

Local anesthetics worsen renal function after ischemia-reperfusion injury in rats

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Lee, H. Thomas, Igor E. Krichevsky, Hua Xu, Ayuko Ota-Setlik, Vivette D. D'Agati, and Charles W. Emala. Local anesthetics worsen renal function after ischemia-reperfusion injury in rats. *Am J Physiol Renal Physiol* 286: F111–F119, 2004. First published September 30, 2003; 10.1152/ajprenal.00108.2003.—Local anesthetics are widely used during the perioperative period, even in patients with preexisting renal disease. However, local anesthetics have been shown to cause cell death in multiple cell lines, including human kidney proximal tubule cells. We questioned whether local anesthetics potentiate renal dysfunction after ischemia-reperfusion (I/R) injury in vivo. Rats were implanted with subcutaneous miniosmotic pumps that continuously delivered lidocaine ($2 \text{ mg}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$), bupivacaine ($0.4 \text{ mg}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$), tetracaine ($1 \text{ mg}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$), or saline vehicle, and 6 h later the rats were subjected to 30 min of renal ischemia or to sham operation. Renal function was assessed by measurement of plasma creatinine at 24 and 48 h after renal I/R injury in the presence or absence of chronic infusions of local anesthetics and correlated to histological changes indicative of necrosis. The degree of renal apoptosis was assessed by three methods: 1) DNA fragmentation detected by terminal deoxynucleotidyl transferase biotin-dUTP nick-end labeling staining, 2) DNA laddering detected after agarose gel electrophoresis, and 3) morphological identification of apoptotic tubules at the corticomedullary junction. We also measured the expression of the proinflammatory markers ICAM-1 and TNF- α . Continuous local anesthetic infusion with renal I/R injury resulted in an increased magnitude and duration of renal dysfunction compared with the saline-infused I/R group. Additionally, both apoptotic and necrotic renal cell death as well as inflammatory changes were significantly potentiated in local anesthetic-treated rat kidneys. Local anesthetic infusion alone without I/R injury had no effect on renal function. We conclude that local anesthetics potentiated renal injury after I/R by increasing necrosis, apoptosis, and inflammation.

acute renal failure; apoptosis; bupivacaine; inflammation; lidocaine; necrosis; tetracaine

ACUTE RENAL FAILURE (ARF) secondary to ischemia-reperfusion (I/R) injury continues to be a significant clinical problem (2, 17, 26). Traditionally, renal cell death in ARF was attributed to necrosis, but recent studies demonstrate that apoptosis accounts for a significant component of renal dysfunction in ARF (21, 25).

Patients with impaired preoperative renal function undergoing aortovascular surgery are at greatest risk for developing perioperative ARF (26). Local anesthetics are widely used in clinical practice, even in patients with impaired preoperative renal function. Epidural infusions of local anesthetic are rou-

tinely used for intraoperative and postoperative analgesia (frequently lasting several days) in patients undergoing major abdominal and vascular procedures. During induction of general anesthesia for endotracheal intubation, intravenous lidocaine is given routinely to blunt the sympathetic reflex to direct laryngoscopy. Local anesthetics are used to provide surgical anesthesia and analgesia in peripheral and central nervous system nerve blocks (spinal and epidural anesthesia). In the intensive care unit, lidocaine is frequently used as an antiarrhythmic agent.

Several in vitro studies found that local anesthetics increase cell death via apoptosis in neuronal, lymphocytic, and osteoblastic cell lines (1, 16, 24). Moreover, we have recently determined that local anesthetics induce apoptosis in human proximal tubule (HK-2) cells by the activation of caspases and via inhibition of ERK mitogen-activated protein kinase and protein kinase B (20). Based on these in vitro findings, we aimed to determine whether local anesthetics increase apoptosis and worsen renal failure in rats after moderate I/R injury.

