Published in final edited form as:

Ann Surg. 2012 March ; 255(3): 492–503. doi:10.1097/SLA.0b013e3182441767.

Isoflurane postconditioning protects against intestinal ischemia-reperfusion injury and multi-organ dysfunction via transforming growth factor-beta1 generation

Minjae Kim, M.D.¹, Sang Won Park, Ph.D.¹, Mihwa Kim, B.S.¹, Vivette D. D'Agati, M.D.², and H. Thomas Lee, M.D., Ph.D.¹

¹Department of Anesthesiology, College of Physicians and Surgeons of Columbia University, New York, NY, 10032

²Department of Pathology, College of Physicians and Surgeons of Columbia University, New York, NY, 10032

Abstract

Objective—This study examined volatile anesthetic-mediated protection against intestinal ischemia-reperfusion injury (IRI).

Background—Intestinal IRI is a devastating complication in the perioperative period leading to systemic inflammation and multi-organ dysfunction. Volatile anesthetics, including isoflurane, have anti-inflammatory effects. We aimed to determine whether isoflurane, given after intestinal ischemia, protects against intestinal IRI and the mechanisms involved in this protection.

Methods—After IACUC approval, mice were anesthetized with pentobarbital and subjected to 30 min of superior mesenteric artery ischemia, followed by 4 hrs of equianesthetic doses of pentobarbital or isoflurane. Five hrs after reperfusion, small intestine tissues were analyzed for morphological injury, apoptosis, neutrophil infiltration, pro-inflammatory mRNAs, and TGF- β 1 levels. We also assessed hepatic and renal injury after intestinal IRI.

Results—Intestinal IRI with pentobarbital led to significant small intestinal dysfunction with increased mucosal injury, TUNEL-positive cells, neutrophil infiltration, and pro-inflammatory mRNAs as well as elevated plasma alanine aminotransferase and creatinine levels. Isoflurane exposure after IRI led to significant attenuation of intestinal, hepatic, and renal injuries. Furthermore, the protective effects of isoflurane were abolished by treatment with a TGF- β 1 neutralizing antibody prior to induction of IRI. Finally, isoflurane exposure led to increased TGF- β 1 levels in intestinal epithelial cells and in plasma.

Conclusions—Our findings demonstrate that isoflurane postconditioning protects against small intestinal injury as well as hepatic and renal dysfunction after severe intestinal IRI via induction of intestinal epithelial TGF- β 1. Our findings support therapeutic applications of volatile anesthetics

Corresponding Author: H. Thomas Lee, M.D., Ph.D., Associate Professor, Department of Anesthesiology, Anesthesiology Research Laboratories, Columbia University, P&S Box 46 (PH-5), 630 West 168th Street, New York, NY 10032-3784, Tel: (212) 305-1807, Fax: (212) 305-8980, t1128@columbia.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Disclosure

The authors of this manuscript have no conflicts of interest to disclose.

during the intraoperative as well as postoperative periods and imply an important role of TGF- β 1 signaling in modulating multi-organ injury.

Introduction

Intestinal ischemia-reperfusion injury (IRI) is a major and frequent clinical problem in perioperative settings such as vascular surgery,¹ trauma,² and liver³ and intestinal⁴ transplantation. In addition, acute mesenteric ischemia is a dire surgical emergency with a mortality rate approaching 60–90%.⁵ Intestinal IRI leads to intestinal epithelial barrier dysfunction with increased permeability and bacterial translocation. In addition, the intestine has been proposed as the source of pro-inflammatory cytokines that can lead to the development of the systemic inflammatory response syndrome and sepsis with subsequent multi-organ failure.⁶

Volatile anesthetics are ubiquitously administered in the perioperative period as an essential part of general anesthesia. In addition to their anesthetic effects, volatile anesthetics may be anti-inflammatory and cytoprotective but their role in intestinal IRI has never been explored. We have previously demonstrated that volatile anesthetics reduce renal proximal tubule cell necrosis *in vitro*⁷ and protect against renal IRI *in vivo*⁸ by release of TGF- β 1,^{9, 10} a potent anti-inflammatory and anti-apoptotic molecule.¹¹

In this study, we questioned whether isoflurane, the most commonly used volatile anesthetic, would protect mice against small intestinal injury and multi-organ dysfunction after intestinal IRI. We hypothesized that isoflurane increases release of TGF- β 1 in the small intestine to protect against intestinal IRI. We demonstrate that isoflurane reduced small intestinal mucosal injury, apoptosis, pro-inflammatory mRNA upregulation, and neutrophil infiltration as well as injury to the liver and kidney via induction of intestinal TGF- β 1.

Materials and Methods

Materials

Isoflurane [2-chloro-2-(difluoromethoxy)-1,1,1-trifluoro-ethane] was purchased from Abbott Laboratories (North Chicago, IL). Unless otherwise specified, all other reagents were purchased from Sigma (St. Louis, MO).

Murine model of intestinal IRI

All animal protocols were approved by the IACUC of Columbia University (New York, NY). Male C57BL/6 mice (Harlan, Indianapolis, IN; 20 to 25 g) were initially anesthetized with intraperitoneal pentobarbital (Henry Schein Veterinary Co., Indianapolis, IN; 50 mg/kg body weight, or to effect) and after laparotomy, the superior mesenteric artery (SMA) was identified and clamped with a microaneurysm clip for 30 min. After unclamping of the SMA, intraperitoneal administration of 0.5 mL warm saline and abdominal closure, the mice were then exposed to an additional 4 hrs of equipotent doses of either pentobarbital via intermittent intraperitoneal administration 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] of isoflurane (1.2%) as described previously.⁸ Briefly, mice were placed in an airtight 10 L chamber on a warming blanket with inflow and outflow hoses located at the top and bottom of the chamber, respectively. Isoflurane was delivered in room air at 5 L/min using an agent-specific vaporizer (Datex-Ohmeda, Madison, WI). The vaporizer was set to maintain chamber concentration of isoflurane at 1.2% monitored by an infrared analyzer sampling gas at the outflow hose. The mice were placed on a heating pad under a warming light to maintain

body temperature $\sim 37^{\circ}\text{C}$. In pilot studies, we monitored the hemodynamic effects of anesthetics via a carotid artery catheter and found no significant differences in systemic arterial pressures between pentobarbital and isoflurane exposure.

To test the effects of TGF- $\beta 1$ neutralization, a monoclonal TGF- $\beta 1$ neutralizing antibody (MAB240; R&D Systems, Minneapolis, MN) or an isotype-control antibody (mouse IgG₁, MAB002; R&D Systems) was administered to mice 5 mg/kg intravenously 10 min prior to intestinal ischemia. Samples (including plasma and tissue) were collected 5 hrs after sham operation or IRI.

