Isoflurane Protects Human Kidney Proximal Tubule Cells against Necrosis via Sphingosine Kinase and Sphingosine-1-Phosphate Generation

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Key Words

Kidney injury, acute • Renal failure, acute • Proximal tubule • Anesthetics, volatile

Abstract

Background/Aims: We previously showed that the inhalational anesthetic isoflurane protects against renal ischemia reperfusion injury in part via sphingosine kinase (SK)-mediated synthesis of sphingosine-1-phosphate (S1P). In this study, we tested the hypothesis that isoflurane directly targets renal proximal tubule cells via SK activation, S1P synthesis and activation of S1P receptors to initiate cytoprotective signaling. **Methods and Results:** Isoflurane-mediated phosphorylation of extracellular signal-regulated kinase (ERK) and Akt and induction of HSP70 in human kidney proximal tubule (HK-2) cells were inhibited by dimethylsphingosine (DMS), an SK inhibitor, and VPC23019, an S1P_{1/3} receptor selective antagonist, in HK-2 cells. A selective S1P₁ receptor agonist, SEW2781, mimicked isoflurane-induced phosphorylation of ERK and Akt and induction of HSP70. Moreover, isoflurane-mediated protection against H₂O₂-induced necrosis of HK-2 cells was significantly attenuated by an S1P_{1/3} receptor antagonist, VPC23019, and by SK inhibitors DMS or 4-[[4-(4-chlorophenyl)-2-thiazolyl]amino]phenol. Finally, overexpression of the SK1 enzyme in HK-2 cells protected against

H₂O₂-induced necrosis. **Conclusions:** Collectively, our study demonstrates that S1P released via isoflurane-mediated SK1 stimulation produces direct anti-necrotic effects probably via S1P₁ receptor-mediated cytoprotective signaling (ERK/Akt phosphorylation and HSP70 induction) in HK-2 cells. Our findings may help to unravel the cellular signaling pathways of volatile anesthetic-mediated renal protection and lead to new therapeutic applications of volatile anesthetics during the perioperative period.

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Introduction

Perioperative acute kidney injury (AKI) due to renal ischemia and reperfusion (IR) injury is a frequent and devastating complication that lacks effective therapy [1]. Development of perioperative AKI leads to high mortality and is frequently complicated by many other life-threatening conditions including respiratory failure, sepsis and a multi-organ dysfunction syndrome [2]. Unfortunately, the mortality and morbidity rate from peri-

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operative AKI subsequently leading to renal failure has changed little over the past 50 years [1].

Volatile anesthetics are administered to virtually all patients subjected to general anesthesia in the United States. Volatile anesthetics, in addition to their anesthetic effects, have non-anesthetic properties in many organ systems including the kidney [3, 4]. We demonstrated previously that volatile anesthetics including isoflurane (2-chloro-2-(difluoromethoxy)-1,1,1-trifluoro-ethane) at a minimum alveolar concentration (MAC) of 1 (defined as the percent concentration in the alveolus of an inhaled anesthetic agent required to prevent 50% of subjects from moving in response to a painful stimulus when used as the sole anesthetic) protected against renal injury in vivo by reducing necrosis and inflammation [3, 5, 6]. Subsequently, we demonstrated that the volatile anesthetic isoflurane activates sphingosine kinase-1 (SK1) with increased sphingosine-1-phosphate (S1P) synthesis to mediate the renal protective effects in vivo [7].

We have also demonstrated previously that volatile anesthetics (0.5–2 MAC) provided significant cytoprotection and produced anti-necrotic and anti-inflammatory effects on cultured renal proximal tubules via direct activation of cytoprotective kinases (ERK, MAPK and Akt) and the induction of HSP70 [8]. In our previous in vivo studies we did not identify the specific cell type(s), e.g. proximal tubules versus leukocytes, targeted by SK activation in mediating the renal protective effects of volatile anesthetics. Reduction of renal IR injury via S1P receptor activation has been demonstrated by others [9–11] and the major mechanisms involved are proposed to be attenuation of the inflammatory response during and after IR via S1P₁ receptor activation. S1P₁ receptor-mediated modulation of the inflammatory response is well known including effects on lymphocyte egress and subsequent lymphopenia. However, it remains to be determined whether volatile anesthetic-mediated SK1 activation and S1P release reduce renal IR injury via direct protective effects on renal tubule cells. Specifically, it is not known whether volatile anesthetic-mediated S1P release provides direct renal tubule cell protection via S1P₁ receptor activation.

