Anti-inflammatory and antinecrotic effects of the volatile anesthetic sevoflurane in kidney proximal tubule cells

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Lee, H. Thomas, Mihwa Kim, Michael Jan, and Charles W. Emala. Anti-inflammatory and antinecrotic effects of the volatile anesthetic sevoflurane in kidney proximal tubule cells. Am J Physiol Renal Physiol 291: F67–F78, 2006. First published February 14, 2006; doi:10.1152/ajprenal.00412.2005.—Renal ischemia-reperfusion (IR) injury is a major clinical problem without effective therapy. We recently reported that volatile anesthetics protect against renal IR injury, in part, via their anti-inflammatory properties. In this study, we demonstrate the anti-inflammatory and antinecrotic effects of sevoflurane in cultured kidney proximal tubule cells and probe the mechanisms of sevoflurane-induced renal cellular protection. To mimic inflammation, human kidney proximal tubule (HK-2) cells were treated with tumor necrosis factor-α (TNF-α; 25 ng/ml) in the presence or absence of sevoflurane. In addition, we studied the effects of sevoflurane pretreatment on hydrogen peroxide (H2O2)-induced necrotic cell death in HK-2 or porcine proximal tubule (LLC-PK1) cells. We demonstrate that sevoflurane suppressed proinflammatory effects of TNF-α evidenced by attenuated upregulation of proinflammatory cytokine mRNA (TNF-α, MCP-1) and ICAM-1 protein and reduced nuclear translocation of the proinflammatory transcription factors NF-κB and AP-1. Sevoflurane reduced necrotic cell death induced with H2O2 in HK-2 cells as well as in LLC-PK1 cells. Sevoflurane treatment resulted in phosphorylation of prosurvival kinases, ERK and Akt, and increased de novo HSP-70 protein synthesis without affecting the synthesis of HSP-27 or HSP-32. We conclude that sevoflurane has direct anti-inflammatory and antinecrotic effects in vitro in a renal cell type particularly sensitive to injury following IR injury. These mechanisms may, in part, account for volatile anesthetics’ protective effects against renal IR injury.

acute renal failure; HK-2 cells; inflammation; necrosis; perioperative period

THE RISK OF ACUTE RENAL FAILURE following intraoperative renal ischemia and reperfusion (IR) is significantly increased in patients with impaired preoperative renal function (33), and the onset of acute renal failure is associated with increased mortality and morbidity (1, 54). Unfortunately, the prognosis of acute renal failure in the perioperative period has changed little for the last 40 years (32, 37). The vast majority of patients undergoing surgery are exposed to volatile anesthetics such as sevoflurane during the intraoperative period. We recently demonstrated that volatile anesthetics have protective effects against renal IR injury in rats (29). This in vivo protection was associated with significantly less necrosis, better preservation of renal tubular architecture as well as reduced inflammation, and reduced neutrophil influx into the kidney. However, it is unclear whether volatile anesthetics reduce necrosis and inflammation of the kidney via direct protective effects on renal tubule cells and/or via indirect effects (e.g., via reducing proinflammatory leukocyte influx and attenuating inflammation caused by these cells). Specifically, it is not known whether volatile anesthetics have direct antinecrotic and/or direct anti-inflammatory effects in vitro in cultured proximal tubule cells. Moreover, the renal cell type potentially protected by volatile anesthetics is not known.

We aimed to determine whether volatile anesthetics directly reduce oxidant-induced necrosis (induced with H2O2) and inflammation (induced with TNF-α and H2O2) in vitro. We studied sevoflurane [2,2,2-trifluoro-1-(trifluoromethyl) ethyl ether] as a representative volatile anesthetic as this anesthetic is a widely used inhalational anesthetic in the US. We used cultures of human renal proximal tubules (HK-2 cells) as this is the renal cell type most susceptible to necrosis after renal IR injury (30, 51). We also utilized a cultured pig kidney cell line typical of normal kidney tubular epithelium (LLC-PK1) to confirm the antinecrotic renal effects of sevoflurane in another proximal tubule cell type. We hypothesized that as we demonstrated in vivo, sevoflurane treatment would protect against oxidant-mediated necrotic injury as well as reduce inflammation in renal tubular cells. In addition, we also aimed to determine the signaling pathways of sevoflurane-mediated protection against oxidant injury in renal proximal tubular cells. Increased cell survival after IR injury has been demonstrated via activation of prosurvival kinases (ERK and Akt) as well as by increased synthesis of heat shock proteins (e.g., HSP-70) which act as cellular chaperones to protect against injury. Among members of the HSP family, HSP-27, -32 (also known as heme oxygenase-1), and -70 are well-known protectants against renal IR injury (31, 43, 66). Therefore, we determined whether sevoflurane activated ERK and Akt and tested the ability of sevoflurane to upregulate the expression of heat shock proteins (HSP-27, -32, or -70) in renal tubular cells.