Histological analysis of intestine, liver, and kidney injury after intestinal IRI

For histological preparations, small intestine (jejunum and ileum), kidney, and liver tissues collected from mice were washed in ice-cold PBS and fixed overnight in 10% formalin. After automated dehydration through a graded alcohol series, tissues were embedded in paraffin, sectioned at 5 microns and stained with hematoxylin-eosin (H&E). Intestinal H&E sections were graded for intestinal IRI-induced mucosal injury using the Chiu score.¹² Kidney H&E sections were graded for the severity (score: 0–3) of four components: 1) cortical tubular vacuolization, 2) peritubular leukocyte infiltration, 3) cortical tubular simplification, and 4) proximal tubule hypereosinophilia/single cell necrosis. The four components were added together to create a composite renal injury score (scale: 0–12). Liver H&E sections were given a semiquantitative score for the percent of hepatic parenchyma showing hepatocyte vacuolization by systematic evaluation of all 20X histologic fields encompassing the total cross sectional area of each specimen. All H&E sections were evaluated by a pathologist (V.D.D.) blinded to the samples.

Detection of apoptosis after intestinal IRI

Apoptosis was detected in the small intestine (jejunum and ileum), kidney, and liver with terminal deoxynucleotidyl transferase biotin-dUTP nick end-labeling (TUNEL) staining, as described previously.¹³ In situ labeling of fragmented DNA was performed with TUNEL stain (green fluorescence) using a commercially available in situ cell death detection kit (Roche, Indianapolis, IN) according to the instructions provided by the manufacturer. TUNEL-positive cells per high power field (hpf, 400X) were counted to quantify apoptosis.

Assessment of small intestine neutrophil infiltration after IRI

Neutrophil infiltration in the small intestine after IRI was determined with immunohistochemistry as described previously.¹⁴ with a monoclonal antibody against PMN (clone 7/4, AbD Serotec, Raleigh, NC). A primary antibody that recognized IgG_{2a} (MCA1212, AbD Serotec) was used as a negative isotype control in all experiments. Neutrophils per hpf were counted to quantify the degree of neutrophil infiltration.

Measurement of pro-inflammatory mRNA expression after intestinal IRI

Renal, hepatic, and intestinal inflammation in mice were determined by measuring mRNA encoding markers of inflammation, including IL-17A, intercellular adhesion molecule 1 (ICAM-1), monocyte chemoattractive protein 1 (MCP-1), macrophage inflammatory protein 2 (MIP-2), and tumor necrosis factor- α (TNF- α) (Table 1). Inflammation in cell culture (described below) was determined by measuring mRNA encoding the pro-inflammatory markers IL-8 and TNF- α (Table 1). Semiquantitative real-time RT-PCR was performed as described.^{9, 15}

Vascular permeability of small intestine, kidney, and liver after intestinal IRI

Changes in vascular permeability of the small intestine (jejunum and ileum), kidney, and liver were assessed by quantitating extravasation of Evans blue dye (EBD) into the tissue as described previously.¹⁶

Intestinal epithelial permeability after IRI

Intestinal epithelial permeability was assessed by enteral administration of FITC-dextran 4000 (200 µg/kg) 10 min before induction of intestinal IRI as described.¹⁷

Plasma alanine aminotransferase (ALT) activity and creatinine levels after intestinal IRI

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

Cell culture

Rat intestinal epithelial cells¹⁸ (IEC-6, ATCC, Manassas, VA) were grown in DMEM high glucose with 10% fetal bovine serum (FBS), 40 µg/ml insulin, and antibiotics (100 U/ml penicillin G, 100 µg/ml streptomycin, and 0.25 µg/ml amphotericin B) (Invitrogen, Carlsbad, CA). Cells were passaged in 75-cm² cell culture flasks at 37°C in a 100% humidified atmosphere of 5% CO₂-95% air, plated in 6-well plates when 80% confluent, and used in the experiments described below when confluent. Human dermal microvascular endothelial cells¹⁹ (HMEC-1, ATCC) were grown in MCDB131 medium with 10% FBS, 2.5 mM L-glutamine, 10 ng/ml epidermal growth factor, 1 µg/ml hydrocortisone, 100 µg/ml G418, and antibiotics (Invitrogen).

Induction of endothelial inflammation *in vitro*

To test the effects of isoflurane on the inflammatory response *in vitro*, HMEC-1 cells were treated with vehicle or TNF-α (10 ng/ml) 30 min prior to exposure to isoflurane in an air-tight, 37°C, humidified modular incubator chamber connected to an in-line agent-specific calibrated vaporizer (Datex-Ohmeda, Madison, WI) to deliver isoflurane [2.5% or 2 MAC] mixed with 95% air-5% CO₂ (carrier gas) at 10 L/min, as described previously.⁷ Exposure to isoflurane lasted 16 hrs. Control cells were exposed to carrier gas in a modular incubator chamber.

Induction of IEC-6 cell necrosis and measurement of cell viability with lactate dehydrogenase (LDH)

After exposure to carrier gas or isoflurane 2.5% for 5 hrs, necrotic injury in IEC-6 cells was induced with exposure to 500 µM H₂O₂ for 4 hrs. LDH released into cell culture media was measured as described.²⁰

TGF-β1 enzyme-linked immunosorbent assay (ELISA)

To test whether isoflurane anesthesia selectively increases TGF-β1, mice were anesthetized for 4 hrs with pentobarbital or isoflurane (without operation) and small intestinal and plasma TGF-β1 levels were measured using an ELISA kit from Promega (Madison, WI) with appropriate acidification of samples for activation of latent TGF-β1. We also exposed IEC-6 cells to carrier gas or to isoflurane (2.5%) for 5 hrs and measured TGF-β1 formed in the media as described above.

Statistical analysis

The data were analyzed with a two-tailed Student's *t*-test when comparing means between two groups. One-way ANOVA plus Tukey's *post hoc* multiple comparison test was used when comparing multiple groups. The ordinal values of the Chiu scores and renal injury scores 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 $\pm$ SEM.

Results

Isoflurane reduces small intestinal mucosal injury after IRI

As previously demonstrated, in our model there were no significant differences in systemic hemodynamics between mice exposed to pentobarbital or isoflurane.²¹ In Figure 1, we demonstrate the protective effects of isoflurane anesthesia in the small intestine. There were no differences in the normal histology of sham-operated mice exposed to pentobarbital (Figure 1A, representative of 4 experiments) and isoflurane (*data not shown*). Intestinal IRI and 4 hrs of pentobarbital exposure led to severe damage to the intestinal mucosa with sloughing of the villous tips (Figure 1B, ileum shown) and lifting of the epithelium from the lamina propria and the development of subepithelial Gruenhagen's spaces (Figure 1C, ileum shown). In contrast, the ilea of mice exposed to 4 hrs of isoflurane after intestinal IRI were protected from severe injury (Figure 1D). The Chiu scoring system was used to grade intestinal mucosal injury after intestinal IRI.¹² Compared to pentobarbital-exposed mice, mice exposed to isoflurane after intestinal IRI had a significant reduction in the Chiu score in the ileum (Figure 1E). In the jejunum, there were no differences in the Chiu scores between mice exposed to pentobarbital or isoflurane after intestinal IRI.