MATERIALS AND METHODS

Implantation of Miniosmotic Pumps and Renal I/R Injury

All protocols were approved by the Institutional Animal Care and Use Committee of Columbia University. Adult male Sprague-Dawley rats (225–275 g, Harlan Sprague-Dawley, Indianapolis, IN) were used. They had free access to rodent chow and water. Rats were anesthetized with intraperitoneal (ip) pentobarbital sodium (50 mg/kg or to effect) and implanted with subcutaneous miniosmotic pumps (model 2ML1, Alzet) that continuously delivered 10 $\mu\text{l/h}$ of 5% lidocaine ($2 \text{ mg}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$), 1% bupivacaine ($0.4 \text{ mg}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$), 2.5% tetracaine ($1 \text{ mg}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$), or saline vehicle. The doses of local anesthetics delivered mimicked clinically administered doses for continuous epidural infusion for a 70-kg person during and after abdominal and vascular surgical procedures. Some rats were infused with 0.5% bupivacaine instead of 1% bupivacaine. Six hours later (the time required for osmotic pump priming), rats were reanesthetized with pentobarbital sodium. After 500 U of heparin were given ip, rats were placed on an electric heating pad under a warming light. Body temperature was monitored with a rectal probe and maintained at 37°C. They were allowed to spontaneously breathe room air. After a laparotomy, rats were subjected to 30-min left renal ischemia after right nephrectomy. The duration of ischemia was shown in pilot studies to produce reversible and moderate renal dysfunction in rats. Some rats were subjected to only sham operation (anesthesia, laparotomy, and right nephrectomy) and received vehicle (saline) infusion, and others received a sham operation plus local anesthetic

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infusions. While some animals underwent reperfusion for 48 h for the measurement of plasma creatinine (Cr), other groups of animals were killed at 24 h for the measurement of plasma creatinine and to obtain kidney tissue for all of the *in vitro* measurements described [histology, terminal deoxynucleotidyl transferase biotin-dUTP nick-end labeling (TUNEL) assay, DNA laddering, semiquantitative RT-PCR, and immunoblotting]. Rats were killed with an overdose of ip pentobarbital sodium.

In some rats (sham-operated, vehicle-treated I/R, and 1% bupivacaine-infused I/R rats), noninvasive systolic and diastolic blood pressures (SBP and DBP, respectively) and heart rate (HR) were measured by the tail-cuff method (model XBP 1000, Kent Scientific, Torrington, CT) in awake rats before renal ischemia (6 h after pump implantation), during anesthesia but before renal ischemia, during anesthesia and 15 min after renal ischemia, 24 h after renal ischemia, and 48 h after renal ischemia. Six consecutive measurements were made for each rat at each time point.

Rat Renal Cortical Membrane Preparation and Immunoblot Analyses

Twenty-four hours after renal ischemic injury, rat kidney cortical tissues were dissected on ice and immediately placed in ice-cold RIPA buffer (150 mM NaCl, 50 mM Tris-HCl, 1 mM EDTA, 1% Triton X-100, pH 7.4) and homogenized for 10 s on ice. The samples were centrifuged for 10 min at 1,000 *g*. The supernatant was collected and used for immunoblotting. ICAM-1 expression was detected in supernatants by immunoblotting (18, 19) using a monoclonal antibody (sc-8439) (Santa-Cruz Biotechnology) diluted 1:500.

Measurement of Plasma Creatinine and Local Anesthetics

Plasma creatinine levels were measured spectrophotometrically using a commercially available quantitative colorimetric assay (Sigma). Plasma lidocaine and bupivacaine levels achieved at 48 h were measured by HPLC at the Nathan Kline Institute (New York).

Histological Examinations to Detect Necrosis and Apoptosis

For histological preparation, explanted kidneys were bisected along the long axis and cut into three equal-size slices. Kidney slices were obtained 24 h after treatment from sham, saline-, or local anesthetic-treated rats and were fixed in 10% formalin solution overnight. After automated dehydration through a graded alcohol series, transverse kidney slices were embedded in paraffin, sectioned at 5 μ m, and stained with hematoxylin-eosin (H&E). Morphological assessment was performed by an experienced renal pathologist (VDD) who was unaware of the treatment that each animal had received. A grading scale of 0–4, as outlined by Jablonski et al. (13), was used to assess the degree of renal tubular necrosis after renal I/R injury. Renal tubular apoptosis was assessed by counting the number of apoptotic bodies in proximal tubules in the outer stripe of the corticomedullary junction (expressed as the mean number of apoptotic bodies per tubule). This area is the most severely injured area after renal I/R

injury. At least 25–30 tubules were counted per field, and 6 fields were examined per slide.