MATERIALS AND METHODS

Cell culture. HK-2 and LLC-PK1 cells (immortalized human proximal tubule and porcine renal tubular cell lines, respectively; American Type Culture Collection, Manassas, VA) were grown and passaged in culture medium (keratinocyte serum-free medium plus 5 ng/ml epidermal growth factor and 40 mg/ml bovine pituitary extract for HK-2 cells and medium 199 plus 5% serum for LLC-PK1 cells) 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. These cell lines have been characterized extensively and retain the phenotypic and functional characteristics of proximal tubule cells in culture (16, 51). They were plated...
in 6- or 24-well plates when 80% confluent and used in the experiments described below when confluent.

Exposure of cultured renal tubule cells to sevoflurane. For the sevoflurane treatment, HK-2 cells were placed in an air-tight, 37°C, humidified modular incubator chamber (Billups-Rothenberg, Del Mar, CA) with inflow and outflow connectors. The inlet port was connected to the in-line agent-specific calibrated sevoflurane vaporizer (Datex-Ohmeda) to deliver sevoflurane [0.55–4.4% or 0.25–2 minimum alveolar concentration (MAC)] mixed with 95% air-5% CO2 at 10 l/min. The outlet port was connected to a Datex-Ohmeda 5250 RGM gas analyzer that measured sevoflurane concentrations. Exposure to sevoflurane lasted 1–16 h. Control cells were exposed to 95% air-5% CO2 in a modular incubator chamber.

Induction of proximal tubule cell necrosis with H2O2. After indicated pretreatment with or without 2.2% (1 MAC) sevoflurane (1–16 h), confluent monolayers of HK-2 or LLC-PK1 proximal tubule cells grown in 6- or 24-well plates were treated with 1–5 mM H2O2 for 2–8 h at 37°C in a 95% air-5% CO2 incubator. We demonstrated previously that H2O2-induced necrosis in HK-2 or LLC-PK1 cells is dose and time dependent (26). We chose to utilize 1–5 mM H2O2 as published experiments demonstrated that these doses killed renal tubular cells rapidly (~70% total LDH released within 1 h). Lower doses (~0.5–2 mM) killed cells more slowly (3–16 h). The doses we proposed are comparable to those employed previously in both the characterization of this cell line and in its validation as a cell culture model of oxidant injury (51).

To determine whether inhibition of Akt or ERK reduced or prevented sevoflurane-mediated proximal tubule protection, an inhibitor of MEK1 (50 μM PD-98059 for 30 min) or phosphatidylinositol 3-kinase (PI3K) (100 nM wortmannin for 30 min) was added before 16-h sevoflurane treatment. Some cells were treated with inhibitors of signaling intermediates only (no sevoflurane or H2O2) to determine the effects of these inhibitors on cell survival. To determine whether inhibition of ERK or Akt at the time of H2O2 injury (after 16-h sevoflurane pretreatment) prevented sevoflurane-mediated proximal tubule protection, some cells were treated with an inhibitor of MEK1 or PI3K after sevoflurane treatment but before H2O2 injury. The doses of inhibitors were determined in preliminary experiments to selectively block MEK1 or PI3K by preventing ERK1/2 or Akt phosphorylation, respectively. Our preliminary studies indicate that our treatment protocols with TNF-α and H2O2 cause negligible apoptosis (data not shown).

Induction of proximal tubule cell inflammation with TNF-α and H2O2. In addition to oxidant stress, TNF-α has been implicated in initiating an inflammatory process after acute renal failure (47, 48). We therefore used TNF-α and H2O2 to mimic an inflammatory process occurring in vivo after renal IR injury. Confluent monolayers of HK-2 cells were treated with 10 ng/ml recombinant TNF-α (Calbiochem) or 100–500 μM H2O2 and exposed to 2.2% sevoflurane or air plus 5% CO2 in a modular incubator chamber for 2–16 h. The dose of TNF-α was determined in preliminary experiments to produce consistent inflammatory changes in renal tubular cells (data not shown).

Measurement of cell viability with LDH. LDH released into cell culture media as indexes of cell death was measured using an LDH assay kit from Promega (Madison, WI). LDH released into the media was expressed as the percentage of total cellular LDH per well measured after the cells were lysed with 1% Triton X-100.

Immunoblot analyses. We measured the activation (via phosphorylation) of extracellular signal-regulated protein kinase (ERK) mitogen-activated protein kinase (MAPK) and Akt (protein kinase B, PKB) by immunoblotting after treatments with 2.2% sevoflurane. Phospho-ERK and phospho-Akt blots were stripped and reprobed for total ERK and Akt. The densitometric quantification was expressed as a ratio of phosphorylated over total forms. We also measured expression of ICAM-1 protein in HK-2 cells after TNF-α or H2O2 treatment and the modulation of ICAM-1 protein expression following sevoflurane exposure. Immunoblotting was performed as described previously (26, 28). In brief, the HK-2 cells in six-well plates were washed twice with Hank’s balanced salt solution (HBSS) and scraped with 100 μl of calcium-free HBSS plus protease inhibitors (2 μg/ml leupeptin and 2 μg/ml aprotinin). Aliquots were used for protein assay and the remaining were mixed with an equal volume of sample buffer (62.5 mM Tris, pH 6.8, 2% SDS, 10% glycerol, and 5% β-mercaptoethanol, final concentration). Twenty to forty micrograms of each sample were electrophoresed at room temperature through discontinuous 10% SDS-polyacrylamide gels at 80 V for 4 h and subjected to immunoblot analysis. The primary antibodies for phospho-ERK1/2, HSP-70, HO-1 (HSP-32), and ICAM-1 were from Santa Cruz Biotechnology. Antibodies for phospho-Akt and HSP-27 were from Cell Signaling Technologies. The antibody for the inducible form of HSP-70 was from Stressgen Biotechnologies. 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 BioImaging System (Upland, CA) and a personal computer. The band intensities of the immunoblots were within the linear range of exposure for all experiments.