Isoflurane reduces small intestinal apoptosis after IRI

Compared to sham-operated mice (Figure 2A, representative of 4 experiments), mice exposed to pentobarbital after intestinal IRI showed many TUNEL-positive cells in the distal tips of villi in the small intestine (Figure 2B, ileum shown). In contrast, there was a reduction in TUNEL-positive cells in the ileum of mice exposed to isoflurane after intestinal IRI (Figure 2C). Quantification of apoptotic cells revealed that compared to pentobarbital exposure after intestinal IRI, isoflurane exposure reduced the number of apoptotic cells in the ileum, with no reduction of apoptotic cells in the jejunum (Figure 2D).

Isoflurane reduces small intestinal neutrophil infiltration after IRI

In contrast to sham-operated mice (Figure 3A, representative of 4 experiments), pentobarbital exposure after intestinal IRI led to increased numbers of neutrophils in the small intestine (Figure 3B, arrows highlight areas of neutrophil infiltration, ileum shown). However, with isoflurane exposure after intestinal IRI, there was a reduction in neutrophil infiltration in the ileum (Figure 3C). The protective effects of isoflurane were not uniform throughout the small intestine as isoflurane exposure significantly reduced neutrophil infiltration in the ileum, but not jejunum, after intestinal IRI (Figure 3D).

Isoflurane reduces small intestinal pro-inflammatory mRNA expression after IRI

Four hrs of pentobarbital exposure after intestinal IRI led to increased mRNA expressions of the pro-inflammatory markers ICAM-1, MCP-1, MIP-2, IL-17A, and TNF- α in the ileum compared to sham-operated mice (Figure 4, representative of 4 experiments). With isoflurane exposure after IRI, there were significant reductions in the mRNA expressions of ICAM-1 and MCP-1 in the ileum compared to pentobarbital exposure, with no change in the mRNA expressions of MIP-2, IL-17A, or TNF- α (Figure 4).

Isoflurane decreases small intestinal vascular permeability after intestinal IRI

Pentobarbital exposure after intestinal IRI caused significant increases in vascular permeability as measured by increased EBD content²² in the jejunum and ileum compared to sham-operated mice (Figure 5). With isoflurane exposure after intestinal IRI, vascular permeability was significantly decreased in the ileum, but not in the jejunum.

Isoflurane does not reduce small intestinal epithelial permeability after IRI

Compared to sham-operated mice (serum FITC-dextran 9.7 ± 3.4 $\mu\text{g/mL}$, $N=4$), mice exposed to pentobarbital after intestinal IRI had increased intestinal permeability (serum FITC-dextran 351.9 ± 74.3 $\mu\text{g/mL}$, $N=6$, $P<0.05$ vs. sham-operated group). With isoflurane exposure after intestinal IRI (serum FITC-dextran 371.0 ± 70.7 $\mu\text{g/mL}$, $N=6$, $P=0.86$), intestinal permeability was not different from mice exposed to pentobarbital after IRI.

Isoflurane does not reduce intestinal epithelial cell necrosis

In addition, we exposed IEC-6 cells to carrier gas or isoflurane 2.5% for 5 hrs prior to treatment with $500 \mu\text{M H}_2\text{O}_2$. LDH release in cells exposed to carrier gas (% LDH Released = 20.3 ± 4.3 , $N=6$) and isoflurane (% LDH Released = 24.5 ± 5.3 , $N=6$, $P=0.55$) were not significantly different.

Isoflurane protects against acute hepatic and renal injury after intestinal IRI in mice

Five hrs after intestinal IRI, mice exposed to pentobarbital anesthesia developed significant hepatic and renal dysfunction as indicated by a rise in plasma ALT (Figure 6A) and creatinine (Figure 7A) levels above sham values. Isoflurane exposure after intestinal IRI protected both hepatic and renal function as evidenced by a significant decline in plasma ALT (Figure 6A) and creatinine (Figure 7A) levels. In the liver, intestinal IRI with pentobarbital exposure resulted in significant vacuolization that was concentrated in perivascular (portal) areas (Figure 6B, left panels, arrows denote portal area) compared to sham-operated mice (Supplemental Figure 1). With isoflurane exposure after intestinal IRI, there were significant decreases in vacuolization in the liver (Figure 6B, right panels; Figure 6C). In the kidney, pentobarbital exposure after intestinal IRI led to increased proximal tubular simplification (Figure 7B, asterisks), proximal tubular hypereosinophilia/single cell necrosis (Figure 7D, arrows), and vacuolization (Figure 7F) as well as peritubular leukocyte infiltration compared to sham-operated mice (Supplemental Figure 2). Isoflurane exposure after intestinal IRI led to reductions in these markers of injury (Figures 7C, E, G) as reflected in a significant reduction in total renal injury scores (Figure 7H).

Isoflurane's effects on pro-inflammatory mRNA expression, apoptosis, and vascular permeability in the kidney and liver after intestinal IRI

Compared to sham-operated mice, mice exposed to pentobarbital after intestinal IRI had increased mRNA expressions of TNF- α and MCP-1 in the liver and TNF- α and ICAM-1 in the kidney. With isoflurane exposure after intestinal IRI, there were no significant differences in these mRNA expressions. In addition, we did not find evidence of increased apoptosis (as measured by morphology on H&E and TUNEL-staining) in the kidneys and livers of mice after exposure to pentobarbital or isoflurane after intestinal IRI compared to sham-operated mice. Vascular permeability in the liver as measured by EBD extraction did not differ between sham-operated mice and mice exposed to pentobarbital or isoflurane after intestinal IRI. In the kidney, however, compared to sham-operated mice exposed to pentobarbital (EBD = 40.7 ± 0.7 $\mu\text{g EBD/g dry tissue}$, $N=3$) or isoflurane (EBD = 36.5 ± 5.4 $\mu\text{g EBD/g dry tissue}$, $N=3$), there was an increase in EBD extraction with pentobarbital exposure after intestinal IRI (EBD = 141.5 ± 25.6 $\mu\text{g EBD/g dry tissue}$, $N=5$, $P<0.05$ vs. both

sham groups). Isoflurane exposure after intestinal IRI led to a ~50% reduction in EBD in the kidney (EBD=65.2±9.8 µg EBD/g dry tissue, N=5, P<0.05 vs. pentobarbital intestinal IRI).

