Isoflurane mediates protection from renal ischemia-reperfusion injury via sphingosine kinase and sphingosine-1-phosphate-dependent pathways

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Kim M, Kim M, Kim N, D’Agati VD, Emala CW, Sr, Lee HT. Isoflurane mediates protection from renal ischemia-reperfusion injury via sphingosine kinase and sphingosine-1-phosphate-dependent pathways. Am J Physiol Renal Physiol 293: F1827–F1835, 2007. First published September 26, 2007; doi:10.1152/ajprenal.00290.2007.—The inhalational anesthetic isoflurane has been shown to protect against renal ischemia-reperfusion (IR) injury. Previous studies demonstrated that isoflurane modulates sphingolipid metabolism in renal proximal tubule cells. We sought to determine whether isoflurane stimulates sphingosine kinase (SK) activity and synthesis of sphingosine-1-phosphate (S1P) in renal proximal tubule cells to mediate renal protection via the S1P signaling pathway. Isoflurane anesthesia reduced the degree of renal failure and necrosis in a murine model of renal IR injury. This protection with isoflurane was reversed by SK inhibitors (DMS and SKI-II) as well as an S1P1 receptor antagonist (VPC23019). In addition, mice deficient in SK1 enzyme were not protected from IR injury with isoflurane. SK activity as well as SK1 mRNA expression increased in both cultured human proximal tubule cells (HK-2) and mouse kidneys after exposure to isoflurane. Finally, isoflurane increased the generation of S1P in HK-2 cells. Taken together, our findings indicate that isoflurane activates SK in renal tubule cells and initiates S1P→S1P1 receptor signaling to mediate the renal protective effects. Our findings may help to unravel the cellular signaling pathways of volatile anesthetic-mediated renal protection and lead to new therapeutic applications of inhalational anesthetics during the perioperative period.

acute renal failure; inflammation; necrosis; reverse transcriptase-polymerase chain reaction; volatile anesthetics

ACUTE RENAL FAILURE (ARF) continues to be a major contributor to morbidity and mortality in the perioperative period. A recent review found that the reported incidence of ARF after major vascular surgery ranged from 1.7 to 25% (52) and in a study of 2,672 patients undergoing coronary artery bypass grafting, development of ARF was associated with a 14-fold increase in mortality (10). Despite continued advances in the understanding of the pathophysiology of ARF, a recent meta-analysis of randomized clinical trials found no conclusive evidence that any of the currently available treatments, including pharmacological and fluid hydration regimens, offered renal protection during surgery (58).

We previously reported that certain volatile anesthetics, including isoflurane [2-chloro-2-(difluoromethoxy)-1,1,1-trifluoro-ethane (isoflurane) was purchased from Abbott Laboratories (North Chicago, IL). N,N-dimethylsphingosine (DMS) was purchased from Biomol International (Plymouth Meeting, MA). 4-[4-(4-Chlorophenyl)-2-thiazoly]amino]phenol (SKI-II) was purchased from Tocris Bioscience (Ellisville, MO). (R)-Phosphoric acid mono-[2-amino-2-(3-oxyl-phenylcarbamoyl)-ethyl] ester (VPC23019) was purchased from Avanti Polar Lipids (Alabaster, AL). α-Erythro-[3-3H]sphingosine was purchased from American Radiolabeled Chemicals (St. Louis, MO).

Murine model of renal IR injury. All animal protocols were approved by the Institutional Animal Care and Use Committee of Columbia University (New York, NY). Male C57BL/6 (Harlan, Indianapolis, IN; 20 to 25 g) or SK1 knockout mice (kindly provided by R. L. Proia, National Institutes of Health, Bethesda, MD; 20 to 25 g) were anesthetized with intraperitoneal pentobarbital sodium (50 mg/kg body wt, or to effect), intraperitoneal ketamine-xylazine (100–10 mg/kg, or to effect), or isoflurane [1.2% or 1 minimum alveolar concentration (MAC, defined as the concentration of volatile anesthetic in the lungs that is needed to prevent movement in 50% of subjects in response to a painful stimulus)] as described previously (31). The generation and initial characterization of SK1 knockout mice have been described

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previously (1). These mice are conegenically derived on a C57BL/6 background.