Semiquantitative RT-PCR for proinflammatory cytokines. RNA from HK-2 cells was extracted and RT-PCR reactions for proinflammatory mRNAs and HSPs (HSP-27, HO-1, HSP-70) were performed with the one-step RT-PCR kit as described previously (Access RT-PCR System, Promega) (29). The quantitative accuracy of our RT-PCR technique was first confirmed for each primer pair used. The PCR cycle number for each primer pair was optimized for linear increases in densitometric band intensity measurements with increasing PCR cycles from 14–22. The starting amount of RNA (0.25–1 μg) was also optimized for linear increase in densitometric band intensity measurements at an optimized cycle of PCR. We used primers designed to recognize human sequences based on sequences of proinflammatory genes (Table 1; Sigma Genosys, Woodlands, TX). They were chosen to yield expected PCR products of 200 to 600 bp, have a 50–60% GC content, and all span an intron to distinguish mRNA products from genomic DNA contamination. Five microliters of the RT-PCR product were analyzed on a 6% acrylamide gel stained with SYBR green (Invitrogen, Carlsbad, CA) for analysis with a UVP BioImaging System. Semiquantitative analysis of mRNA expression gene was accomplished by obtaining the ratio of the band density of the mRNA’s of interest to that of GAPDH (a housekeeping gene) from the same sample.

Nuclear protein extraction. HK-2 cells were scraped in 500 μl of buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 1.5 mM MgCl2, 20% glycerol, 0.2 mM PMSF, 0.5 mM DTT; Protease Inhibitor Cocktail, Roche, Indianapolis, IN) for 10 min at 4°C. The cells were homogenized using a polytron homogenizer for 5 s to release the nuclei into solution and centrifuged at 18,000 g for 5 min at 4°C. The supernatant was discarded and the pellet was resuspended in 50 μl of buffer B (20 mM HEPES, pH 7.9, 1.5 mM MgCl2, 0.5 mM EDTA, 25% glycerol, 0.1% Triton X-100, 0.2 mM PMSF, 0.5 mM DTT; Protease Inhibitor Cocktail) and incubated for 1 h at 4°C with occasional swirling to extract nuclear protein. The solubilized pellet was centrifuged at 16,000 g for 15 min and the supernatant containing nuclear proteins was used for EMSAs.

EMSA. EMSA was performed using the Gel Shift Assay System (Promega). The oligonucleotides for NF-κB and AP-1 (Promega) were used as templates and were end-labeled with 10 μCi of γ-32P ATP (Perkin Elmer Life Technology) and purified using a G-25 Spin Column (Amersham Biosciences). Ten micrograms of the nuclear extract were incubated with 1 μl of the labeled probe for 20 min at room temperature and electrophoresed on a 4% polyacrylamide gel (200 V at 4°C). Two micrograms of Hela nuclear extract (Promega) were used for positive control and 1 μl of a TransCruz polyclonal antibody for p65 subunit of NF-κB or antibody for c-fos (for AP-1;
Fig. 1. LDH release after H$_2$O$_2$ injury in HK-2 cells (A; $n = 6$) and LLC-PK$_1$ cells (B, C, D; $n = 9$) with or without sevoflurane (Sevo) pretreatment (2.2% for 16 h). Released LDH from cells was expressed as a percentage of total LDH (LDH released plus intracellular LDH). A: LDH release in HK-2 cells pretreated with 2.2% sevoflurane/air or air/5% CO$_2$ for 16 h and treated with 5 mM H$_2$O$_2$ for 0–8 h. B: LDH release in LLC-PK$_1$ cells pretreated with 2.2% sevoflurane/air + 5% CO$_2$ or air + 5% CO$_2$ for 16 h and treated with 5 mM H$_2$O$_2$ for 0–8 h. C: LDH release in LLC-PK$_1$ cells pretreated with 2.2% sevoflurane/air + 5% CO$_2$ or air + 5% CO$_2$ for 16 h and treated with 1, 2, or 5 mM H$_2$O$_2$ for 8 h. D: LDH release in LLC-PK$_1$ cells pretreated with 0.25–4.4% sevoflurane/air + 5% CO$_2$ or air + 5% CO$_2$ for 16 h and treated with 5 mM H$_2$O$_2$ for 0–8 h. E: LDH release in LLC-PK$_1$ cells pretreated with 2.2% sevoflurane/air + 5% CO$_2$ or air + 5% CO$_2$ for 1–16 h and treated with 5 mM H$_2$O$_2$ for 4 h. Error bars represent SE. A–E: *$P < 0.05$ vs. H$_2$O$_2$ injury without sevoflurane pretreatment.