The protective effects of isoflurane are mediated via TGF-β1

To evaluate the role of TGF-β1 in mediating the protective effects of isoflurane after intestinal IRI, we treated some animals with an isotype-control (IgG₁) antibody or a TGF-β1 neutralizing antibody prior to induction of intestinal IRI. Similar to our previous findings, mice treated with IgG₁ (Figure 8A) or TGF-β1 neutralizing antibody (Figure 8C) prior to intestinal IRI with pentobarbital exposure developed severe intestinal mucosal injury with sloughing of the villous tips in the ileum. Isoflurane exposure led to preservation of ileal mucosa in mice treated with IgG₁ (Figure 8B) but not TGF-β1 neutralizing antibody (Figure 8D) prior to intestinal IRI, indicating that the protective effects of isoflurane after intestinal IRI were mediated via induction of TGF-β1.

To further confirm the importance of TGF-β1 in isoflurane-mediated protection, we assessed apoptosis in mice treated with IgG₁ or TGF-β1 neutralizing antibody. Mice treated with IgG₁ (Figure 8E) or TGF-β1 neutralizing antibody (Figure 8G) prior to intestinal IRI with pentobarbital exposure showed many TUNEL-positive cells in the distal villous tips in the ileum. Isoflurane exposure led to a reduction in TUNEL-positive cells in mice treated with IgG₁ (Figure 8F) but not TGF-β1 neutralizing antibody (Figure 8H).

The IgG₁ or TGF-β1 neutralizing antibodies had no effects on hepatic (Figure 8I) or renal (Figure 8J) function in sham-operated mice. Mice treated with IgG₁ or TGF-β1 neutralizing antibody and exposed to pentobarbital after intestinal IRI developed significant hepatic (Figure 8I) and renal (Figure 8J) dysfunction 5 hrs after IRI. Isoflurane exposure protected mice treated with IgG₁ antibody prior to intestinal IRI but failed to protect mice treated with TGF-β1 neutralizing antibody (Figures 8I–J),

Isoflurane induces TGF-β1 in mouse intestine and plasma

Isoflurane anesthesia increased TGF-β1 levels in both the plasma (Figure 9A) and small intestine (Figure 9B) of sham mice compared to mice anesthetized with pentobarbital. In IEC-6 cells, isoflurane exposure increased TGF-β1 levels compared to carrier gas exposure (Figure 9C).

Isoflurane exposure decreases pro-inflammatory mRNA expression in HMEC-1 cells

As isoflurane exposure reduced small intestinal vascular permeability in mice after intestinal IRI, we wanted to test the protective effects of isoflurane in a model of inflammation in cultured microvascular endothelial cells. There was increased expression of pro-inflammatory mRNAs (IL-8 and TNF-α) in HMEC-1 cells exposed to carrier gas after TNF-α treatment compared to vehicle-treated cells (Figure 10). With isoflurane exposure after TNF-α treatment, there was a significant reduction in pro-inflammatory mRNA expression.

Discussion

The major findings of this study are that a clinically relevant concentration of isoflurane (1 MAC) administered after intestinal IRI reduced intestinal injury and decreased inflammation and apoptosis while improving vascular permeability. In addition, isoflurane led to an improvement in multi-organ dysfunction in both the liver and kidneys after intestinal IRI. These effects of isoflurane were mediated by the TGF-β1 pathway as isoflurane failed to protect mice treated with a TGF-β1 neutralizing antibody prior to intestinal IRI. Moreover, isoflurane exposure led to higher TGF-β1 levels in the small intestinal epithelia and plasma of mice.

Intestinal IRI is a serious clinical problem in the perioperative period and settings such as intestinal⁴ or liver transplantation³ require an obligate period of intestinal ischemia that is an important determinant of short- and long-term outcomes. Furthermore, acute mesenteric ischemia is associated with an exceedingly high mortality rate despite surgical intervention.⁵ The ischemia and reperfusion phases have distinct pathophysiological features with the majority of mucosal injury occurring during the reperfusion phase,²³ and gut-derived factors play an important role in mediating the systemic inflammatory state and multi-organ dysfunction in settings such as trauma and shock.²⁴ Therefore, once an ischemic event has been identified, strategies to diminish the cascade of events leading to intestinal and multi-organ injury during the reperfusion phase may help to improve morbidity and mortality.

The direct role of volatile anesthetics in intestinal IRI has not been previously established. We demonstrate that exposure to isoflurane during the reperfusion phase of intestinal IRI (isoflurane postconditioning) reduced mucosal injury in the small intestine as well as apoptosis, neutrophil infiltration, and vascular permeability. Interestingly, these markers of injury after IRI were generally worse in the ileum compared to the jejunum, and isoflurane exposure was protective only in the ileum, but not jejunum, 5 hrs after IRI. Though the SMA provides the main blood supply for both the jejunum and ileum, it is possible that collateral flow, such as from the celiac axis, could supply areas such as the proximal jejunum while the ileum was more dependent on SMA blood flow, resulting in greater injury. Indeed, a previous study found that the degree of ischemic injury varied by intestinal segment in a murine model of SMA occlusion, with the greatest damage found in the distal jejunum and proximal ileum.²⁵ We sampled tissue from the proximal areas of the jejunum and ileum, which is consistent with our findings of greater injury in the proximal ileum compared to the proximal jejunum. We measured pro-inflammatory mRNA expression in the ileum after intestinal IRI and found that isoflurane reduced the expressions of ICAM-1 and MCP-1 but not of TNF- α , IL-17A, or MIP-2. ICAM-1 is an important mediator of neutrophil transendothelial migration²⁶ and MCP-1 acts as a chemoattractant for monocytes and lymphocytes to areas of injury.²⁷ This indicates that isoflurane modulates the inflammatory response in IRI through these leukocyte-specific pathways and may be the reason why other markers of tissue injury and inflammation, such as TNF- α , did not change.

Microvascular injury leading to end-organ hypoperfusion is a prominent feature of intestinal IRI resulting in vascular injury not only in the intestines but also in the lungs,²⁸ liver,²⁹ and kidneys.³⁰ We confirmed that intestinal IRI caused microvascular injury in the small intestine. However, isoflurane exposure led to a significant reduction in vascular permeability as well as pro-inflammatory mRNA induction in the ileum, suggesting that isoflurane may have protective effects on the endothelium. It is difficult to isolate the effects of isoflurane on specific cell types when analyzing whole tissue (RT-PCR), so we used a model of TNF- α -induced inflammation in HMEC-1 cells and found that isoflurane reduced pro-inflammatory mRNA expression in these cells, further confirming the protective effects of isoflurane on the microvasculature. Anesthetics have differential effects on the mesenteric microcirculation after hemorrhage³¹ and isoflurane improved end-organ perfusion after hemorrhagic shock compared to ketamine-based intravenous anesthesia.³²

Anesthetics may cause hemodynamic changes and regional disturbances in blood flow. However, we previously demonstrated that compared to pentobarbital, isoflurane did not significantly alter systemic hemodynamics.²¹ There was no evidence of direct protective effects of volatile anesthetics on intestinal epithelial cells as isoflurane failed to reduce intestinal epithelial permeability after IRI *in vivo* and rat intestinal epithelial cells (IEC-6) were not protected by isoflurane against H₂O₂-induced cell necrosis *in vitro*. After IRI, the greatest injury, as well as the greatest protection with isoflurane exposure, occurred in the distal villous tips (histopathology, apoptosis, neutrophil infiltration). IEC-6 cells are derived

from intestinal crypts¹⁸ and may not represent the population that is protected by isoflurane. Taken together, these results imply that isoflurane protected the intestine through indirect means, such as better preservation of intestinal blood flow and oxygenation, rather than through direct cytoprotection of epithelial cells.