For studies involving ischemia and reperfusion, mice were subjected to midline laparotomy, right nephrectomy, 30 min of left renal ischemia, and closure of the abdomen in two layers. Isoflurane-anesthetized mice were returned to the chamber to breathe isoflurane for an additional 3 h while pentobarbital sodium-anesthetized mice were returned to their cages to recover from the anesthesia. The mice were placed on a heating pad under a warming light to maintain body temperature between 36 and 38°C. Body temperature was monitored with a rectal probe and maintained at 37°C in all mice. In some mice, a carotid artery catheter was placed to measure systemic blood pressures at 1, 2, and 3 h after induction of anesthesia.

**Effect of SK antagonists and S1P receptor antagonist on renal IR injury.** To test the effect of SK inhibition on renal IR injury, the SK inhibitors DMS and SKI-II were administered to mice undergoing renal IR injury. DMS (5 mg/kg) was given intraperitoneally 30 min pre- and postischemia. SKI-II (50 mg/kg) was administered subcutaneously 15 min pre- and 4 h postischemia. In addition, VPC23019, a specific S1P1/S1P3 receptor antagonist (10-fold more selective at the S1P1 vs. the S1P3 receptor), was administered to test the effects of S1P1 receptor blockade. VPC23019 (1 mg/kg) was given intraperitoneally 10 min preischemia and 10 min preraperfusion.

**Measurement of plasma creatinine.** Renal function was assessed by measuring plasma creatinine 24 h after ischemia using a colorimetric method based on the Jaffe reaction (22).

**Histological detection of necrosis.** Morphologic assessment was performed by an experienced renal pathologist who was unaware of the study. A renal injury score grading scale of 0 to 4, as outlined by Jablonski et al. (23), was used to assess the degree of renal tubular necrosis in the outer medullary cortex. The upper aqueous phase was collected and counted in a liquid scintillation counter (Packard BioScience, Meriden, CT).

**In vitro enzyme preparations from HK-2 cells.** For whole cell assays, HK-2 cells (treated with either 2.5% isoflurane or carrier gas) were scraped in lysis buffer for the SK activity assay. For separation of membrane and cytosolic fractions, cells were scraped in buffer E and the lysate was spun at 1,000 g for 5 min. The supernatant was then spun at 100,000 g for 30 min to separate the fractions. The pellet was resuspended in lysis buffer. The protein concentrations of both fractions were determined.

**SK activity assay.** SK activity was measured as described by Vessey et al. (54). Briefly, enzyme preparations from kidneys (20 μg total protein) or HK-2 cells (120–240 μg total protein) were incubated in assay buffer (0.05% Triton X-100, 250 mM KCl, 0.05 μM [3H]sphingosine (20 Ci/mmol; American Radiolabeled Chemicals), 5 mM ATP, 10 mM MgCl2, 100 mM Tris pH 8.0) in a total volume of 100 μl at 20°C for 15 (renal cortices) or 60 min (HK-2 cells). These incubation times yielded a linear increase in SK activity (data not shown). [3H]S1P was extracted using 1.2 ml of methanol:chloroform:water (80:10:10). The top phase was aspirated, and the bottom aqueous phase was collected and counted in a liquid scintillation counter (Packard BioScience, Meriden, CT). To verify the product of our extraction, aliquots of the upper aqueous phases were resolved on silica 60 Å TLC plates (Whatman, Florham Park, NJ) using 1-butanol:methanol:acetic acid:ddH2O (80:20:10:20). The plates were scraped in 1-cm increments and counted in a liquid scintillation counter. We found that the peaks from our extraction had the same relative mobility (Rf) as purified [3H]S1P (data not shown).

**RT-PCR for SK1 mRNA.** Samples from mouse renal cortices (21 h following 3 h of exposure to either 1.2% isoflurane or pentobarbital sodium) or HK-2 cells (following exposure to 3, 6, or 16 h of 2.5% isoflurane) were collected and total RNA was extracted for semiquantitative RT-PCR analysis as described previously (31). The expression of SK1 mRNA was measured using primers designed based on published GenBank sequences for mice and humans (Table 1). Primers were purchased from Sigma Genosys (The Woodlands, TX).