Table 1. Primer sequences

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Sevoflurane reduces necrosis in HK-2 cells. HK-2 or LLC-PK1 cells pretreated with sevoflurane (0.55–4.4% or 0.25–2 MAC) released significantly less LDH following 5 mM H$_2$O$_2$ treatment compared with control cells exposed to 95% air-5% CO$_2$ for 16 h (Fig. 1, A and B). Specifically, HK2 cells treated for 8 h with 5 mM H$_2$O$_2$ released 25.2 ± 2.3% of total cellular LDH which was reduced to 18.1 ± 2.1% by 16 h of 2.2% sevoflurane pretreatment (n = 6, P < 0.05; Fig. 1A). Likewise, following 8 h of 5 mM H$_2$O$_2$, LLC-PK1 cells released 52.5 ± 2.0% of total cellular LDH which was reduced to 36.6 ± 0.8% in cells pretreated with 16 h of 2.2% (1 MAC) sevoflurane (n = 9, P < 0.05; Fig. 1B).

Following 16 h of 2.2% sevoflurane pretreatment, cells were exposed to 1, 2, or 5 mM H$_2$O$_2$ for 8 h at 37°C in a humidified 5% CO$_2$ incubator. 2.2% Sevoflurane pretreatment significantly decreased the amount of total cellular LDH released at each dose of H$_2$O$_2$ (Fig. 1C; LDH released with 1, 2, or 5 mM H$_2$O$_2$ treatment = 13.1 ± 1.5%, n = 9, P < 0.05), 19.3 ± 1.9% (n = 9, P < 0.05), and 36.6 ± 0.8% (n = 6, P < 0.05) after sevoflurane treatment, respectively, vs. LDH released = 24.5 ± 2.1% (n = 6), 32.5 ± 3.9% (n = 6), and 52.5 ± 2.0% (n = 6) without sevoflurane treatment. To evaluate the dose-dependent protection of sevoflurane against H$_2$O$_2$-induced necrosis in LLC-PK1 cells, LLC-PK1 cells were pretreated with 0.55, 1.1, 2.2, or 4.4% sevoflurane for 16 h before 4 h of 5 mM H$_2$O$_2$. Significant reduction in total cellular LDH release was achieved with 0.25–4.4% sevoflurane pretreatment (Fig. 1D). To evaluate the pretreatment time course of sevoflurane-mediated protection against H$_2$O$_2$-induced necrosis in LLC-PK1 cells, sevoflurane pretreatment was done for 2, 4, 10, or 16 h before 4 h of 5 mM H$_2$O$_2$. Significant reduction in total cellular LDH release was achieved with 4, 10, or 16 h of sevoflurane pretreatment (Fig. 1E). In contrast, sevoflurane pretreatment for 1–2 h did not protect against H$_2$O$_2$-induced necrosis.

Sevoflurane treatment results in activation of prosurvival kinases ERK and Akt. 2.2% sevoflurane treatment increased phosphorylation of ERK$_{1/2}$ and Akt within 1 h in HK-2 cells (Fig. 2, A and B) and LLC-PK1 cells (data not shown). ERK and Akt phosphorylation peaked at 4 h and then returned to baseline after the 4-h time point (Fig. 2B). The band intensities for total ERK and Akt did not change with increased phosphorylation of these proteins (data not shown).

Sevoflurane-induced protection is abolished by inhibition of Akt or ERK. Pretreatment with wortmanin or with PD-98059 before sevoflurane treatment blocked sevoflurane-mediated protection against H$_2$O$_2$ in HK-2 cells (Fig. 3) as well as LLC-PK1 cells (data not shown). LDH released from cells treated only with wortmanin or PD-98059 was similar to vehicle-treated cells. Inhibition of ERK or Akt at the time of H$_2$O$_2$ injury (after 16-h sevoflurane pretreatment) failed to prevent sevoflurane-mediated protection against H$_2$O$_2$ injury (data not shown).