Previous studies demonstrate differential protective effects among volatile anesthetics in a model of renal IRI⁸ or myocardial ischemia,³³ suggesting that different cellular mechanisms may be involved. Isoflurane, but not halothane, improved mesenteric blood flow following esophageal ileocoloplasty in humans³⁴ and isoflurane improved tissue oxygenation after colonic resection and anastomosis compared to desflurane.³⁵ Volatile anesthetics are lipophilic molecules³⁶ and differences in lipid solubility may play a role in their differential effects in IRI.⁸ Further studies are needed to determine the effects of other volatile anesthetics in intestinal IRI. Anesthetic toxicity is a potential concern including nephrotoxicity and hepatotoxicity. However, isoflurane is minimally metabolized and has not been shown to have significant nephrotoxicity³⁷ or hepatotoxicity.³⁸

TGF- β 1 is an important regulator of wound healing, immunity, and inflammation.³⁹ TGF- β 1 was protective in a model of stroke⁴⁰ and in myocardial IRI, TGF- β 1 reduced inflammatory injury to the endothelium,⁴¹ while a deficiency of TGF- β 1 rendered mice more vulnerable to renal IRI.⁴² TGF- β 1 administration after intestinal ischemia preserved mesenteric blood flow, despite reduced mean arterial pressures, by reducing splanchnic vascular resistance via release of nitric oxide.⁴³ We also previously demonstrated that volatile anesthetics increased release of TGF- β 1 which was protective in renal proximal tubules *in vitro*⁹ and in renal IRI *in vivo*.¹⁰ Given this knowledge, we tested whether there was a role for TGF- β 1 in mediating the protective effects of isoflurane following intestinal IRI. We found that injection of a TGF- β 1 neutralizing antibody prior to intestinal IRI reversed the protection that was mediated by isoflurane. Taken together, we theorize that isoflurane may mediate protection against intestinal IRI through release of TGF- β 1. The precise cause and effect relationship between isoflurane and TGF- β 1 in intestinal IRI will need to be explored in future studies.

Intestinal IRI leads not only to injury in the intestine itself, but affects distant organs such as the liver, kidney, and lungs.^{6, 28} In our study, isoflurane reduced injury to both the liver and kidneys after intestinal IRI as evidenced by improved histological findings (vacuolization in the liver, renal injury scores) and markers of organ function (plasma ALT and creatinine levels). Isoflurane reduced markers of inflammation (RT-PCR) and apoptosis (TUNEL) in the intestine, but we did not find a reduction in these markers in the liver or kidney after intestinal IRI. Based on this evidence, we postulate that isoflurane does not protect the liver and kidneys directly after intestinal IRI. Rather, isoflurane, via release of TGF- β 1, reduces the production and release of cytokines, free radicals, and other inflammatory mediators in the small intestine, thereby reducing the systemic inflammatory effects in distant organs such as the liver and kidney. There may be some concern about the effects of volatile anesthetics on hepatic blood flow as desflurane, but not isoflurane, was found to reduce total hepatic blood flow in dogs.⁴⁴ However, in the liver, we found that while pentobarbital led to significant tissue injury adjacent to the vascular areas (portal vein), isoflurane reduced injury in these areas, suggesting that isoflurane exposure improved hepatic blood flow or reduced pro-inflammatory mediator release into the liver from the small intestine, or a combination of both.

As volatile anesthetics are delivered systemically, it is difficult to determine in an *in vivo* study whether they mediate their protective effects directly on the intestine or via systemic effects such as modulation of leukocyte migration and infiltration. Based on the available evidence, it is likely that isoflurane has a multitude of effects, including both direct

cytoprotective effects in the intestine with activation of pro-survival signaling pathways, as well as systemic anti-inflammatory mechanisms including peripheral lymphopenia and reduction of pro-inflammatory cytokines.

In conclusion, we demonstrated that isoflurane induces TGF- β 1 signaling in the small intestine to reduce intestinal mucosal injury, apoptosis, neutrophil infiltration, and endothelial injury after intestinal IRI, as well as damage to the liver and kidneys. Further elucidation of the mechanisms of protection may lead to advancements in the treatment of intestinal and multi-organ dysfunction following intestinal IRI.

Acknowledgments

This work was supported by National Institutes of Health Grants R01 DK-058547, R01 GM-067081, and T32 GM-008464.

References

1. Zhang WW, Kulaylat MN, Anain PM, et al. Embolization as cause of bowel ischemia after endovascular abdominal aortic aneurysm repair. *J Vasc Surg.* 2004; 40(5):867–872. [PubMed: 15557898]
2. Thuijls G, de Haan JJ, Derikx JP, et al. Intestinal cytoskeleton degradation precedes tight junction loss following hemorrhagic shock. *Shock.* 2009; 31(2):164–169. [PubMed: 18650780]
3. Vincenti M, Behrends M, Dang K, et al. Induction of intestinal ischemia reperfusion injury by portal vein outflow occlusion in rats. *J Gastroenterol.* 2010
4. Farmer DG, Venick RS, Colangelo J, et al. Pretransplant predictors of survival after intestinal transplantation: analysis of a single-center experience of more than 100 transplants. *Transplantation.* 2010; 90(12):1574–1580. [PubMed: 21107306]
5. Kassahun WT, Schulz T, Richter O, et al. Unchanged high mortality rates from acute occlusive intestinal ischemia: six year review. *Langenbecks Arch Surg.* 2008; 393(2):163–171. [PubMed: 18172675]
6. Mallick IH, Yang W, Winslet MC, et al. Ischemia-reperfusion injury of the intestine and protective strategies against injury. *Dig Dis Sci.* 2004; 49(9):1359–1377. [PubMed: 15481305]
7. Lee HT, Kim M, Jan M, et al. Anti-inflammatory and antinecrotic effects of the volatile anesthetic sevoflurane in kidney proximal tubule cells. *Am J Physiol Renal Physiol.* 2006; 291(1):F67–F78. [PubMed: 16478975]
8. Lee HT, Ota-Setlik A, Fu Y, et al. Differential protective effects of volatile anesthetics against renal ischemia-reperfusion injury in vivo. *Anesthesiology.* 2004; 101(6):1313–1324. [PubMed: 15564938]
9. Lee HT, Kim M, Kim J, et al. TGF-beta1 release by volatile anesthetics mediates protection against renal proximal tubule cell necrosis. *Am J Nephrol.* 2007; 27(4):416–424. [PubMed: 17622749]
10. Lee HT, Chen SW, Doetschman TC, et al. Sevoflurane protects against renal ischemia and reperfusion injury in mice via the transforming growth factor-beta1 pathway. *Am J Physiol Renal Physiol.* 2008; 295(1):F128–F136. [PubMed: 18434384]
11. Shi Y, Massague J. Mechanisms of TGF-beta signaling from cell membrane to the nucleus. *Cell.* 2003; 113(6):685–700. [PubMed: 12809600]
12. Chiu CJ, McArdle AH, Brown R, et al. Intestinal mucosal lesion in low-flow states. I. A morphological, hemodynamic, and metabolic reappraisal. *Arch Surg.* 1970; 101(4):478–483. [PubMed: 5457245]
13. Kim M, Park SW, Kim M, et al. Isoflurane Activates Intestinal Sphingosine Kinase to Protect against Renal Ischemia-Reperfusion-induced Liver and Intestine Injury. *Anesthesiology.* 2011; 114(2):363–373. [PubMed: 21245730]
14. Lee HT, Gallos G, Nasr SH, et al. A1 adenosine receptor activation inhibits inflammation, necrosis, and apoptosis after renal ischemia-reperfusion injury in mice. *J Am Soc Nephrol.* 2004; 15(1):102–111. [PubMed: 14694162]