Therefore, in this study we tested the hypothesis that isoflurane directly protects human kidney proximal tubule (HK-2) cells in culture against oxidant (H₂O₂)-induced necrosis via SK1 activation and release of S1P with subsequent activation of S1P receptors (effects completely independent of S1P-mediated lymphopenia observed in vivo). We used cultures of human renal proximal tubules as this is the renal cell type most susceptible to ne-

crosis after renal IR injury [12, 13]. In addition, we determined whether lentivirus-mediated overexpression of the SK-1 enzyme would provide increased resistance against oxidant-induced necrosis in HK-2 cells.

Materials and Methods

2-Chloro-2-(difluoromethoxy)-1,1,1-trifluoro-ethane (isoflurane) was purchased from Abbott Laboratories (North Chicago, Ill., USA). N,N-Dimethylsphingosine (DMS) was purchased from Biomol International (Plymouth Meeting, Mass., USA). 4-[[4-(4-Chlorophenyl)-2-thiazolyl]amino]phenol (SKI-II) was purchased from Tocris Bioscience (Ellisville, Mo., USA). (R)-Phosphoric acid mono-[2-amino-2-(3-octyl-phenylcarbamoyl)-ethyl] ester (VPC23019) was purchased from Avanti Polar Lipids (Alabaster, Ala., USA). 5-[4-Phenyl-5-(trifluoromethyl)thiophen-2-yl]-3-[3-(trifluoromethyl)phenyl]1,2,4-oxadiazole (SEW-2871) was obtained from Tocris Bioscience. Unless otherwise specified, all other reagents were purchased from Sigma (St. Louis, Mo., USA).

HK-2 Cell Culture and Generation of SK1-Overexpressing HK-2 Cells

HK-2 cells (immortalized human proximal tubular cell line, American Type Culture Collection, Manassas, Va., USA) were grown and passaged in culture medium (50:50 mixture of DMEM low glucose and F12 plus 5% serum) and antibiotics (100 U/ml of penicillin G, 100 µg/ml of streptomycin, and 0.25 µg/ml of amphotericin B) at 37°C in a 100% humidified atmosphere of 5% CO₂-95% air. All of the treatments were performed after cells were maintained for 24 h in serum-free basal media.

We generated HK-2 cells overexpressing wild-type SK1 [14] by infecting HK-2 cells with SK1 encoding lentivirus. Lentivirus encoding wild-type SK1 were produced by subcloning the SK1 cDNA into a modified shuttle vector CMV-pLL3.7 where the insert expression is driven by a CMV promoter followed by an IRES-enhanced green fluorescent protein (EGFP) sequence for simultaneous co-expression of 2 independent proteins; the EGFP reporter gene and SK1 fused to a hemagglutinin (HA) tag. Lentivirus was produced by a triple transfection of CMV-SK1-pLL3.7 (10 µg), pVSVG (Vesicular stomatitis virus G, Invitrogen, Carlsbad, Calif., USA; 7 µg), and pΔ8.9 (from Dr. Van Parjs, MIT, Cambridge Mass., USA; 5 µg) as described [15]. In brief, CMV-SK1-pLL3.7 and the 2 packaging vectors were cotransfected into 80–90% confluent HEK-293FT cells in 10-cm tissue culture plates using 20 µl of Lipofectamine 2000 (Invitrogen) in serum-free OptiMEM medium according to the manufacturer's recommendations. Supernatant was collected after 48 h and passed through a 0.45-µm filter to remove cells and debris. An approximate viral titer was determined by infecting HEK293 cells with serial dilutions of the final virus suspension and counting the number of fluorescent cells 48 h after infection. We typically obtained titers of 2–5 × 10⁶ infectious units/ml starting from one 10-cm plate of HEK293FT cells. Cell culture medium was removed from HK-2 cells growing on 6-well plates and replaced with 1.5 ml of lentivirus medium and incubated at 37°C. After 48 h, the lentivirus medium was replaced with cell culture medium and infected HK-2 cells were propagated and used for studies.