### Table 1. Primer sequences

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<th>Primers</th>
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SK, sphingosine kinase.
To verify the results of our semiquantitative RT-PCR analysis, we also performed quantitative real-time RT-PCR analysis on the mouse kidneys and one time point (6-h isoflurane treatment) of the HK-2 cell samples. The results were consistent with the semiquantitative analysis (data not shown). Q-RT-PCR was performed with the MyQ Real Time Detection System (Bio-Rad, Hercules, CA) using SYBR Green I Brilliant Mastermix (Stratagene, La Jolla, CA). The cDNA template was synthesized using Omniscript Reverse Transcriptase and oligo-dT primer (Qiagen, Valencia, CA). Specificity of the amplification was checked by melting curve analysis and by agarose gel electrophoresis. All reactions were performed in duplicate with appropriate negative controls. The Ct values were determined by using Mx3000P software. Values were normalized for GAPDH mRNA.

**Protein determination.** Protein content was determined with the Pierce Chemical bicinchoninic acid protein assay reagent with bovine serum albumin as a standard.

**Statistical analysis.** The data were analyzed with Student’s t-test when comparing means between two groups. One-way ANOVA plus Dunnett’s post hoc multiple comparison test was used when comparing multiple groups. The ordinal values of the Jablonski scale renal injury score were analyzed by the Mann-Whitney nonparametric test. In all cases, a probability statistic <0.05 was taken to indicate significance. All data are expressed throughout the text as means ± SE.

**RESULTS**

**Isoflurane protects against renal IR injury in mice.** We determined in our preliminary studies that systemic arterial blood pressure did not differ significantly between 1.2% isoflurane- and pentobarbital sodium-anesthetized mice. The average systolic arterial pressures for the pentobarbital sodium-anesthetized group at 1, 2, and 3 h after induction of anesthesia were 135 ± 7, 141 ± 9, and 145 ± 10 mmHg (n = 3), respectively. The average systolic arterial pressures for the isoflurane-anesthetized group at 1, 2, and 3 h after induction of anesthesia were 132 ± 6, 136 ± 8, and 139 ± 6 mmHg (n = 3), respectively. Finally, we maintained the temperature (measured with an infrared temperature sensor during laparotomy and reperfusion under anesthesia) at 37°C at all times. The average temperatures for the pentobarbital sodium-anesthetized group at 1, 2, and 3 h after induction of anesthesia were 37 ± 1, 36 ± 1, and 36 ± 1°C (n = 4), respectively. The average temperatures for the isoflurane-anesthetized group at 1, 2, and 3 h after induction of anesthesia were 37 ± 1, 37 ± 1, and 36 ± 1°C (n = 4), respectively.

Twenty-four hours after renal IR injury, C57BL/6 mice anesthetized with pentobarbital sodium developed significant renal dysfunction indicated by a rise in plasma creatinine (Cr = 2.4 ± 0.2 mg/dl, n = 11, vs. sham 0.5 ± 0.0 mg/dl, n = 3, P < 0.01; Fig. 1A). Mice anesthetized with ketamine-xylazine also developed significant renal dysfunction (Cr = 3.1 ± 0.3 mg/dl, n = 3) indicating that the worsening of renal function was not a function of barbiturate anesthesia. In contrast, mice treated with 1.2% isoflurane (1 MAC) during renal ischemia and the first 3 h of reperfusion (Iso IR) had significantly lower plasma creatinine values compared with pentobarbital sodium-anesthetized mice (Cr = 1.3 ± 0.1, n = 6, P < 0.01; Fig. 1A).

The renal protection mediated by isoflurane was abrogated upon treatment with the SK inhibitors DMS (14, 16) (Cr = 2.7 ± 0.1 mg/dl, n = 6, *P < 0.01 vs. Iso IR; Fig. 1B) and SKI-II (18) (Cr = 3.5 ± 0.1 mg/dl, n = 5, *P < 0.01 vs. Iso IR; Fig. 1B), as well as the S1P1 receptor antagonist VPC23019 (13) (Cr = 2.6 ± 0.3 mg/dl, n = 7, *P < 0.01 vs. Iso IR; Fig. 1B).