Quantitative accuracy of the RT-PCR technique. All PCR methods by definition involve amplification of an original target and are thus at risk of losing quantitative accuracy. Both the RNA input and cycle number of PCR gave a result that was within the range of a linear response. Total RNA was isolated from freshly isolated proximal tubules and the quantitative accuracy of the RT-PCR technique was first confirmed. RT-PCR reactions for ICAM-1 mRNA are shown as a representative study; however, tests for quantitative nature of RT-PCR technique were performed for all of the primers used in this study. The PCR cycle for each primer pair was optimized for linear increases in densitometric measurements with increasing PCR cycles from 14–22 (Fig. 4, left). The starting amount of RNA (0.25–1 µg) was also optimized for linear increase in...
Sevoflurane decreases proinflammatory mRNA expression in HK-2 cells. Concomitant sevoflurane treatment caused time (Fig. 5)- and dose-dependent (data not shown) attenuation of proinflammatory mRNA upregulation in response to TNF-α. Figure 5A shows representative gel images of RT-PCR results for TNF-α, ICAM-1, and MCP-1 in HK-2 cells after TNF-α treatment or after TNF-α plus sevoflurane (representative of 5 experiments). Figure 5B shows densitometric quantifications of relative band intensities from RT-PCR reactions for each indicated mRNA treated with TNF-α or with TNF-α plus 2.2% sevoflurane for 16 h. Treatment with TNF-α for 16 h increased TNF-α, ICAM-1, and MCP-1 mRNA expression in HK-2 cells by 2.0 ± 0.1 (n = 9)-, 1.7 ± 0.1 (n = 9)-, and 1.9 ± 0.3 (n = 5)-fold, respectively, and treatment with 2.2% sevoflurane reduced expression to 1.6 ± 0.1 (n = 9, P < 0.05)-, 1.2 ± 0.1 (n = 6, P < 0.05)-, and 1.0 ± 0.1 (n = 5, P < 0.05)-fold, respectively. Similarly, sevoflurane reduced expression of proinflammatory mRNA in response to H₂O₂ treatment (data not shown).

Protein expression of ICAM-1 is suppressed with sevoflurane in HK-2 cells. TNF-α (Fig. 6) or H₂O₂ (data not shown) treatment increased protein expression of ICAM-1 in HK-2 cells which was reduced by 2.2% sevoflurane for 16 h (n = 4) consistent with our result of sevoflurane’s effects on ICAM-1 mRNA expression (Fig. 5B).

Sevoflurane selectively increases HSP-70 mRNA and protein. As shown in Fig. 7, A and B, HSP-70 mRNA was significantly increased at 4, 8, or 16 h after sevoflurane treatment in HK-2 cells. HSP-70 protein expression also increased significantly after 1, 2, 4, 8, or 16 h of sevoflurane treatment in HK-2 cells (Fig. 7, C and D). In contrast, there was no significant upregulation of HSP-27, HO-1 (HSP-32), and HSP-90 mRNA or protein expression after sevoflurane treatment (Figs. 7, A-D). Upregulation of HSP-70 is not specific for sevoflurane as other inhalational anesthetics including isoflu-
rane and halothane also upregulated HSP-70 mRNA and protein expression (data not shown). Similar selective upregulation of HSP-70 mRNA and protein occurred in LLC-PK1 cells after sevoflurane treatment (data not shown). Pretreatment with wortmannin or with PD-98059 blocked sevoflurane-mediated upregulation of HSP-70 protein (Fig. 7E). De novo gene transcription and protein synthesis are required for HSP-70 upregulation with sevoflurane treatment as actinomycin D (an inhibitor of new gene transcription, 10 μg/ml, 30-min pretreatment before sevoflurane) and cycloheximide (an inhibitor of new protein synthesis, 10 μg/ml, 30-min pretreatment before sevoflurane), respectively, blocked HSP-70 upregulation (Fig. 7F).

Sevoflurane suppresses TNF-α-induced NF-κB and AP-1 translocation in HK-2 cells. TNF-α treatment for 16 h dose dependently increased nuclear translocation of NF-κB and AP-1 in HK-2 cells (Fig. 8, A and C). Exposure to 2.2% sevoflurane for 16 h during TNF-α treatment decreased NF-κB (Fig. 8, A and B) and AP-1 translocation (Fig. 8, C and D). Figure 8, A and C, shows representative blots of NF-κB and AP-1 EMSA after TNF-α treatment or after TNF-α plus sevoflurane for 16 h (representative of 5 experiments). Figure 8, B and D, shows densitometric quantifications of relative band intensities from NF-κB as well as AP-1 EMSA TNF-α treatment or after TNF-α plus sevoflurane for 16 h. Treatment with 10 ng/ml TNF-α for 16 h increased nuclear translocation of NF-κB (1.9 ± 0.2-fold, n = 8, P < 0.05 vs. saline-treated group) and AP-1 (1.7 ± 0.2-fold, n = 5, P < 0.05 vs. saline-treated group) and these increases in proinflammatory transcription factors were significantly attenuated by 2.2% sevoflurane (NF-κB = 1.4 ± 0.1-fold, n = 9, P < 0.05, and AP-1 = 1.2 ± 0.1, n = 5, P < 0.05; Fig. 8, B and D). Figure 9 shows a dose-dependent reduction of TNF-α-induced NF-κB translocation with increasing concentrations of sevoflurane at 0.28, 0.55, 1.1, and 2.2% for 16 h thus further confirming the direct inhibitory effect of sevoflurane on NF-κB (representative of 6 experiments). Similar dose-dependent inhibition of AP-1 translocation by sevoflurane was observed (data not shown).