Exposure of HK-2 Cells to Isoflurane

For isoflurane treatment, HK-2 cells in 10-cm plates were placed in an airtight, 37°C, humidified modular incubator chamber (Billups-Rothenberg, Inc., Del Mar, Calif., USA) with inflow and outflow connectors as described previously [8, 16]. The inlet port was connected to an in-line calibrated agent specific vaporizer (Datex-Ohmeda) to deliver varying concentrations of isoflurane (2.5%) mixed with carrier gas (95% air and 5% CO₂) at 10 liters/min. The outlet port was connected to a Datex-Ohmeda 5250 RGM gas analyzer that measured volatile anesthetic concentrations. We previously demonstrated that clinically relevant concentrations of volatile anesthetics (0.5–2 MAC) produced cytoprotective signaling in HK-2 cells [8]. For this study, we exposed HK-2 cells to 1–2 MAC or 1.2–2.5% isoflurane for 4–14 h at 37°C. Control cells were exposed to carrier gas in an identical modular incubator chamber.

Some HK-2 cells were pretreated with a selective S1P₁ receptor agonist (SEW-2781, 1 μM) for either 5–180 min or for 1–24 h instead of isoflurane treatment. Separate cohorts of HK-2 cells were pretreated for 30 min with either a selective S1P_{1/3} receptor antagonist VPC23019 (10–100 μM), or sphingosine kinase inhibitors; DMS (10–100 μM) or SKI-II (5 μM), and exposed to 2.5% isoflurane. In preliminary experiments, we were able to demonstrate that DMS and SKI-II inhibited SK activity [7] in a concentration-dependent fashion in mouse kidney and HK-2 cell extracts.

Induction of HK-2 Cell Necrosis

In preliminary studies, we determined that isoflurane treatment failed to attenuate apoptosis, induced with tumor necrosis factor-α (TNF-α, 10 ng/ml) plus cycloheximide (5 μg/ml) for 16 h, in HK-2 cells (assessed with DNA laddering, PARP and caspase 3 immunoblotting). Confluent HK-2 cells, EGFP HK-2 cells and EGFP-SK1 HK-2 cells were incubated with 2.5% isoflurane for 14 h and subjected to necrotic injury with 2 mM H₂O₂ for 0–6 h as described [17]. We determined in preliminary studies that the ability of H₂O₂ to kill HK-2 cells is dose- and time-related. High (e.g. 1–10 mM) doses of H₂O₂ killed cells rapidly (within 1 h), whereas lower doses (e.g. 1–10 μM) killed cells more slowly. Our preliminary studies also showed that our treatment with H₂O₂ (for 1–6 h) causes negligible apoptosis (PARP and caspase 3 immunoblotting and DNA laddering).

Measurement of Cell Viability with LDH

LDH released into cell culture media as indices of rapid, necrotic cell death were measured using an LDH assay kit from Promega (Madison, Wisc., USA). Released LDH from cells was expressed as a percentage of total cellular LDH (LDH released into the media following cell lysis with 1% Triton-X for 5 min).

Immunoblot Analyses

Immunoblotting analyses were performed as described previously [18]. The primary antibodies for phospho-ERK1/2, total ERK and β-actin were from Santa Cruz Biotechnologies (Santa Cruz, Calif., USA). The primary antibody for total HSP70, phospho-Akt and total Akt1 were from Cell Signaling Technologies (Danvers, Mass., USA). The primary antibodies for S1P₁ and S1P₃ receptors were obtained from Cayman Chemical (Ann Arbor, Mich., USA). The primary antibodies for S1P₂, S1P₄ and S1P₅ receptors were from GenWay Biotech, Inc. (San Diego, Calif., USA).