Detection of DNA Fragmentation

DNA laddering. Twenty-four hours after renal ischemic injury, renal cortices were dissected and extracted DNA (Wizard, Promega, Madison, WI) was electrophoresed at 70 V in a 2.0% agarose gel in Tris-acetate-EDTA buffer. The gel was stained with ethidium bromide and photographed under UV illumination. DNA ladder markers (100 bp) were added to each gel as a reference for the analysis of internucleosomal DNA fragmentation.

In situ TUNEL assay. Fixed rat kidney sections obtained at 24 h of treatment were deparaffinized in xylene and rehydrated through graded ethanols to water. *In situ* labeling of fragmented DNA was performed with TUNEL (green fluorescence) using a commercially available *in situ* cell death detection kit (Roche) according to the manufacturer's instruction. To visualize the total number of cells in the field, kidney sections were also stained with propidium iodide (red fluorescence).

Renal Cortical Myeloperoxidase Assay

Myeloperoxidase (MPO) is an enzyme present in leukocytes and is an index of tissue leukocyte infiltration (30). Twenty-four hours after renal ischemic injury, renal cortex (~200 mg) was dissected and homogenized for 30 s in 2 ml of 50 mM potassium phosphate buffer, pH 7.4, at 4°C. The samples were centrifuged for 15 min at 16,000 *g* at 4°C, and the resultant pellet was resuspended in 2 ml of 50 mM potassium phosphate buffer, pH 7.4, with 0.5% hexadecyltrimethyl ammonium bromide at 4°C. The samples were sonicated for 30 s and centrifuged at 16,000 *g* for 15 min at 4°C. Fifty microliters of supernatant were mixed with 750 μ l of 45 mM potassium phosphate buffer, pH 6.0, containing 0.167 mg/ml *o*-dianisidine and 0.3% H₂O₂. Absorbance (460 nm) was measured over a period of 5 min (unit of enzyme activity = Δ OD \cdot min⁻¹ \cdot mg protein⁻¹), and the relative MPO activity was expressed as the percentage of the sham-operated group. The remaining supernatant was used to determine protein concentrations.

Semiquantitative RT-PCR for Proinflammatory Cytokines and Proapoptotic Genes

Twenty-four hours after renal ischemic injury, renal cortices were dissected and total RNA was extracted using TRIzol (Invitrogen, Carlsbad, CA) reagent. RNA concentrations were determined with spectrophotometric readings at 260 nm and run on agarose gels to verify equal loading and RNA quality. RT-PCR was performed to analyze the expression of proinflammatory (TNF- α , IL-1 β , and ICAM-1) genes. Primers were designed based on published GenBank sequences for the rat (Table 1). Primer pairs were chosen to yield expected PCR products of 200–600 bp and to amplify a genomic region that spans one or two introns to eliminate the confounding effect of amplifying contaminating genomic DNA. Primers were

Table 1. Primer sequence

Primer	Accession No. (Rat)	Sequence (Sense/Antisense)	Product Size, bp	Annealing Temperature, °C
TNF- α	AF329986	5'-TACTGAACTTCGGGGTGATTGGTCC-3' 5'-CAGCCTTGTCCTTGAAGAGAACC-3'	295	65
ICAM-1	NM_012967	5'-TGTTTCCTGCCTCTGAAGC-3' 5'-CTTCGTTTGTGATCCTCCG-3'	409	60
IL-1 β	NM_031512	5'-CTGAAAGCTCTCCACCTC-3' 5'-TGCTGATGTACCAGTTGGGG-3'	292	56
GAPDH	AF106860	5'-ACCCACAGTCCATGCCATCAC-3' 5'-CACCACCTGTTGCTGTAGCC-3'	450	65

Table 2. Creatinine values after renal ischemia-reperfusion injury

	24 h	48 h
Sham	1.0 ± 0.1 (n=4)	0.9 ± 0.2 (n=4)
Saline + IR	2.1 ± 0.4 (n=4)*	0.9 ± 0.1 (n=4)
5% Lidocaine + IR	4.3 ± 0.3 (n=10)*†	4.3 ± 0.8 (n=6)*†
0.5% Bupivacaine + IR	2.2 ± 0.5 (n=4)*	1.4 ± 0.3 (n=4)
1% Bupivacaine + IR	4.3 ± 0.3 (n=8)*†	3.3 ± 0.9 (n=6)*†
2.5% Tetracaine + IR	4.8 ± 0.2 (n=11)*†	3.2 ± 0.6 (n=6)*†
Sham + 1% bupivacaine	0.8 ± 0.1 (n=4)	0.9 ± 0.1 (n=4)

