced by TNF- α ³⁷; we demonstrated that AKI induces production of IL-17A in Paneth cells, causing increased concentrations of additional cytokines, including TNF- α ,³¹ a potent mediator of the inflammatory cascade seen after AKI.³⁸ Herein, we show that isoflurane up-regulated SK1 in intestinal crypts, revealing a potential mechanism for the decrease in IL-17A and TNF- α , both produced in intestinal crypts, seen after isoflurane exposure. MIP-2 is a chemokine involved in inflammation and immunoregulation and is a potent regulator of neutrophil chemotaxis.³⁹ MCP-1 mediates inflammation⁴⁰ and ICAM-1 regulates neutrophil retention⁴¹ after AKI. Isoflurane exposure reduced IL-6 expression after renal IRI in the liver but not in the small intestine, suggesting a divergent role of this cytokine in the liver and intestine. Indeed, IL-6 plays a role in liver inflammation and progression to hepatocellular carcinoma⁴² but protects enterocytes against cell death and apoptosis and protected mice against intestinal IRI.⁴³

The protective effects of volatile anesthetics have been shown in multiple organ systems, including the brain,⁴⁴ heart,⁴⁵ and lung.³³ In our model, mice were protected from AKI-induced liver and intestine injury with exposure to isoflurane after renal IRI (*i.e.*, postconditioning). Clinically, volatile anesthetics can be administered outside of the operating room in the intensive care unit,⁴⁶ and this may potentially have therapeutic benefits in patients with AKI. We demonstrated that the protective effects of volatile anesthetics on the kidney and cultured human proximal tubule cells were mediated *via* the SK/S1P pathway^{17,18}; recently, isoflurane postconditioning protected neonatal rats from hypoxic-ischemic brain injury *via* a mechanism dependent on SK/S1P signaling.⁴⁴ Given this knowledge, we tested whether there was a role for SK/S1P signaling in protecting against extrarenal organ dysfunction after renal IRI. We found that administration of SKI-II reversed the isoflurane-mediated protection from renal IRI. In addition, we demonstrated that mice lacking the SK1 enzyme were not protected against liver and intestinal injury after renal IRI with isoflurane. Because of the inherent concerns regarding the use of genetic knockout mice (*e.g.*, alterations in the expression of unrelated proteins), we used both a pharmacologic inhibitor and genetic knockout mice to study the role of SK in renal IRI. By using immunofluorescence imaging, we determined that the small-intestinal crypts had the greatest increase in staining for SK1. Taken together, these data indicate that activation of the SK1/S1P pathway in small-intestinal crypts is involved in mediating the protective effects of isoflurane on the liver and intestine after renal IRI.

The lysophospholipid S1P has multiple roles in cellular signaling and balances against the proapoptotic effects of sphingosine and ceramide *via* the “sphingolipid rheostat.”⁴⁷ S1P protected intestinal cells from apoptosis *via* serine/threonine-specific protein kinase (Akt) activation⁴⁸ and mediated the protective effects of glucocorticoids on renal mesan-

gial cells.⁴⁹ S1P reduced IRI injury in a rat model of lung transplantation⁵⁰ and protected against renal IRI injury in mice.⁵¹ S1P binds to specific G protein-coupled receptors, of which five are known (S1P1–5).⁸ FTY720, a sphingosine analog that is phosphorylated *in vivo*, has produced lymphopenia through its actions on the S1P1 receptor by reducing lymphocyte egress from lymph nodes and has shown protection in models of liver,⁵² bowel,⁵³ and renal⁵⁴ ischemia.

SK, the enzyme catalyzing the conversion of sphingosine to S1P, is an important regulator of the sphingolipid rheostat. Many agents are known to stimulate SK activity, including agonists of growth factor receptors (*e.g.*, platelet-derived growth factor [PDGF], vascular endothelial growth factor [VEGF], nerve growth factor [NGF], and epidermal growth factor [EGF]), transforming growth factor β , and TNF- α .⁵⁵ Of the two known isoforms, SK1 mediates cytoprotection, whereas SK2 is generally thought to be proapoptotic.⁵⁶ Indeed, SK1 mediated ischemic postconditioning in isolated mouse hearts⁵⁷ and was protective *via* down-regulation of c-Jun N-terminal kinase (JNK) activity.⁵⁸ Mice lacking the SK1 enzyme had poor recovery from anaphylaxis and delayed histamine clearance, whereas mice lacking the SK2 enzyme had rapid recovery from anaphylaxis.⁵⁹ In certain models, such as Crohn disease, SK had a deleterious effect because SK inhibition⁶⁰ or mice lacking the SK1 enzyme⁶¹ had reduced inflammation and colon damage.

Anesthetics can cause hemodynamic changes and regional disturbances in blood flow. Previously, it was demonstrated that, compared with pentobarbital, volatile anesthetics did not significantly alter systemic blood pressure or renal blood flow.¹⁷ Certain volatile anesthetics, such as methoxyflurane, undergo renal metabolism with direct nephrotoxic effects due to inorganic fluoride. However, isoflurane is minimally metabolized and has not been linked to fluoride nephrotoxicity.⁶²

A limitation of our study is that volatile anesthetics may have both local and systemic effects for reducing the severity of hepatic and intestinal injury after renal IRI; therefore, it is difficult to determine in an *in vivo* study whether volatile anesthetics mediate their protective effects directly on small-intestinal crypts or *via* systemic effects on the migration and infiltration of lymphocytes and neutrophils. The effects are likely multifactorial, including both direct cytoprotective effects on hepatocytes and intestinal epithelial cells with activation of prosurvival signaling pathways, such as the extracellular-signal-regulated kinase (ERK) and Akt pathways, and systemic antiinflammatory mechanisms, including peripheral lymphopenia, preservation of endothelial barrier integrity, and reduction of proinflammatory cytokines.

In conclusion, we demonstrated that isoflurane activates the SK1/S1P signaling pathway in small-intestinal crypts to reduce hepatic and intestinal injury, apoptosis, and proinflammatory mRNA up-regulation after renal IRI. Further elucidation of the mechanisms of protection may lead to advancements in the treatment of extrarenal organ dysfunction after renal IRI.

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