The antibody for the inducible form of HSP-70 was from Stressgen Biotechnologies (San Diego, Calif., USA). The primary antibody for SK1 was from ECM Biosciences (Versailles, Ky., USA). Phospho-ERK or phospho-Akt blots were stripped and reprobed for total ERK or total Akt1. The secondary antibody (goat anti-rabbit or anti-mouse IgG conjugated to horseradish peroxidase at 1:5,000 dilution) was detected with enhanced chemiluminescence immunoblotting detection reagents (Amersham), with subsequent exposure to a CCD camera coupled to a UVP Bio-imaging System (Upland, Calif., USA) and a personal computer. The band intensities of the immunoblots were within the linear range of exposure for all experiments.

High-Pressure Liquid Chromatography Detection of S1P

Confluent HK-2 cells were sonicated in PBS (pH 7.2). Aliquots were used for protein assay and the remainder of HK-2 cellular lysates were processed for high-pressure liquid chromatography (HPLC) to measure S1P levels as described by Min et al. [19] with two steps of sample pretreatment: enzymatic dephosphorylation of S1P by alkaline phosphatase (100 U/sample, Sigma) and subsequent analysis of o-phthalaldehyde derivatives of the liberated sphingosine bases by HPLC. By introducing C17 S1P (Avanti Polar Lipids, Inc.) as an internal standard, S1P present in a sample can be quantified on a C18 reversed-phase column with a simple mobile phase of acetonitrile:deionized distilled water (89:11, v/v) and expressed as fold increase over the carrier gas-treated group.

Reverse Transcription-Polymerase Chain Reactions for SK1 and S1P₁₋₅ Receptors

We detected mRNA expression of S1P receptor subtypes 1–5 with reverse transcription-polymerase chain reaction (RT-PCR) as described previously using the Access RT-PCR System (Promega) [17]. We also tested for the overexpression of SK1 mRNA after lentivirus infection in HK-2 cells. SK1 and S1P₁₋₅ primers were designed based on published GenBank sequences for human (table 1) and to amplify a genomic region that spans one or more introns to eliminate the confounding effect of amplifying contaminating genomic DNA. For each experiment, we also performed semiquantitative RT-PCR under conditions yielding linear results for GAPDH (table 1) to confirm equal RNA input.

SK1 Activity Assay

SK1 activity was measured as described by Vessey et al. [20] with some modification as described [7].

Statistical Analysis

The data were analyzed with Student's t test when comparing means between 2 groups or with one-way analysis of variance plus Bonferroni post hoc multiple comparison test to compare mean values across multiple treatment groups. In all cases, a probability statistic <0.05 was taken to indicate significance. All data are expressed throughout the text as mean ± SEM.

Protein Determination

Protein content was determined with the Pierce Chemical (Rockford, Ill., USA) bicinchoninic acid protein assay reagent with BSA as a standard.

Fig. 1. Total RNA from HK-2 cells was extracted and subjected to RT-PCR. Molecular weight (M) markers are shown on the left. Messenger RNAs encoding 5 subtypes of S1P receptors (R) are present in human proximal tubule (HK-2) cells. Representative of 4 experiments.

Fig. 2. Isoflurane (2.5% for 3, 6 or 14 h) increases S1P synthesis in HK-2 cells ($n = 5$) compared to carrier gas-treated cells ($n = 6$). PMA (100 μ M for 6 h, $n = 5$) was used as a positive control. * $p < 0.05$ vs. carrier gas-treated cells.

Table 1. Primer sequences

Primers	Species	Size, bp	Sequence, sense/antisense	Annealing, °C/cycle No.
GAPDH	Human	450	5'-ACCACAGTCCATGCCATCAC-3' 5'-CACCACCCTGTTGCTGTAGCC-3'	65/15
SK-1	Human	330	5'-ATCTCCTTCACGCTGATGC-3' 5'-GTGCAGAGACAGCAGGTTCA-3'	66/26
S1P1	Human	434	5'-CGAGAGCACTACGCAGTCAG-3' 5'-AGCAGCAGATGAGAATGAAC-3'	64/19
S1P2	Human	249	5'-GCCTAGCCAGTTCTGAAAGC-3' 5'-GAGTGGAACCTTGCTGTT-3'	58/22
S1P3	Human	428	5'-ATCTGGCATTCGAGCGCAGG-3' 5'-GGCAATCAAAACCATCAGGT-3'	64/27
S1P4	Human	361	5'-ACATCACGCTGAGTGACCTG-3' 5'-AAGAGGATGTAGCGCTTGGA-3'	66/27
S1P5	Human	390	5'-ACCAGGGCGCAGACCTTG-3' 5'-AGTGAGTGCCACGAAGACG-3'	62/27