15. Lee HT, Park SW, Kim M, et al. Acute kidney injury after hepatic ischemia and reperfusion injury in mice. *Lab Invest.* 2009; 89(2):196–208. [PubMed: 19079326]
16. Park SW, Kim M, Chen SW, et al. Sphinganine-1-phosphate attenuates both hepatic and renal injury induced by hepatic ischemia and reperfusion in mice. *Shock.* 2010; 33(1):31–42. [PubMed: 19752779]
17. Hart ML, Henn M, Kohler D, et al. Role of extracellular nucleotide phosphohydrolysis in intestinal ischemia-reperfusion injury. *FASEB J.* 2008; 22(8):2784–2797. [PubMed: 18353866]
18. Quaroni A, Wands J, Trelstad RL, et al. Epithelioid cell cultures from rat small intestine. Characterization by morphologic and immunologic criteria. *J Cell Biol.* 1979; 80(2):248–265. [PubMed: 88453]
19. Ades EW, Candal FJ, Swerlick RA, et al. HMEC-1: establishment of an immortalized human microvascular endothelial cell line. *J Invest Dermatol.* 1992; 99(6):683–690. [PubMed: 1361507]
20. Kim M, Kim M, Park SW, et al. Isoflurane protects human kidney proximal tubule cells against necrosis via sphingosine kinase and sphingosine-1-phosphate generation. *Am J Nephrol.* 2010; 31(4):353–362. [PubMed: 20234131]
21. Kim M, Kim N, Kim M, et al. Isoflurane mediates protection from renal ischemia-reperfusion injury via sphingosine kinase and sphingosine-1-phosphate-dependent pathways. *Am J Physiol Renal Physiol.* 2007; 293(6):F1827–F1835. [PubMed: 17898040]
22. Awad AS, Ye H, Huang L, et al. Selective sphingosine 1-phosphate 1 receptor activation reduces ischemia-reperfusion injury in mouse kidney. *Am J Physiol Renal Physiol.* 2006; 290(6):F1516–F1524. [PubMed: 16403835]
23. Parks DA, Granger DN. Contributions of ischemia and reperfusion to mucosal lesion formation. *Am J Physiol.* 1986; 250(6 Pt 1):G749–G753. [PubMed: 3717337]
24. Deitch EA, Xu D, Kaise VL. Role of the gut in the development of injury- and shock induced SIRS and MODS: the gut-lymph hypothesis, a review. *Front Biosci.* 2006; 11:520–528. [PubMed: 16146750]
25. Stallion A, Kou TD, Latifi SQ, et al. Ischemia/reperfusion: a clinically relevant model of intestinal injury yielding systemic inflammation. *J Pediatr Surg.* 2005; 40(3):470–477. [PubMed: 15793720]
26. Yang L, Froio RM, Sciuto TE, et al. ICAM-1 regulates neutrophil adhesion and transcellular migration of TNF-alpha-activated vascular endothelium under flow. *Blood.* 2005; 106(2):584–592. [PubMed: 15811956]
27. Carr MW, Roth SJ, Luther E, et al. Monocyte chemoattractant protein 1 acts as a T-lymphocyte chemoattractant. *Proc Natl Acad Sci U S A.* 1994; 91(9):3652–3656. [PubMed: 8170963]
28. Breithaupt-Faloppa AC, Vitoretti LB, Coelho FR, et al. Nitric oxide mediates lung vascular permeability and lymph-borne IL-6 after an intestinal ischemic insult. *Shock.* 2009; 32(1):55–61. [PubMed: 18838940]
29. Horie Y, Yamagishi Y, Kato S, et al. Low-dose ethanol attenuates gut ischemia/reperfusion-induced liver injury in rats via nitric oxide production. *J Gastroenterol Hepatol.* 2003; 18(2):211–217. [PubMed: 12542608]
30. LaNoue JL Jr, Turnage RH, Kadesky KM, et al. The effect of intestinal reperfusion on renal function and perfusion. *J Surg Res.* 1996; 64(1):19–25. [PubMed: 8806468]
31. Brookes ZL, Brown NJ, Reilly CS. Differential effects of intravenous anaesthetic agents on the response of rat mesenteric microcirculation in vivo after haemorrhage. *Br J Anaesth.* 2002; 88(2):255–263. [PubMed: 11883388]
32. Englehart MS, Allison CE, Tieu BH, et al. Ketamine-based total intravenous anesthesia versus isoflurane anesthesia in a swine model of hemorrhagic shock. *J Trauma.* 2008; 65(4):901–908. discussion 908-9. [PubMed: 18849809]
33. Preckel B, Schlack W, Comfere T, et al. Effects of enflurane, isoflurane, sevoflurane and desflurane on reperfusion injury after regional myocardial ischaemia in the rabbit heart in vivo. *Br J Anaesth.* 1998; 81(6):905–912. [PubMed: 10211018]
34. Jacob L, Boudaoud S, Payen D, et al. Isoflurane, and not halothane, increases mesenteric blood flow supplying esophageal ileocoloplasty. *Anesthesiology.* 1991; 74(4):699–704. [PubMed: 2008951]