**DISCUSSION**

The major findings of the current study are that clinically relevant concentrations (0.25–2 MAC) of sevoflurane reduce...
inflammation as well as necrosis in human and porcine renal tubule (HK-2 and LLC-PK1) cells. In addition, sevoflurane reduces proinflammatory transcription factors NF-κB and AP-1 as well as proinflammatory mRNA and protein expression. Reduction in necrosis and inflammation with sevoflurane were associated with activation of prosurvival kinases ERK and Akt (PKB) as well as selective increased synthesis of HSP-70 dependent on ERK and Akt activation.

We demonstrated recently that volatile anesthetics reduced necrosis and inflammation in rats after renal IR injury in vivo (29). Renal IR under sevoflurane anesthesia resulted in drastic improvements in renal function, improved preservation of renal proximal tubular architecture, reduced necrosis, and resulted in near-complete inhibition of neutrophil influx. However, with in vivo studies, it is difficult to determine whether the protective effects were due to direct renal tubular effects and/or due to effects on leukocyte/neutrophil activation and infiltration. Therefore, in the current study, we questioned whether a volatile anesthetic produced direct anti-inflammatory and protective effects on renal proximal tubule cells in culture. We focused on cultures of proximal tubules as these cells are the primary site of injury in renal IR (30, 61). This region is marginally oxygenated under normal physiological conditions with a high basal metabolic demand (8, 30). Therefore, with IR

Fig. 7. A: representative gel images of semi-quantitative RT-PCR results of heat shock proteins (HSP)-32 (HO-1), -27, -70, and GAPDH from HK-2 cells treated with 2.2% sevoflurane for 0–16 h. Representative image of 6 experiments is shown. C: representative immunoblots of HSP-32, -27, and -70 from HK-2 cells treated with 2.2% sevoflurane for 0–16 h. Representative blot of 6 experiments is shown. B and D: densitometric quantifications of relative band intensities from RT-PCR reactions compared with GAPDH band intensity (B) and immunoblots (D). *P < 0.05 vs. 0 hour. E: increase in HSP-70 protein expression with sevoflurane is blocked with 50 μM PD-98059 (PD) or 100 nM Wort. A duplicate sample experiment is shown representative of 4 independent experiments. F: increase in HSP-70 protein expression with sevoflurane is blocked with 10 μg/ml actinomycin D (ActD) or with 10 μg/ml cycloheximide (CXM). A duplicate sample experiment is shown representative of 4 independent experiments.
injury, proximal tubules in the outer medullary zone suffer the most damage. Our study shows that sevoflurane had direct anti-inflammatory as well as direct antinecrotic effects in in vitro cultures of proximal tubule cells.

In this study, we injured HK-2 cells with H2O2 to produce necrosis. We demonstrate that H2O2 causes dose-dependent necrosis in both HK-2 and LLC-PK1 cells (Fig. 1). Oxygen free radical generation with resultant oxidant tissue stress is a major cause of renal reperfusion injury (12, 42). During reperfusion after ischemia, reactive oxygen species, such as superoxide anion, hydroxyl radical, and H2O2, are generated. These reactive oxygen species cause lipid peroxidation of the renal cell membrane with resultant intracellular calcium overload and subsequent necrotic cell death (42, 52, 53). We demonstrate in this study that as we observed in vivo, sevoflurane treatment protects against oxidant-mediated injury in human renal proximal tubule cells. Our data demonstrate that the antinecrotic effect of sevoflurane is not specific to human cells as LLC-PK1 cells are protected against necrosis as well. We again demonstrate that induction of necrosis in in vitro immortalized proximal tubule cells requires high doses of H2O2 (1–5 mM) as we reported previously (26).

A typical general anesthetic case requires ~1 MAC defined as the percent inhalational agent required to stop 50% of subjects from moving in response to a surgical stimulus. We demonstrate that clinically relevant concentrations of sevoflurane (0.25–2 MAC) produced concentration-dependent and

Fig. 8. Representative gel image of NF-κB (A) and AP-1 (C) electrophoretic mobility shift assay (EMSA) of nuclear extracts from HK-2 cells treated with increasing doses of TNF-α (0.1–10 ng/ml) in 95% air-5% CO2 or in 2.2% sevoflurane. Hela nuclear extracts were used as a positive control. Representative images of 5 experiments are shown. B and D: densitometric quantifications of relative band intensities from NF-κB (n = 9) and AP-1 EMSA (n = 5). *P < 0.05 vs. TNF-α.

Fig. 9. Representative gel image of NF-κB EMSA of nuclear extracts from HK-2 cells treated with increasing doses of sevoflurane (0–2.2% for 16 h). Representative image of 6 experiments is shown.
significant protection against proximal tubule necrosis. Moreover, we show that 4-h pretreatment with 1 MAC (2.2%) sevoflurane produced significant protection against necrosis. Our study provides evidence that pretreatment with sevoflurane results in a significant change in the cultured renal tubule cells that persists for several hours following clearance of the anesthetic. This raises the exciting possibility that beneficial anesthetic effects persist after clearance of the agent due to induced changes in intracellular signaling in proximal tubules. Taken together, our findings may have clinical significance for patients undergoing general anesthesia in the operating room.