Fig. 3. A A selective S1P_{1/3} receptor antagonist VPC23019 (VPC, 10 or 100 μ M) attenuates isoflurane (2.5%)-induced ERK and Akt phosphorylation (4 h treatment) and HSP70 induction (14 h treatment). Representative of 4 independent experiments. **B** Inhibition of sphingosine kinase (SK) with dimethylsphingosine (DMS, 10 or 100 μ M) also attenuated the isoflurane-mediated phosphoryla-

tion of ERK and Akt and induction of HSP70 in HK-2 cells. Representative of 4 independent experiments. Bottom panel of each figure shows densitometric quantifications of relative band intensities for immunoblotting experiments ($n = 4$). * $p < 0.05$ vs. vehicle-treated cells in carrier gas group. # $p < 0.05$ vs. vehicle-treated cells in isoflurane group. Means $\pm$ SE.

Results

Expression of S1P₁₋₅ Receptors in HK-2 Cells

With RT-PCR, we show that mRNAs for all 5 S1P receptor subtypes (S1P₁₋₅) are present in HK-2 cells (fig. 1, representative of 4 experiments). No bands were detected after RT-PCRs in water blanks with S1P₁₋₅ primers or with GAPDH primers. Moreover, with immunoblotting we also determined that proteins for all 5 S1P receptor subtypes are present in HK-2 cells (data not shown).

Isoflurane Increases S1P Synthesis in HK-2 Cells

We utilized HPLC to measure the formation of S1P in HK-2 cells after carrier gas (95% room air plus 5% CO₂ for 14 h) or isoflurane treatment. We demonstrate that a clinically relevant concentration of isoflurane increases S1P synthesis in HK-2 cells in a time-dependent manner (fig. 2). Isoflurane (2.5% or 2 MAC) treatment for 3, 6 or 14 h increased S1P formed in HK-2 cell lysates to 1.6 ± 0.08 -fold ($n = 5$, $p < 0.05$), 2.2 ± 0.3 -fold ($n = 5$, $p < 0.05$) and 3.0 ± 0.55 -fold ($n = 5$, $p < 0.05$), respectively, compared to the carrier gas-treated group (fig. 2). The protein

Fig. 4. A selective $S1P_1$ receptor agonist (SEW-2871, 1 μ M) phosphorylates ERK MAPK and Akt (5–180 min treatment, **A**) and upregulates HSP70 (1–24 h treatment, **B**) in HK-2 cells. Representative of 4 separate experiments. Bottom of each panel shows densitometric quantifications of relative band intensities for immunoblotting experiments ($n = 4$). * $p < 0.05$ vs. time zero. Means $\pm$ SE.

kinase C activator, phorbol 12-myristate 13-acetate (PMA), was used as a positive control for SK stimulation and $S1P$ synthesis. PMA (100 μ M for 6 h) increased $S1P$ synthesis compared to the carrier gas-treated group (fig. 2).

$S1P_{1/3}$ Receptor Antagonism or SK Inhibition Attenuates Isoflurane-Mediated Phosphorylation of ERK MAPK and Akt and Induction of HSP70

We have previously demonstrated that volatile anesthetics induce ERK/Akt phosphorylation and HSP induction that peak at 4 and 14 h, respectively [18]. HK-2 cells were first treated with a selective $S1P_{1/3}$ receptor antagonist VPC23019 (10 or 100 μ M) or with vehicle (1% DMSO) and exposed to 2.5% isoflurane or to carrier gas (95% room air plus 5% CO_2) for 4 h (to induce ERK and Akt phosphorylation, $n = 4$) or 14 h (to induce HSP70 protein synthesis, $n = 4$). VPC23019 attenuated the increases in ERK MAPK and Akt phosphorylation and HSP70 induction in HK-2 cells (fig. 3A). Furthermore, inhibition of SK with DMS (10 or 100 μ M, $n = 4$) also attenuated the iso-

flurane-mediated phosphorylation of ERK and Akt as well as HSP70 in HK-2 cells (fig. 3B).