**Fig. 1.** Plasma creatinine values after ischemia-reperfusion (IR) injury. A: plasma creatinine (Cr; mg/dl; 24 h after ischemia) from sham-operated mice (Sham) and mice subjected to IR under pentobarbital sodium anesthesia (PB IR), ketamine-xylazine anesthesia (Ket-Xyl IR), or isoflurane anesthesia (Iso IR). B: plasma creatinine from mice subjected to IR under isoflurane anesthesia with the S1P1 receptor antagonist VPC23019 (Iso+VPC23019 IR) or the sphingosine kinase (SK) inhibitor N,N-dimethylsphingosine (Is+DMS IR) and SKI-II (Iso+SKI-II IR), or under pentobarbital sodium anesthesia with VPC23019 (PB+VPC23019 IR), DMS (PB+DMS IR), or SKI-II (PB+SKI-II IR). C: plasma creatinine from SK1 knockout mice subjected to IR under isoflurane anesthesia (SK1KO Iso IR) or under pentobarbital sodium (SK1KO PB IR) anesthesia. †P < 0.01 vs. PBS IR group. *P < 0.01 vs. Iso IR group. Error bars represent SE. Statistical analysis performed using 1-way ANOVA with Dunnett’s post hoc multiple comparison test.
Pentobarbital sodium-anesthetized mice treated with the SK inhibitors or S1P1 receptor antagonist had creatinine values of 2.3 ± 0.2 mg/dl (DMS; n = 7, P < 0.05 vs. Iso IR), 2.8 ± 0.4 mg/dl (SKI-II; n = 5, P < 0.01 vs. Iso IR), and 2.4 ± 0.2 mg/dl (VPC23019; n = 7, P < 0.05 vs. Iso IR; Fig. 1B). The compounds themselves had no effect on creatinine values in sham mice (data not shown).

In addition to pharmacological inhibition, we utilized a strain of mice that were deficient in SK1 enzyme. The generation and characterization of these mice have been described previously (1) and they are congenically derived on a C57BL/6 background. Mice lacking SK1 enzyme were not protected previously (1) and they are congenically derived on a C57BL/6 strain of mice that were deficient in SK1 enzyme. The generation and characterization of these mice have been described previously (1) and they are congenically derived on a C57BL/6 background. Mice lacking SK1 enzyme were not protected previously (1) and they are congenically derived on a C57BL/6 strain of mice that were deficient in SK1 enzyme.

Isoflurane anesthesia reduces renal tubular necrosis. In Fig. 2, the renal-protective effects of isoflurane anesthesia are further supported by representative histological slides. Thirty minutes of renal ischemia followed by 24 h of reperfusion under pentobarbital sodium anesthesia resulted in significant renal injury as evidenced by severe tubular necrosis, medullary congestion and hemorrhage, and the development of proteinaceous casts (Fig. 2B). Isoflurane anesthesia significantly attenuated this drastic necrosis after IR injury (Fig. 2C). When the mice were treated with inhibitors of SK (DMS and SKI-II; Fig. 2, D and E) or with the S1P1 receptor antagonist (VPC23019; Fig. 2F), the protective effects of isoflurane were not seen.

The Jablonski scale renal injury histology grading scores are shown in Fig. 3. Thirty minutes of renal ischemia under pentobarbital sodium anesthesia and 24 h of reperfusion resulted in severe acute tubular necrosis as demonstrated by the renal injury score (2.8 ± 0.4, n = 8). In contrast, renal injury was less severe with isoflurane anesthesia (0.8 ± 0.3, n = 4, P < 0.01 vs. pentobarbital sodium). Mice anesthetized with pentobarbital sodium without subsequent IR injury had normal renal histology as demonstrated by a renal injury score of 0.0 ± 0.0 (n = 5).

Isoflurane increases SK activity in vivo. Mice were anesthetized with 1.2% isoflurane or pentobarbital sodium for 3 h (without IR injury) and allowed to recover for 21 h and their renal cortices were extracted for SK activity measurement. Mice anesthetized with isoflurane demonstrated higher renal SK activity compared with those mice anesthetized with pentobarbital sodium (Fig. 4). Kidneys of isoflurane-anesthetized mice exhibited an increase in renal SK activity in the membrane fractions (1.17 ± 0.06-fold, n = 6, P < 0.05) compared with pentobarbital sodium-anesthetized mice (1.00 ± 0.05-fold, n = 6), while the cytosolic fractions did not exhibit such an increase (Fig. 4). The kidneys of mice were also collected immediately after exposure to isoflurane or pentobarbital sodium for 3 h (no recovery) but there were no differences in renal SK activity between the two treatment groups (data not shown).