Values are means ± SE. n, No. of rats; IR, ischemia-reperfusion. * $P < 0.05$ vs. sham group. † $P < 0.05$ vs. saline + IR group.

purchased from Sigma Genosys (The Woodlands, TX). RT-PCR was performed using the Access RT-PCR System (Promega), which is designed for a single tube reaction for first-strand cDNA synthesis (48°C for 45 min) using AMV reverse transcriptase, and subsequent PCR using Tfl DNA polymerase. PCR cycles included a denaturation step of 94°C for 30 s, followed by an optimized annealing temperature (Table 1) for 1 min, followed by a 1-min extension period at 68°C. All PCR reactions were completed with a 7-min incubation at 68°C to allow enzymatic completion of incomplete cDNAs. The PCR cycle number for each primer pair was optimized to yield linear increases in the densitometric measurements of resulting bands with increasing cycles of PCR (15–30 cycles). The starting amount of RNA was also optimized to yield linear increases in the densitometric measurements of resulting bands at an established number of PCR cycles. Based on these preliminary experiments 0.5–1.0 µg of total RNA was used as a template for all RT-PCR reactions. The number of PCR cycles yielding linear results was 21, 24, and 22 for ICAM-1, TNF-α, and IL-1β, respectively. For each experiment, we also performed semi-quantitative RT-PCR under conditions yielding linear results for GAPDH (15 cycles) to confirm equal RNA input. The products were

resolved in a 6% polyacrylamide gel and stained with Syber green (Roche, Indianapolis, IN), and the band intensities were quantified using a Fluor-S Multi Imager (Bio-Rad, Hercules, CA).

Protein Determination

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

Materials

Solutions were made daily. Pentobarbital sodium was purchased from Henry Schein Veterinary (Indianapolis, IN). All local anesthetics were obtained from Sigma (St. Louis, MO) and were dissolved in PBS.

Statistical Analysis

A one-way analysis of variance was used to compare mean values across multiple treatment groups with a Dunnett post hoc multiple comparison test (e.g., sham vs. I/R). The ordinal values of the Jablonski scale were analyzed by the Kruskal-Wallis nonparametric test with Dunn posttest comparison between groups. In all cases, a probability statistic < 0.05 was taken to indicate significance. All data are expressed throughout the text as means ± SE.

RESULTS

Renal Function Assessment and Plasma Levels of Local Anesthetics After Chronic Infusion

Thirty minutes of renal ischemia with saline infusion (vehicle for local anesthetics) caused moderate renal dysfunction at 24 h ($Cr = 2.1 \pm 0.4$ mg/dl, $n = 4$, vs. sham-operated rat $Cr = 1.0 \pm 0.1$ mg/dl, $n = 4$) followed by recovery to a normal creatinine at 48 h after renal ischemia ($Cr = 0.9 \pm 0.1$ mg/dl, $n = 4$, vs. sham-operated rat at 48 h; $Cr = 0.9 \pm 0.2$ mg/dl,