Selective $S1P_1$ Receptor Agonist Mimics Volatile Anesthetic Signaling in HK-2 Cells

We have previously demonstrated that volatile anesthetics induce phosphorylation of ERK MAPK and Akt as well as upregulation of HSP70 synthesis [8]. Figure 4 shows that a selective $S1P_1$ receptor agonist (SEW-2871, 1 μ M) acutely but transiently phosphorylates ERK MAPK and Akt proteins (5–180 min treatment; fig. 4A) and upregulates HSP70 protein (1–24 h treatment; fig. 4B) in HK-2 cells ($n = 4$) mimicking the effects of volatile anesthetics in cultured proximal tubule cells.

$S1P_{1/3}$ Receptor Antagonism or SK Inhibition Attenuates Isoflurane-Mediated Protection against Necrosis in HK-2 Cells

We have demonstrated previously that HK-2 cells are subject to rapid necrosis when treated with H_2O_2 in a

dose- (1–5 mM) and time- (1–6 h) dependent manner [21]. Confluent HK-2 cells were incubated with 2.5% isoflurane or with carrier gas for 14 h and subjected to necrotic injury with 2 mM H₂O₂ for 4 h. Carrier gas-treated HK-2 cells subjected to H₂O₂-induced necrosis released significantly increased amounts of intracellular LDH into the cell culture media compared to the vehicle-treated HK-2 cells. Isoflurane-treated cells released significantly less LDH compared to cells exposed to carrier gas after H₂O₂ injury (fig. 5). However, the SK inhibitors (20 μM DMS, n = 6, or 5 μM SKI-II) or a S1P_{1/3} receptor antagonist (VPC23019, 20 μM) prevented this protection by isoflurane without affecting cellular necrosis in carrier gas-treated cells (fig. 5).

Overexpression of SK1 Enzyme Increases HK-2 Cell Resistance against H₂O₂-Induced Necrosis

Utilizing lentivirus infection, we overexpressed EGFP-SK1 or EGFP in HK-2 cells and generated HK-2 cells stably expressing either EGFP or EGFP-SK1. Figure 6 shows increased protein and mRNA expression in EGFP-SK1 lentivirus-infected HK-2 cells compared to EGFP lentivirus-infected HK-2 cells (representative of 3 experiments). SK enzyme activity in EGFP-SK1-overexpressing HK-2 cells were significantly increased compared to the SK activity in EGFP-overexpressing HK-2 cells (3.2 ± 0.3-fold, p < 0.05, n = 4). We subjected control (uninfected) HK-2 cells, EGFP HK-2 cells and EGFP-SK1 HK-2 cells to H₂O₂-mediated necrosis (2 mM for 0–4 h). Figure 7 shows that EGFP-SK1-overexpressing HK-2 cells release significantly less LDH after H₂O₂ injury compared to control HK-2 cells or to EGFP HK-2 cells demonstrating significant resistance to necrosis.

Discussion

The major findings of this study are that in HK-2 cells: (1) a clinically relevant concentration of isoflurane (2 MAC)-mediated phosphorylation of ERK and Akt and induction of HSP70 was inhibited by the SK inhibitor (dimethylsphingosine) and the S1P_{1/3} receptor selective antagonist (VPC23019); (2) the selective S1P₁ receptor agonist SEW2781 mimicked isoflurane-induced phosphorylation of ERK and Akt and induction of HSP70; (3) isoflurane-mediated protection against H₂O₂-induced necrosis was significantly attenuated by the S1P_{1/3} receptor antagonist VPC23019 and by the SK inhibitors DMS or SKI-II, and (4) overexpression of the SK1 enzyme in HK-2 cells protected against H₂O₂-induced necrosis.