We demonstrate in this study for the first time that sevoflurane pretreatment is associated with increased phosphorylation of Akt and ERK MAPK in renal tubule cells. Sevoflurane-related increases in phosphorylation of Akt and ERK have been reported in hippocampal slice cultures (11), cardiomyocytes (57), and vascular smooth muscle cells (70). ERK MAPK and/or Akt activation reduce necrosis after IR injury in several organs (7, 38, 58). For example, ERK activation mediates ischemic preconditioning in the heart (35, 36) and Akt activation improves outcome after IR injury in the heart (58). In addition, isoflurane may protect against myocardial infarction during early reperfusion by activation of phosphatidylinositol-3-kinase and Akt signal transduction (6, 58). However, in renal IR injury, the role of Akt and ERK in proximal tubule cell cytoprotection is not as clear.

Interestingly, 1- to 2-h treatment with sevoflurane failed to significantly protect either HK-2 or LLC-PK1 cells against necrosis. Only 4- to 16-h pretreatments induced significant protection suggesting that newly synthesized cytoprotective protein(s) (e.g., HSP-70) is (are) involved in protection. HSP-70 upregulation begins after ~4 h of sevoflurane pretreatment (Fig. 6) and at least 4-h sevoflurane pretreatment is required for cytoprotection to occur (Fig. 1E). However, we showed that ERK and Akt activation occurred within 1 h of sevoflurane treatment and peaked at 4 h before returning to baseline at 16 h. We also showed that pharmacological inhibition of Akt or ERK phosphorylation blocked sevoflurane-mediated protection against necrosis in HK-2 cells and LLC-PK1 cells. Moreover, activation of ERK and Akt is responsible for sevoflurane-mediated upregulation of HSP-70 as inhibition of ERK or Akt phosphorylation prevented the upregulation of HSP-70. Therefore, we conclude that early (1–4 h) activation of ERK and Akt pathways participates in mediating sevoflurane’s antinecrotic effects by mediating the upregulation of HSP-70. The early increase in pERK and pAkt induced by sevoflurane is both necessary and sufficient to account for the protection measured at the later (16 h) time point. This conclusion is further supported by the fact that inhibition of ERK and Akt after sevoflurane (16 h) treatment failed to prevent sevoflurane-mediated protection against H2O2 injury.

In our previous in vivo study, 1-h pretreatment with volatile anesthetic failed to protect renal function. Volatile anesthetics needed to be administered during ischemia and for 3 h after reperfusion suggesting that brief exposure to volatile anesthetics is not sufficient to induce renal protection. Our in vitro as well as in vivo data suggest that new protein synthesis (e.g., HSP-70) perhaps subsequent to ERK and/or Akt activation (phosphorylation) might be required for sevoflurane-mediated protection in renal cells.

In this study, we used two independent cell lines of renal tubules (HK-2 and LLC-PK1) to confirm that the renal tubular effects of sevoflurane do not occur in a single cell line. HK-2 cells are immortalized adult human proximal tubular cells transfected with E6/E7 genes of the human papilloma virus (HPV16) (51). Transfection with HPV16 has been shown to immortalize epithelial cells of diverse origin without significantly altering their phenotype or function. HK-2 cells have been shown to retain the phenotypic expression and functional characteristics of human proximal tubules (46, 51). Extensive studies have used HK-2 cells to study in vitro renal physiology and pathology (19, 20, 69). LLC-PK1 cells are transformed, stable porcine renal tubule cell line with similar transport characteristic of proximal tubule cells (16, 44).

In renal IR injury, sublethal injury is amplified by the inflammatory cascade that occurs during the reperfusion period (23, 24, 50). The inflammatory response is initiated and elaborated by cytokines (e.g., TNF-α), chemoattractive chemokines (e.g., IL-8, MCP-1, MIP-2), and the expression of adhesion molecules (e.g., ICAM-1, selectins) (3, 9, 10). Recent evidence indicates that elaboration of these adhesion molecules not only occur in nonrenal cells (e.g., endothelial cells, dendritic cells) but also in renal proximal tubules cells. We previously showed in vivo that mRNA for proinflammatory ICAM-1, TNF-α, and chemokines (MCP-1, MIP-2, IL-8, and IP-10) in rat renal cortices is upregulated 16 h after IR injury. However, in vivo, it is difficult to determine the exact source of this chemokine and cytokine generation in the kidney (e.g., proximal tubules, endothelial cells). We now show that renal...
proximal tubule cells can directly upregulate cytokine and chemokine mRNA in response to TNF-α. We also showed recently that volatile anesthetics reduce inflammatory changes after renal IR injury in vivo. Again, with in vivo studies, it is impossible to determine whether the anti-inflammatory response is direct (e.g., renal proximal tubule cells are targeted), indirect (e.g., endothelial cells, dendritic cells are targeted), or both. We show in this study for the first time that sevoflurane has direct anti-inflammatory effects in proximal tubules in culture. Attenuation of the expression of these cytokines and chemokines from proximal tubules by sevoflurane may contribute to the reduction in renal inflammatory injury after IR.