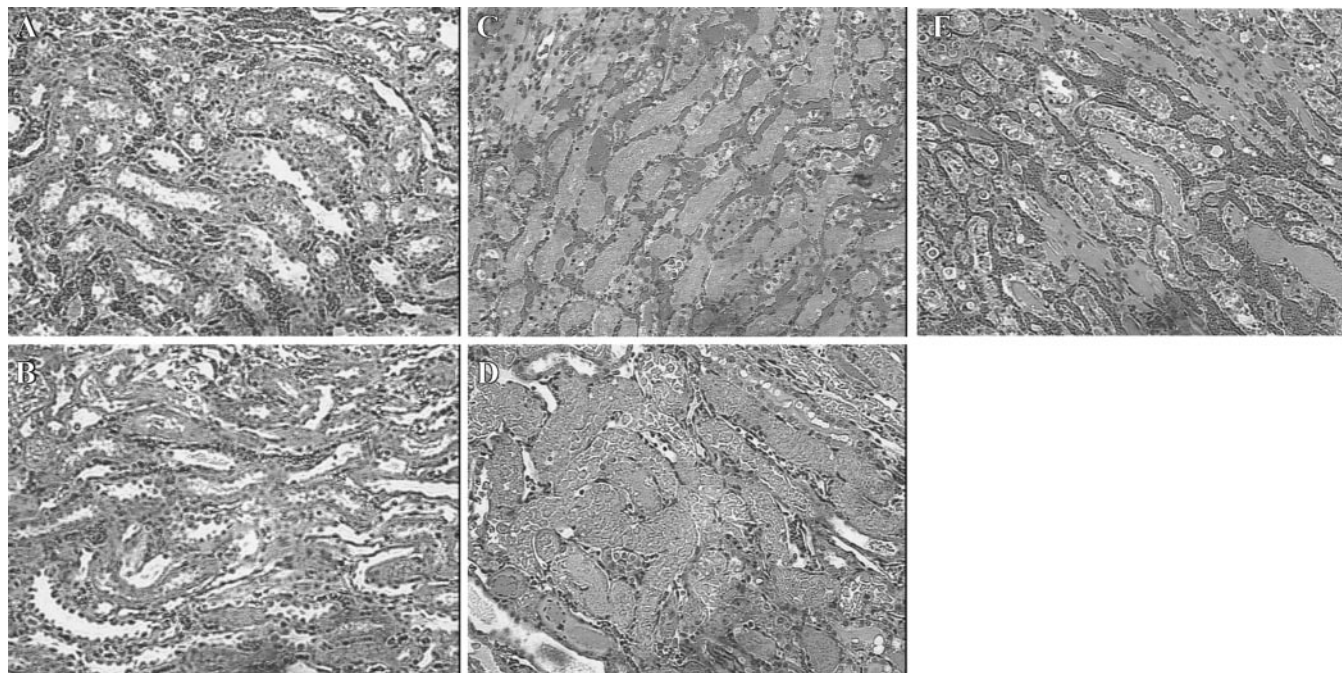


Fig. 1. Representative light microscopic photographs of the corticomedullary junction of the rat kidney. A: sham-operated rats. B: vehicle (saline)-treated rats subjected to ischemia-reperfusion (I/R). C–E: 5% lidocaine-, 1% bupivacaine-, and 2.5% tetracaine-treated rats subjected to I/R, respectively. All 3 local anesthetic treatments increased necrotic damage. Representative of 4 independent experiments. Hematoxylin and eosin staining; magnification: $\times 200$.

$n = 4$, Table 2). Rats receiving 1% bupivacaine infusions and only sham operations had normal Cr at days 1 (0.8 ± 0.1 mg/dl, $n = 4$) and 2 (0.9 ± 0.1 mg/dl, $n = 4$). However, continuous 1% bupivacaine infusions after renal ischemia worsened renal function at 24 and 48 h after injury (Table 2). Similarly, chronic infusions of either 5% lidocaine or 2.5% tetracaine worsened renal function at 24 and 48 h after injury (Table 2). In contrast, continuous infusions of a lower dose of bupivacaine (0.5%) after renal ischemia did not effect renal function at 24 or 48 h after injury compared with the vehicle-treated I/R group (Cr = 2.2 ± 0.5 mg/dl, $n = 4$, and 1.4 ± 0.3 mg/dl, $n = 4$ at 24 and 48 h, respectively, after renal ischemia) (Table 2).

After 48 h, the plasma levels of lidocaine and bupivacaine achieved with 10 μ l/h infusion of 5 and 1% solution, respectively, were 3.1 ± 0.2 ($n = 7$) and 0.9 ± 0.1 μ M ($n = 6$). The plasma level achieved with the infusion of 0.5% bupivacaine (which failed to significantly worsen renal function) was 0.2 ± 0.02 μ M ($n = 4$). Because tetracaine is rapidly metabolized by plasma esterases, we were unable to measure its plasma concentration.