Fig. 5. Inhibition of sphingosine kinase (SK) with dimethylsphingosine (DMS, 20 μM) or with 4-[[4-(4-chlorophenyl)-2-thiazolyl]-aminophenol (SKI-II, 5 μM) or blocking the S1P_{1/3} receptor with VPC-23019 (10 μM) prevents isoflurane-mediated protection against H₂O₂ necrosis in HK-2 cells. HK-2 cells were incubated with 2.5% isoflurane or with carrier gas for 14 h and subjected vehicle treatment or to necrotic injury with 2 mM H₂O₂ for 4 h (n = 6 each). * p < 0.05 vs. vehicle-treated cells. # p < 0.05 vs. carrier gas-treated cells. Means ± SE.

Fig. 6. HK-2 cells infected with EGFP-SK1 encoding lentivirus (EGFP-SK1 cells) for 2 days show increased SK1 protein and mRNA expression compared to EGFP encoding lentivirus-infected cells. Representative of 3 experiments.

Fig. 7. EGFP-SK1-overexpressing HK-2 cells are protected against H_2O_2 -induced necrosis. Control HK-2 cells (HK-2, $n = 5$), EGFP-overexpressing HK-2 cells (EGFP HK-2, $n = 5$) or EGFP-SK1-overexpressing HK-2 cells (EGFP-SK1 HK-2, $n = 5$) were subjected to 0–4 h of 2 mM H_2O_2 treatment. * $p < 0.05$ vs. HK-2 cells at time 0. # $p < 0.05$ vs. HK-2 cells at each time point.

We demonstrated in previous studies that clinically utilized volatile anesthetics, including isoflurane, provided significant protection against renal IR injury in mice and rats with improved renal function and a profound reduction in renal tubular necrosis [3, 6, 7, 22]. Furthermore, we showed that mice anesthetized with isoflurane demonstrated reduced inflammatory changes in the kidney including a reduction in proinflammatory leukocyte influx (neutrophils, lymphocytes and macrophages) as well as decreased proinflammatory mRNA upregulation after renal IR injury in vivo [6]. Our subsequent studies demonstrated that isoflurane stimulates SK enzyme in the murine kidney and this SK activation and release of S1P provided powerful renal protection in a mouse renal IR injury model [7]. The lysophospholipid S1P has been implicated as a cytoprotective signaling molecule balancing against the pro-apoptotic effects of sphingosine and ceramide via the putative ‘sphingolipid rheostat’ [23, 24]. In addition, S1P is involved in the regulation of cellular proliferation, cellular migration and angiogenesis as well as the modulation of lymphocyte migration [25]. S1P binds to specific G protein-coupled receptors, of which five are known (S1P_{1–5}) [26].

However, with in vivo studies, it is difficult to determine whether isoflurane-mediated SK activation and S1P

release cause direct renal tubular protection and/or cause renal protection via modulating the inflammatory response. Indeed, S1P₁ receptor-mediated immune modulation via inducing lymphopenia is well known [27] and several studies demonstrate powerful protective effects of S1P₁ receptor activation in a murine model of renal IR injury [9–11]. S1P receptor activation also produces non-lymphocyte-related effects such as mediating TNF- α activation of endothelial cell adhesion molecules via ERK and NF- κ B [28], reducing endothelial permeability in acute lung injury [29], and protecting endothelial cells from inflammatory and apoptotic stimuli [30]. In vitro, S1P₁ receptor activation in endothelial cells produces attenuation of endothelial apoptosis with subsequent strengthening of endothelial integrity [28, 31]. However, the direct protective effect of S1P₁ receptor activation on renal proximal tubular cells independent of lymphocyte modulation has never been examined. Therefore, until this study, it was unknown whether S1P released with volatile anesthetic treatment can directly target proximal tubule cells, the cell type most susceptible to renal IR injury (S3 segments of proximal tubules).