Nuclear translocation of transcription factors such as NF-κB or AP-1 plays an important role in propagating the inflammatory process. In the nucleus, these transcription factors bind to specific DNA motifs and regulate transcription of target genes including TNF-α, ICAM-1, as well as several key chemokines involved in inflammatory renal tubular damage (MCP-1, MIP-2, and IP-10) (2, 21, 49). We showed in our in vivo study that renal cortical NF-κB nuclear translocation is attenuated by volatile anesthetic treatment. We show in this study that increased NF-κB and AP-1 nuclear translocation in response to a proinflammatory stimulus TNF-α is directly and significantly reduced in HK-2 cells following sevoflurane treatment. Blocking NF-κB or AP-1 transcription attenuates inflammation and reduces cell death in many pathophysiological states including sepsis or IR injury (34, 59, 60). Therefore, the direct reduction of proinflammatory transcription factor activation by sevoflurane may contribute to renal protection.

In this study, we sought to examine other mechanisms of sevoflurane-mediated anti-inflammatory and antinecrotic effects with in vitro approaches. Volatile anesthetics cause upregulation of HSP-70 and -32 in the liver (15, 64). However, it is unclear whether volatile anesthetics modulate HSP expression in the kidney. We focused on three well-known cytoprotective HSPs; HSP-27, HO-1 (HSP-32), and HSP-70 of the diverse family members of heat shock proteins. Our data demonstrate that sevoflurane selectively upregulated mRNA and protein expression of inducible HSP-70 without affecting the expression of HSP-27 or HO-1.

The 70-kDa HSP-70 belongs to a family of highly conserved molecular chaperones that regulate protein folding and subsequent protein structure preservation during normal and stress conditions (5). In addition to promoting protein folding, HSP-70 is a well-known cytoprotective protein. In cultured renal epithelial cells, protection from adenosine 5′-triphosphate (ATP) depletion or heat stress is correlated with HSP-70 levels (39, 40, 62, 63). HSP-70 expression is inversely correlated with myocardial infarct size in rats (17, 18) and overexpression of HSP-70 in mice protects against cardiac ischemia (45). Induction of HSP-70 expression leads to increased resistance against in vivo renal IR injury (55, 65, 66). We demonstrated for the first time that sevoflurane increases HSP-70 mRNA and protein expression in HK-2 cells and LLC-PK1 cells. In addition to activating ERK and Akt, sevoflurane may protect renal proximal tubules against necrosis and inflammation by a HSP-70-mediated mechanism. We demonstrated that inhibition of ERK and Akt phosphorylation blocked both sevoflurane-mediated protection against oxidant-induced necrosis and upregulation of HSP-70. These results support the hypothesis that HSP-70 plays an integral role in sevoflurane-mediated protection against necrosis.

In this study, only the direct effects of sevoflurane on renal proximal tubule cell survival were studied. The effects of sevoflurane on leukocyte infiltration after renal IR may be another potential renal protective mechanism of sevoflurane. A potential limitation in the interpretation of this study is that immortalized renal tubule cells are used instead of primary cultures.

The direct signaling mechanism(s) of sevoflurane-mediated activation of ERK and Akt remains to be determined. However, based on the studies performed in other organs (e.g., heart), we may speculate several possible mechanisms. Volatile anesthetics directly interact with the mitochondrial electron transport chain (14, 22) which may augment the release of reactive oxygen species. Indeed, exposure to volatile anesthetics has been shown to generate minute quantities of reactive oxygen species in the heart (4, 41). Although large quantities of reactive oxygen species cause direct cellular injury contributing to the pathogenesis of IR injury, a small amount of reactive oxygen species triggers activation of several kinases including ERK and Akt (4). In addition, volatile anesthetics may activate several G protein-coupled receptors including A1 adenosine receptors as selective blockade of these receptors abolished the cardiac protective effects of volatile anesthetic-mediated preconditioning (13, 25, 68). Volatile anesthetics may also bind and directly activate G proteins as pertussis toxin prevented volatile anesthetic-mediated cardiac protection in dogs (56). Therefore, volatile anesthetics may trigger the phosphorylation of ERK and Akt via activation of G1-coupled receptor-mediated pathways.

In conclusion, our study shows that sevoflurane directly reduced necrosis and inflammation in proximal tubules in vitro via mechanisms involving reduction of proinflammatory transcription factors (NF-κB and AP-1) and reduction in proinflammatory mRNAs. Sevoflurane also activated prosurvival kinases ERK and Akt and upregulation of HSP-70 (Fig. 10). Further elucidation of the signaling pathways of sevoflurane-mediated activation of cytoprotective pathways may lead to effective therapies against renal IR injury and acute renal failure.

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