35. Muller M, Schindler E, Roth S, et al. Effects of desflurane and isoflurane on intestinal tissue oxygen pressure during colorectal surgery. *Anaesthesia*. 2002; 57(2):110–115. [PubMed: 11871946]
36. Antkowiak B. How do general anaesthetics work? *Naturwissenschaften*. 2001; 88(5):201–213. [PubMed: 11482433]
37. Cousins MJ, Skowronski G, Plummer JL. Anaesthesia and the kidney. *Anaesth Intensive Care*. 1983; 11(4):292–320. [PubMed: 6359948]
38. Schieble TM, Costa AK, Heffel DF, et al. Comparative toxicity of halothane, isoflurane, hypoxia, and phenobarbital induction in monolayer cultures of rat hepatocytes. *Anesthesiology*. 1988; 68(4):485–494. [PubMed: 3354886]
39. Mantel PY, Schmidt-Weber CB. Transforming growth factor-beta: recent advances on its role in immune tolerance. *Methods Mol Biol*. 2011; 677:303–338. [PubMed: 20941619]
40. Prehn JH, Backhauss C, Kriegelstein J. Transforming growth factor-beta 1 prevents glutamate neurotoxicity in rat neocortical cultures and protects mouse neocortex from ischemic injury in vivo. *J Cereb Blood Flow Metab*. 1993; 13(3):521–525. [PubMed: 8097519]
41. Lefer AM, Ma XL, Weyrich AS, et al. Mechanism of the cardioprotective effect of transforming growth factor beta 1 in feline myocardial ischemia and reperfusion. *Proc Natl Acad Sci U S A*. 1993; 90(3):1018–1022. [PubMed: 8381531]
42. Guan Q, Ngan CY, Du C. Expression of transforming growth factor-beta1 limits renal ischemia-reperfusion injury. *Transplantation*. 2010; 89(11):1320–1327. [PubMed: 20458271]
43. Thomas GR, Thibodaux H. Transforming growth factor-beta 1 inhibits postischemic increases in splanchnic vascular resistance. *Biotechnol Ther*. 1992; 3(3–4):91–100. [PubMed: 1303730]
44. Merin RG, Bernard JM, Doursout MF, et al. Comparison of the effects of isoflurane and desflurane on cardiovascular dynamics and regional blood flow in the chronically instrumented dog. *Anesthesiology*. 1991; 74(3):568–574. [PubMed: 2001036]

E

Figure 1. Isoflurane protects against small intestinal injury after intestinal ischemia-reperfusion injury (IRI)

Representative photomicrographs of ileum from 4 experiments (hematoxylin and eosin staining, magnifications of 200X). Compared to sham-operated mice exposed to pentobarbital (A), intestinal IRI followed by 4 hrs of exposure to pentobarbital led to severe damage to the intestinal mucosa and sloughing of the villous tips (B, magnified in inset) and lifting of the epithelium from the lamina propria and the development of subepithelial Gruenhagen's spaces (C, inset shows Gruenhagen's space). In contrast, the intestines of mice exposed to 4 hrs of 1.2% isoflurane after intestinal IRI were protected from severe injury (D). (E) Small intestinal histology was evaluated with Chiu scores (scale 0–5) in the jejunum and ileum following intestinal IRI with exposure to pentobarbital or isoflurane. Tissues were collected 5 hrs after intestinal IRI. * $P < 0.05$ vs. pentobarbital IRI group. Data presented as mean $\pm$ SEM.

D

Figure 2. Isoflurane protects against apoptosis after intestinal ischemia-reperfusion injury (IRI) Representative fluorescence photomicrographs (of 4 experiments, magnifications of 200X) illustrating apoptotic nuclei (TUNEL fluorescence staining, green) in the ilea of sham-operated mice exposed to pentobarbital (A) and mice exposed to 4 hrs of pentobarbital (B) or 1.2% isoflurane (C) after intestinal IRI. (D) Apoptotic cells per high powered field (hpf) were quantified in the jejunum and ileum of mice following intestinal IRI with exposure to pentobarbital or isoflurane. Tissues were collected 5 hrs after intestinal IRI. * $P < 0.05$ vs. pentobarbital IRI group. Data presented as mean $\pm$ SEM.

Pentobarbital Sham

Pentobarbital IRI

Isoflurane IRI

Figure 3. Isoflurane reduces neutrophil infiltration after intestinal ischemia-reperfusion injury (IRI)

Representative photomicrographs (of 4 experiments, magnifications of 200X) of immunohistochemistry illustrating neutrophil infiltration in the ilea of sham-operated mice exposed to pentobarbital (A) and mice exposed to 4 hrs of pentobarbital (B, inset shows Gruenhagen's space with neutrophil infiltration, arrows highlight areas of neutrophil infiltration) or 1.2% isoflurane (C) after intestinal IRI. Secondary antibody conjugated to horseradish peroxidase was developed with diaminobenzidine to stain neutrophils dark brown. (D) Neutrophils per high powered field (hpf) were quantified in the jejunum and ileum of mice following intestinal IRI with exposure to pentobarbital or isoflurane. Tissues were collected 5 hrs after intestinal IRI. * $P < 0.05$ vs. pentobarbital IRI group. Data presented as mean $\pm$ SEM.

Figure 4. Isoflurane reduces some pro-inflammatory mRNA expression after intestinal ischemia-reperfusion injury (IRI)

Mice were subjected to sham operation or intestinal IRI followed by exposure to 4 hrs of pentobarbital (PB) or 1.2% isoflurane (Iso). Ileal tissues were collected 5 hrs after IRI and pro-inflammatory mRNA expression was measured using semiquantitative RT-PCR. Densitometric quantifications of band intensities relative to GAPDH from RT-PCR reactions. N=4 per group. #P<0.05 vs. sham group. *P<0.05 vs. pentobarbital IRI group.

Figure 5. Isoflurane reduces vascular permeability after intestinal ischemia-reperfusion injury (IRI)

Quantification of Evans blue dye (EBD) extravasation as an index of vascular permeability of jejunal and ileal tissues in mice 5 hrs after sham operation or intestinal IRI followed by exposure to 4 hrs of pentobarbital or 1.2% isoflurane. N=3–4 per group. #P<0.05 vs. pentobarbital sham group. *P<0.05 vs. pentobarbital IRI group. Data presented as mean ± SEM.

A

B
Pentobarbital IRI

Isoflurane IRI

C

Figure 6. Isoflurane reduces liver injury after intestinal ischemia-reperfusion injury (IRI)
 (A) Plasma alanine aminotransferase (ALT, U/L) was measured in mice exposed to 4 hrs of pentobarbital or 1.2% isoflurane after sham operation (N=4 for each group) or intestinal IRI (N=8 for each group). (B) Representative photomicrographs of liver from 4 experiments (hematoxylin and eosin staining, magnifications of 200X or 600X as indicated) of mice exposed to pentobarbital after intestinal IRI (left panels), and mice exposed to 1.2% isoflurane after IRI (right panels). Arrows indicate portal area. (C) Percent (%) vacuolization scores in the livers of mice exposed to pentobarbital or 1.2% isoflurane after intestinal IRI. #P<0.05 vs. pentobarbital sham mice. *P<0.05 vs. pentobarbital IRI group. Data presented as mean ± SEM.