Isoflurane increases SK activity in vitro. Treatment of HK-2 cells with 3–16 h of 2.5% isoflurane demonstrated a time-

![Figure 2](http://ajprenal.physiology.org/DownloadedFrom/New_Download/102203361013207/F1830_AJP-RenalPhysiol_VOL293DECEMBER2007wwwajprenalorg.png)

**Fig. 2.** Isoflurane reduces renal injury. Representative photomicrographs (hematoxylin and eosin staining, magnification ×200) of the outer medulla of the kidneys of sham-operated mice (A) and mice subjected to renal IR injury under pentobarbital sodium (B) and isoflurane (C) anesthesia, or under isoflurane anesthesia with pretreatment with the SK inhibitors, DMS (D) and SKI-II (E), or with the S1P1 receptor antagonist, VPC23019 (F). Severe tubular dilatation, tubular swelling and necrosis, medullary luminal congestion and hemorrhage are present in the kidneys of mice subjected to IR during pentobarbital sodium anesthesia (B), but these changes are attenuated in animals subjected to IR injury under isoflurane (C). When treated with inhibitors of SK (D,E) or an S1P1 receptor antagonist (F), the protection seen with isoflurane anesthesia is reversed.
dependent increase in SK activity (Fig. 5). When measured in whole cell lysate fractions, SK activity did not increase after 3 h of isoflurane exposure but increased 1.28 ± 0.06-fold (n = 6, P < 0.01 vs. control) and 1.37 ± 0.10-fold (n = 6, P < 0.01 vs. control) after 9 and 16 h of isoflurane exposure, respectively (Fig. 5A).

When SK activity was measured in HK-2 cells separated into membrane and cytosolic fractions, there was a time-dependent increase in SK activity in the membrane fraction (Fig. 5B). In the membrane fraction, there was no increase in SK activity after 3 h of isoflurane exposure but SK activity increased 1.19 ± 0.06-fold (n = 6, P < 0.05 vs. control) and 1.22 ± 0.07-fold (n = 6, P < 0.01 vs. control) after 9 and 16 h of isoflurane exposure, respectively (Fig. 5B). In the cytosolic fractions, there were no changes in SK activity after 3 and 9 h of isoflurane exposure but after 16 h of isoflurane exposure, there was a decrease to 0.90 ± 0.02-fold (n = 6, P < 0.01 vs. control; Fig. 5B).

**Isoflurane increases [3H]S1P formation in HK-2 cells.** Consistent with our findings of increased SK activity with isoflurane exposure, HK-2 cells treated with 2.5% isoflurane for 6 h had higher cellular [3H]S1P levels (1.45 ± 0.01-fold, n = 6, P < 0.01) compared with control (Fig. 6).

**Isoflurane increases synthesis of SK1 mRNA in mice.** We measured renal SK1 mRNA expression in kidneys of mice 21 h after being exposed to isoflurane or pentobarbital sodium to determine whether the increased SK activity levels were correlated with an increase in SK1 mRNA synthesis (Fig. 7A). We found that in mice exposed to isoflurane, renal SK1 mRNA expression increased 1.43 ± 0.10-fold (n = 6, P < 0.01) over mice exposed to pentobarbital sodium (1.00 ± 0.05-fold, n = 6; Fig. 7B).

**Isoflurane increases SK1 mRNA synthesis in HK-2 cells.** In HK-2 cells, there was an increase in SK1 mRNA expression with increasing exposure to 2.5% isoflurane that peaked with
We demonstrated that in rats, volatile anesthetics, including isoflurane, provided significant protection against renal IR injury with improved renal function, reduction in renal tubular necrosis, improved preservation of renal proximal tubular architecture, and inhibition of neutrophil influx (31). Subsequently, we showed that the volatile anesthetic sevoflurane reduced necrosis and inflammation in HK-2 cells (29). This in vitro protection was associated with activation of the prosurvival kinases ERK and Akt. Therefore, it is likely that volatile anesthetics have direct effects on renal tubules to reduce injury (necrosis and inflammation) after IR. However, the signaling mechanisms of volatile anesthetics before ERK and Akt activation are not known. Therefore, in this study, we aimed to determine the proximal signaling pathways involved in this volatile anesthetic-mediated renal protection.