Effects of Bupivacaine Infusion on Blood Pressure and HR

Before anesthesia, bupivacaine-infused rats [SBP = 130 ± 5 mmHg, DBP = 81 ± 6 mmHg, and HR = 394 ± 10 beats/min (bpm), $n = 3$] had similar SBP, DBP, and HR compared with sham-operated (SBP = 135 ± 6 mmHg, DBP = 85 ± 4 mmHg, and HR = 388 ± 11 bpm, $n = 3$) or vehicle-infused I/R rats (SBP = 134 ± 7 mmHg, DBP = 84 ± 3 mmHg, and HR = 405 ± 9 bpm, $n = 3$). The SBP, DBP, and HR for vehicle-treated I/R rats during anesthesia before renal ischemia (SBP = 114 ± 8 mmHg, DBP = 75 ± 7 mmHg, and HR = 418 ± 19 bpm, $n = 3$); 15 min after renal ischemia under anesthesia (SBP = 99 ± 5 mmHg, DBP = 63 ± 6

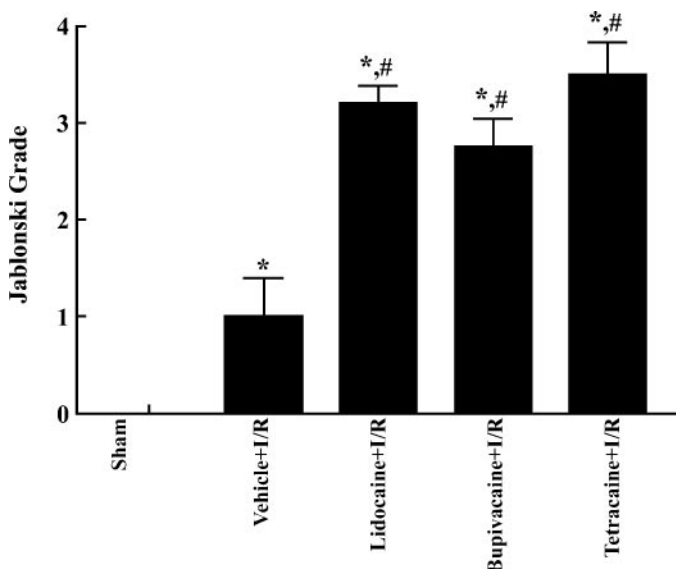


Fig. 2. Jablonski grading scale scores for the histologic appearance of acute tubular necrosis from sham-operated rats (sham; $n = 3$), vehicle-treated rats subjected to I/R (vehicle+I/R; $n = 3$), and local anesthetic-treated rats subjected to I/R (5% lidocaine+I/R, $n = 6$; 1% bupivacaine+I/R, $n = 4$; and 2.5% tetracaine+I/R, $n = 4$). Error bars, SE. * $P < 0.05$ vs. sham. # $P < 0.05$ vs. vehicle+I/R.

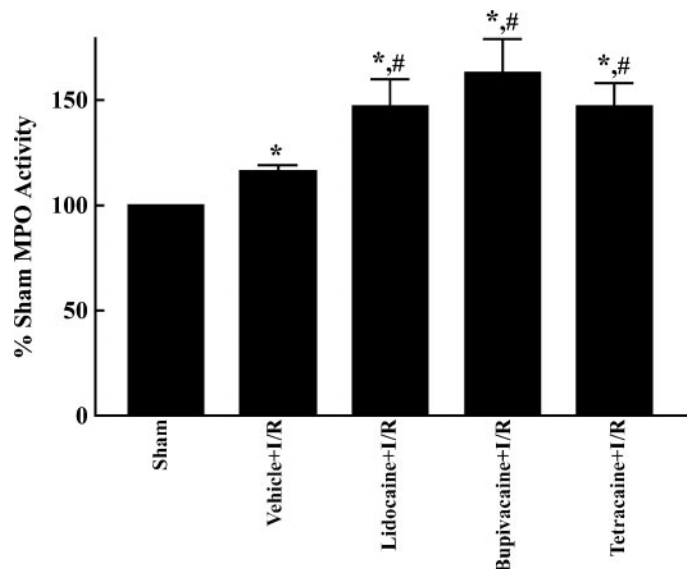


Fig. 3. Myeloperoxidase (MPO) activity in renal cortex of rats after sham operation or I/R injury in rats receiving saline vehicle ($n = 4$), lidocaine ($n = 4$), bupivacaine ($n = 4$), or tetracaine ($n = 4$). Error bars, SE. * $P < 0.05$ vs. sham. # $P < 0.05$ vs. vehicle+I/R.

mmHg, and HR = 374 ± 14 bpm, $n = 3$); 24 h after ischemia while awake (SBP = 124 ± 6 mmHg, DBP = 76 ± 7 mmHg, and HR = 431 ± 9 bpm, $n = 3$); and in awake animals 48 h after renal ischemia (SBP = 122 ± 8 mmHg, DBP = 65 ± 6 mmHg, and HR = 405 ± 18 bpm, $n = 3$) were not significantly different compared with bupivacaine-treated I/R rats.