A

Pentobarbital IRI

Isoflurane IRI

H

Figure 7. Isoflurane reduces kidney injury after intestinal ischemia-reperfusion injury (IRI)

(A) Plasma creatinine (Cr, mg/dL) was measured in mice exposed to 4 hrs of pentobarbital or 1.2% isoflurane after sham operation (N=4 for each group) or intestinal IRI (N=8 for each group). Representative photomicrographs of kidney from 4 experiments (hematoxylin and eosin staining, magnifications of 200X, 400X, or 600X as indicated) of mice exposed to pentobarbital after intestinal IRI highlighting tubular simplification (B, asterisks), hypereosinophilia (D, arrows), and vacuolization (F). Mice exposed to 1.2% isoflurane after IRI had significant reductions in these injuries (C, E, and G). (H) Total renal injury scores (scale 0–12) composed of 4 criteria (scale 0–3 each): 1) cortical tubular vacuolization, 2) peritubular leukocyte infiltration, 3) cortical tubular simplification, and 4) proximal tubular hypereosinophilia/single cell necrosis for kidney sections from mice exposed to pentobarbital or 1.2% isoflurane after intestinal IRI. #P<0.05 vs. pentobarbital sham mice. *P<0.05 vs. pentobarbital IRI group. Data presented as mean ± SEM.

Pentobarbital+IgG₁ IRI

200X

Isoflurane+IgG₁ IRI Pentobarbital+TGF- β Ab IRIIsoflurane+TGF- β Ab IRI

Figure 8. Isoflurane protects via transforming growth factor-β1 after intestinal ischemia-reperfusion injury (IRI)

(A–D) Representative photomicrographs of ileum from 4 experiments (hematoxylin and eosin staining, magnifications of 200X) from mice treated with a TGF-β1 neutralizing antibody (Ab) or an isotype-control Ab (IgG₁) prior to intestinal IRI with exposure to pentobarbital (A, IgG₁; C, TGF-β1 Ab) or isoflurane (B, IgG₁; D, TGF-β1 Ab). (E–H) Representative fluorescence photomicrographs (of 4 experiments, magnifications of 200X) illustrating apoptotic nuclei (TUNEL fluorescence staining, green) in the ilea of mice treated with TGF-β1 Ab or IgG₁ prior to intestinal IRI with exposure to pentobarbital (E, IgG₁; G, TGF-β1 Ab) or isoflurane (F, IgG₁; H, TGF-β1 Ab). (I–J) Plasma alanine aminotransferase

(I; ALT, U/L) and creatinine (J; Cr, mg/dL) from mice treated with TGF- β 1 Ab or IgG₁ prior to sham operation (N=4 for each group) or intestinal IRI (N=8 for each group) followed by exposure to 4 hrs of pentobarbital or isoflurane. Samples (H&E, TUNEL, plasma ALT and Cr) were collected 5 hrs after sham operation or intestinal IRI. #P<0.05 vs. pentobarbital+IgG₁ sham mice. *P<0.05 vs. pentobarbital+IgG₁ IRI group. Data presented as mean $\pm$ SEM.

C

Figure 9. Isoflurane increases transforming growth factor (TGF)-β1 levels in plasma and intestine

Mice were exposed to 4 hrs of pentobarbital or 1.2% isoflurane and TGF-β1 levels were measured in the plasma (A) and intestine (B) using ELISA, with appropriate acidification of samples for activation of latent TGF-β1. N=4 per group. (C) TGF-β1 levels were measured in cultured rat intestinal epithelial cells (IEC-6) after exposure to 5 hrs of carrier gas or 2.5% isoflurane. N=6 per group. *P<0.05 vs. pentobarbital or carrier gas group. Data presented as mean ± SEM.

Figure 10. Isoflurane reduces pro-inflammatory mRNA upregulation in human dermal microvascular endothelial cells (HMEC-1) following exposure to TNF- α

(A) Representative gel images (of 4 experiments) of semiquantitative RT-PCR of IL-8 and TNF- α in HMEC-1 cells treated with vehicle (Veh) or TNF- α (10 ng/mL) 30 min prior to exposure to 16 hrs of carrier gas or 2.5% isoflurane. (B) Densitometric quantifications of band intensities relative to GAPDH from RT-PCR reactions. N=4 per group. *P<0.05 vs. carrier gas vehicle group. Data presented as mean $\pm$ SEM.

Primers used to amplify mRNAs encoding GAPDH and proinflammatory cytokines based on published GenBank sequences for mice.

Table 1

Primers	Accession Number	Sequence (Sense/Antisense)	Product Size (bp)	Cycle Number	Annealing Temp (°C)
Mouse MIP-2	X53798	5' - CCAAGGGTTGACTTCAAGAAC - 3' 5' - AGCGAGGCACATCAGGTACG - 3'	282	28	60
Mouse ICAM-1	X52264	5' - TGTTTCTGCTCTGAAGC - 3' 5' - CTTCGTTTGTGATCCTCCG - 3'	409	21	60
Mouse TNF- α	X02611	5' - TACTGAACTTCGGGTGATTGGTCC - 3' 5' - CAGCCTTGTCCTTGAAGAGAAACC - 3'	290	24	65
Mouse MCP-1	NM_011333	5' - ACCTGCTGCTACTCATTCAC - 3' 5' - TTGAGGTGGTTGTGGAAGAG - 3'	312	22	60
Mouse IL-17A	NM_010552	5' - TCCAGAGGCCCTCAGACTA - 3' 5' - ACACCCACAGCATCTTCTC - 3'	248	32	66
Human TNF- α	NM_000594	5' - CGGACGTGGAGCTGGCCGAGGAG - 3' 5' - CACCAGCTGGTTATCTCTCAGTCTC - 3'	355	24	68
Human IL-8	NM_000584	5' - TTGTGAGGACATGTGGAAGC - 3' 5' - ACACAGCTGGCAATGACAAG - 3'	420	27	60
GAPDH	M32599	5' - ACCACAGTCCATGCCATCAC - 3' 5' - CACCACCCCTGTTGCTGTAGCC - 3'	450	15	65

bp, base pairs; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; ICAM-1, intercellular adhesion molecule-1; IL, interleukin; MCP-1, monocyte chemoattractant protein 1; MIP-2, macrophage inflammatory protein 2; TNF- α , tumor necrosis factor- α . Respective anticipated RT-PCR product size, PCR cycle number for linear amplification and annealing temperatures used for each primer are also provided.