In our current study, we showed that, consistent with our previous results in rats (31), the volatile anesthetic isoflurane protected mice against renal IR injury compared with pentobarbital sodium anesthesia, as demonstrated by reduction in plasma creatinine values as well as reduced necrosis. Pentobarbital sodium, ketamine, and isoflurane anesthesia at doses utilized in our study did not affect systemic blood pressure or renal blood flow in mice or rats (30, 31) making it unlikely that altered hemodynamics explain the differences between groups. In addition, pentobarbital sodium anesthesia did not worsen the degree of renal failure compared with ketamine-xylazine anesthesia, indicating that there was no specific effect of barbiturate anesthesia causing worsening of renal failure. The volatile anesthetic methoxyflurane has been shown to be nephrotoxic due to its metabolism to inorganic fluoride. However, isoflurane is minimally metabolized and has not been linked to fluoride nephrotoxicity in rats or humans (11).

We aimed to determine whether there was a role for SK/S1P signaling in mediating this protection. We found that administration of the SK inhibitors, DMS and SKI-II, reversed the isoflurane-mediated protection from renal IR injury. DMS is a well-known inhibitor of SK enzyme (14, 16), whereas SKI-II is a newly developed SK inhibitor with a higher potency and selectivity (18). We were able to demonstrate that SKI-II inhibited SK activity in a concentration-dependent fashion in mouse kidney and HK-2 cell extracts (M. Kim and H. T. Lee et al., unpublished data). We also examined the role of S1P receptor blockade using the S1P receptor antagonist, VPC23019, which behaves as a competitive antagonist at both the S1P1 and S1P3 receptors, although it is 10-fold selective for S1P1 vs. S1P3 (13). Administration of VPC23019 was also associated with a reversal of isoflurane-mediated renal protection. In addition, we demonstrated that mice lacking SK1 enzyme were not protected against renal IR injury with isoflurane. Due to the inherent concerns regarding the use of genetic knockout mice (e.g., alterations in the expression of unrelated proteins), we used both pharmacological inhibitors as well as genetic knockout mice to study the role of SK in renal IR injury. Taken together, these data indicate that both increased S1P formation, via upregulation of SK1, as well as S1P1 vs. S1P3 receptor activation are involved in mediating the protective effects of isoflurane in renal IR injury.

Renal IR injury is an inflammatory process involving multiple cellular and systemic responses, including complement activation, activation of proinflammatory cytokines and chemokines, and infiltration by leukocytes such as neutrophils,
macrophages, and T cells (6). Early studies focused primarily on the role of neutrophils in renal IR injury as these were the most identified cell type in tissue, but recent data are suggestive of an increasing role for T cells (19), including evidence that CD4/CD8 knockout mice were protected from renal IR injury (46). Leukocyte interactions with endothelial cells occur early in renal IR injury, promoting capillary plugging with erythrocytes and platelets, which interferes with restoration of blood flow upon reperfusion (57). Kidney cells are also directly involved in the inflammatory response, as tubular epithelial cells have been shown to modulate the inflammatory cascade, such as by inducing ICAM-1 expression (9) as well as upregulating complement 5a (15).

Early theories on the mechanisms of action of volatile anesthetics proposed that lipid solubility was the main factor in determining anesthetic potency, suggesting a lipophilic site, such as the plasma membrane, as the site of action (37, 41), although recent evidence points toward more specific molecular targets for anesthetic action (2). Volatile anesthetics have been shown to increase membrane fluidity and activate sphingomyelin hydrolysis to form ceramide, a precursor to S1P, in neuronal extracts (39, 43) and renal cortex (33). Based on this ability of volatile anesthetics to modulate the properties of the lipid bilayer, we posited that the anti-inflammatory and anti-necrotic effects of volatile anesthetics could be mediated by specific signaling pathways related to the plasma membrane.