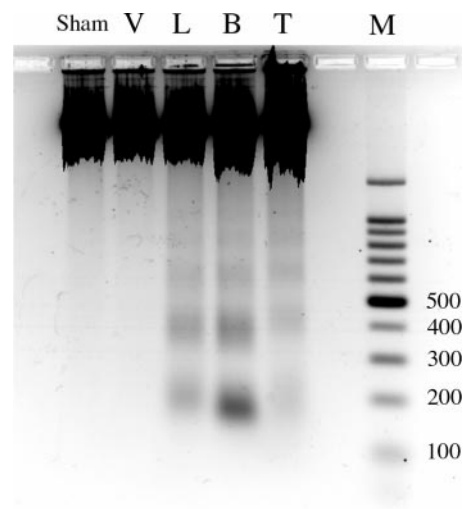


Fig. 4. Representative experiment (of 4) illustrating DNA fragmentation (laddering) in an ethidium bromide-stained agarose gel. Equal amounts of DNA (10 μ g) were loaded in each lane. No laddering was visible in DNA extracted from sham kidneys or in DNA from saline vehicle-treated animals subjected to 30-min renal ischemia and 24 of reperfusion (V). However, infusion of local anesthetics during reperfusion caused DNA fragmentation characteristic of apoptosis. L, 5% lidocaine; B, 1% bupivacaine; T, 2.5% tetracaine. Each lane represents DNA from individual animals. In the last lane, a 100-bp DNA size marker (M) was loaded.