Our previous studies demonstrated direct protective effects of volatile anesthetics on renal proximal tubules suggesting that volatile anesthetics may directly target renal proximal tubule cells to mediate renal protection. We showed previously remarkable protection of the S3 segment of proximal tubules after renal IR injury in rat and mouse models in vivo [6, 22]. Subsequently, we have demonstrated that volatile anesthetics can directly protect proximal tubule cells against necrosis and inflammation in vitro [8]. Volatile anesthetics also produce cytoprotective signaling cascades (ERK MAPK and Akt phosphorylation and HSP70 induction) in cultured proximal tubule cells [8]. Taken together with the findings of our current study, we propose that isoflurane generates cytoprotective signaling (ERK and Akt phosphorylation and HSP70 induction) and protects against necrosis via SK activation and subsequent activation of S1P receptors in HK-2 cells.

We focused on cultures of proximal tubules (S3 segment origin in particular) as these cells are the primary site of injury in renal IR [12, 32]. This region of the proximal tubule is marginally oxygenated under normal physiological conditions with a high basal metabolic demand [12, 33]. Therefore, with IR injury, proximal tubules in the outer medullary zone suffer the most damage. In this study, we injured HK-2 cells with H_2O_2 to produce necrosis. We previously demonstrated that H_2O_2 causes dose-dependent necrosis in HK-2 cells [34]. Oxygen free radical generation with resultant oxidant tissue

stress is a major cause of renal reperfusion injury [35, 36]. During reperfusion after ischemia, reactive oxygen species, such as superoxide anion, hydroxyl radical, and H_2O_2 , are generated. These reactive oxygen species cause lipid peroxidation of the renal cell membrane with resultant intracellular calcium overload and subsequent necrotic cell death [36–38]. Lack of protection against TNF- α - and cycloheximide-induced apoptosis with isoflurane treatment suggests that isoflurane-induced S1P release is insufficient to reduce caspase-mediated apoptosis pathways. This is in contrast with the anti-apoptotic effects of S1P in other cell types including endothelial cells [39, 40].

We demonstrate an important role for SK/S1P signaling in mediating isoflurane-mediated antinecrotic effects in HK-2 cells (fig. 3–5). We found that two well-known SK inhibitors, DMS and SKI-II, reversed the isoflurane-mediated protection from necrosis with H_2O_2 treatment. DMS is a well-known inhibitor of the SK enzyme [5, 21], whereas SKI-II is a newly developed SK inhibitor with a higher potency and selectivity [17]. We were able to demonstrate that SKI-II inhibited SK activity in a concentration-dependent fashion in mouse kidney and HK-2 cell extracts [unpublished data]. We also examined the role of S1P receptor blockade using the S1P_{1/3} receptor antagonist VPC23019. VPC23019 behaves as a competitive antagonist at both the S1P₁ and S1P₃ receptors, although it is ~50-fold more selective for the S1P₁ versus S1P₃ receptor but is completely inactive at the S1P₂ receptor [41]. Pretreatment with VPC23019 was also associated with a reversal of isoflurane-mediated protection against HK-2 cell necrosis. Tak-

en together, these data indicate that both increased S1P formation, via an increase in SK enzyme activity, as well as S1P_{1/3}-receptor activation are involved in mediating the protective effects of isoflurane in HK-2 cell necrosis.

One of the limitations of this study is that the data presented in this study are largely pharmacological, which has the potential to have nonspecific effects. Furthermore, although the overexpression data are quite convincing and establish the link between isoflurane and the S1P₁ receptor, they do not directly address the effect of isoflurane. Isoflurane could still be utilizing additional mechanisms to produce the cytoprotective effects. Studies directly targeting S1P₁ receptor deletion (with S1P₁ receptor knockout mice) or knockdown (with siRNA for S1P₁ receptor) to show elimination of the isoflurane effect should be performed in the future to complement our current studies.

In conclusion, we demonstrated in this study that isoflurane directly activates the SK and S1P receptor signaling pathway in HK-2 cells. Activation of S1P₁ receptors mimicked the signaling pathways of isoflurane in HK-2 cells. We also demonstrated that overexpression of the SK1 enzyme itself can produce protection against H_2O_2 -induced necrosis in HK-2 cells. These effects of isoflurane can mechanistically explain the powerful reduction in necrosis and inflammation and the severity of renal failure after renal IR injury. Further elucidation of the mechanisms of protection as well conclusive elucidation of the specific S1P receptor subtype may lead to advancements in the treatment of renal IR injury and acute renal failure.

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