The lysophospholipid S1P has been implicated as a cytoprotective signaling molecule balancing against the proapoptotic effects of sphingosine and ceramide via the putative “sphingolipid rheostat” (34, 50). In addition, S1P is involved in the regulation of cellular proliferation, cellular migration, and angiogenesis as well as the modulation of lymphocyte migration (49). S1P binds to specific GPCRs, of which five are known (S1P₁-₅) (50), and mediates its anti-apoptotic effects via a G protein-dependent pathway involving Akt and ERK signaling in hepatic myofibroblasts (12), lung epithelium (38), and melanocytes (25). S1P has been shown to signal in an “inside-out” manner by which intracellularly generated S1P acts in an autocrine/paracrine fashion to mediate GPCR-activated signaling (21). S1P has also been shown to inhibit apoptosis by acting as an intracellular second messenger independently of GPCRs, although no specific molecular targets have been found (40, 50). In our study, isoflurane-mediated protection required the effects of increased SK activity or S1P₁ receptor activation, suggesting that increased SK activity in the kidney led to higher levels of S1P that signaled via the S1P₁ receptor to mediate the cytoprotective effects of isoflurane.

S1P plays a role in modulating inflammatory processes, such as mediating TNF-α activation of endothelial cell adhesion molecules via ERK and NF-κB (56), reducing endothelial permeability in acute lung injury (20), and protecting endothelial cells from TNF-α-mediated monocyte interactions (5). The role of S1P receptors in inducing peripheral lymphopenia was discovered with the compound FTY720 (8), a sphingosine analog that is phosphorylated in vivo and has properties of an agonist at all five S1P receptors except S1P₁ (36). Lymphopenia was induced via sequestration of circulating lymphocytes in lymph nodes, but not spleen (8, 36). Further studies implicated S1P₁ as the specific GPCR mediating peripheral lymphopenia (17, 48). Platelets (3) and red blood cells (42) have been shown to be significant sources S1P and it remains to be determined whether volatile anesthetics affect the release of S1P into the circulation.

Two recent studies demonstrated the role of the S1P₁ receptor in mediating protection from renal IR injury and this was associated with lymphopenia as well as the inhibition of the proinflammatory cytokines TNF-α, P-selectin, and ICAM-1 (32) along with reduced vascular permeability (4). These results are consistent with our findings that S1P₁ receptor antagonism abrogates the renal protection mediated by isoflurane, although the exact mechanisms have yet to be elucidated.

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., PDGF, VEGF, NGF, and EGF), TGF-β, and TNF-α (21). Our study is the first to show that isoflurane activates SK. SK activation can occur via several different posttranslational mechanisms, such as phosphorylation, ubiquitination, and palmitoylation, and these effects are typically only seen for a few minutes following stimulation (21). SK activation lasting for longer periods of time has also been demonstrated, likely from upregulation of transcription, following PMA treatment in HEL cells (7) and estrogen treatment in MCF-7 breast cancer cells (51). An important aspect of SK activation is its translocation from the cytosol to the plasma membrane, as it is mainly a cytosolic protein while its substrate, sphingosine, is present in cell membranes (21, 44, 45).

In our study, HK-2 cells exposed to isoflurane exhibited increased SK activity at 9 and 16 h of volatile anesthetic exposure, but not at 3 h, suggesting that there is a time-dependent upregulation of SK transcription that is responsible for the increased SK activity seen after isoflurane exposure. In addition, the increase in SK activity was found in the plasma membrane fraction but not in the cytosolic fraction, suggesting that there is preferential localization of SK to the membrane where it can be in direct contact with its substrate. Our in vitro data are supported by our findings in mice showing that after 3 h of isoflurane exposure and 21 h of recovery, renal SK activity also increased in the membrane fraction relative to the cytosolic fraction. We also demonstrated that there was an increase in SK1 mRNA levels after isoflurane exposure both in vivo and in vitro.

One limitation of our study is that volatile anesthetics may have both local and systemic effects in renal IR injury, so it is difficult to determine in an in vivo study whether volatile anesthetics mediate their protective effects directly on renal tubular cells or via systemic effects on the migration and infiltration of leukocytes and neutrophils. The evidence suggests that the effects are likely multifactorial, including both direct cytoprotective effects on renal tubules with activation of prosurvival signaling pathways, such as the ERK and Akt pathways, as well as systemic anti-inflammatory mechanisms including peripheral lymphopenia, preservation of endothelial barrier integrity, and reduction of proinflammatory cytokines.

In conclusion, we demonstrated that isoflurane activates the SK/S1P signaling pathway to reduce necrosis and inflammation and the severity of renal failure after renal IR injury. Further elucidation of the mechanisms of protection may lead to advancement in the treatment of renal IR injury and ARF.
ACKNOWLEDGMENTS

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