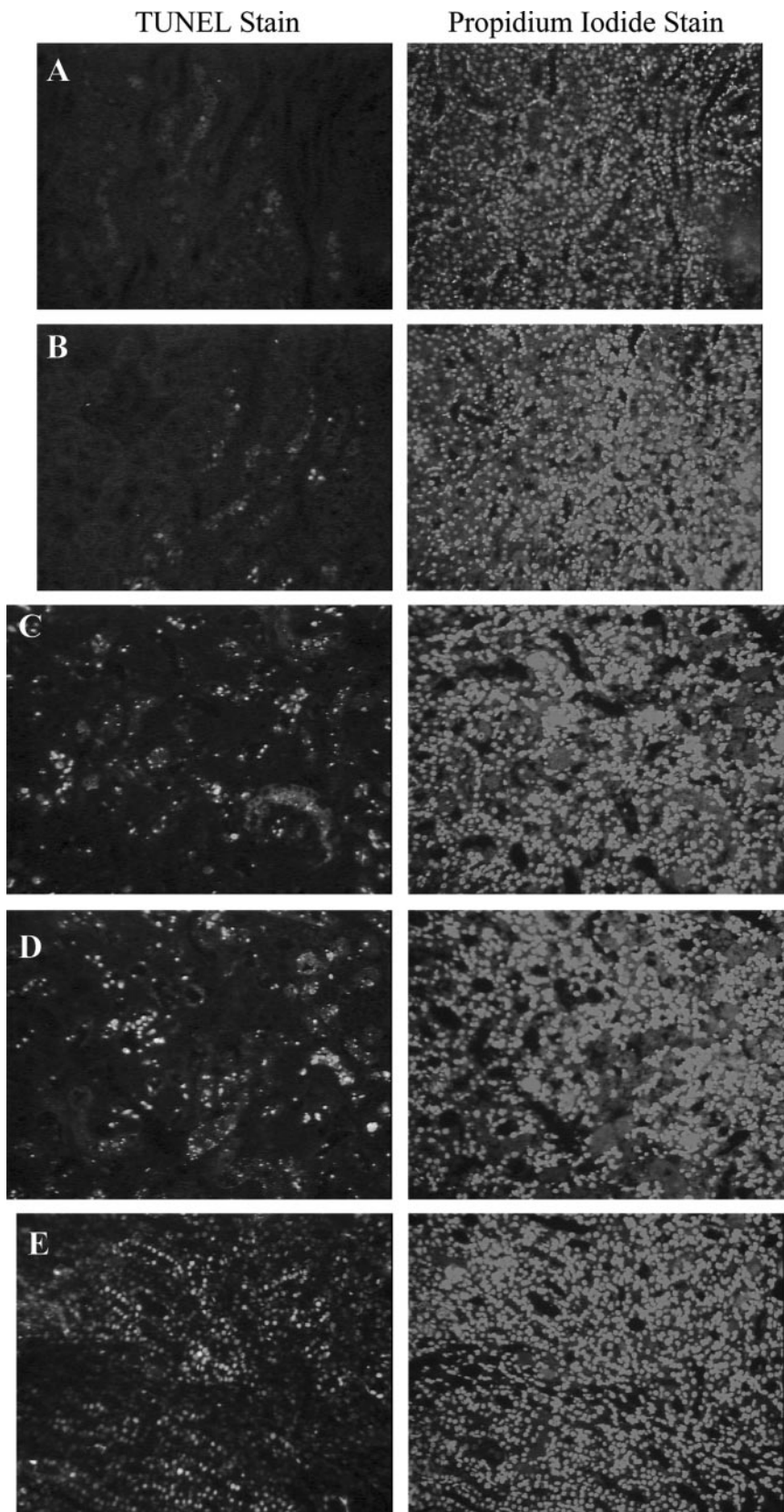


Fig. 5. Representative fluorescent photomicrographs of kidney sections from identical fields illustrate apoptotic nuclei [terminal deoxynucleotidyl transferase biotin-dUTP nick-end labeling (TUNEL) fluorescence stain; *left*] or total nuclei (propidium iodide fluorescence stain; *right*). *A*: sections from sham-operated rats showed rare apoptotic nuclei. *B*: sections from vehicle-infused rats subjected to I/R showed occasional TUNEL-positive staining. *C–E*: 5% lidocaine-, 1% bupivacaine-, or 2.5% tetracaine-infused rats, respectively, subjected to I/R showed a large increase in the number of TUNEL-positive cells. Corticomedullary areas are shown (magnification: $\times 100$). Photomicrographs are representative of 4 independent experiments.

Local Anesthetic Treatment Increases Necrotic Injury After Renal I/R

Exacerbation of renal function with local anesthetic treatment after I/R injury is further supported by representative histological slides (Fig. 1). Vehicle-treated rats subjected to 30 min of renal ischemia (Fig. 1B) followed by 24 h of reperfusion displayed mild renal injury. Rats treated with lidocaine (Fig. 1C), bupivacaine (Fig. 1D), or tetracaine (Fig. 1E) and subjected to renal I/R injury showed significantly worsened renal morphology, as evidenced by severe tubular necrosis, medullary congestion and hemorrhage, and the development of proteinaceous casts. The Jablonski scale histology grading scores for necrotic changes (13) are shown in Fig. 2. Thirty minutes of renal ischemia followed by 24 h of reperfusion in saline-treated rats resulted in mild acute tubular necrosis (Jablonski grade = 1.0 ± 0.4 , $n = 3$, $P < 0.05$ vs. sham-operated group). Rats chronically infused with local anesthetics and subjected to renal I/R demonstrated significantly worsened histological grading to reflect moderate to severe necrosis (Jablonski grade = 3.2 ± 0.2 , $n = 6$, 2.8 ± 0.3 , $n = 4$, and 3.5 ± 0.3 , $n = 4$, for lidocaine-, bupivacaine-, and tetracaine-treated rats, respectively) compared with rats chronically infused with saline vehicle and subjected to renal I/R (Fig. 2). Rats chronically infused with the lower dose of bupivacaine (0.5%) and subjected to I/R injury had Jablonski scores similar to those rats subjected to I/R alone (0.96 ± 0.4 , $n = 4$, vs. 1.0 ± 0.4 , $n = 3$, respectively). Rats treated with 1% bupivacaine for 24 h without I/R had normal renal histology reflected in a Jablonski score of 0 ($n = 3$).

Local Anesthetic-Treated Rats Show Increased Leukocyte Infiltration After I/R

Renal cortical MPO assay. Thirty minutes of renal ischemia and 24 h of reperfusion slightly but significantly increased MPO activity in the renal cortex of rats receiving saline vehicle ($116 \pm 3\%$ of sham-operated rats, $n = 4$). Local anesthetic-treated rats subjected to renal I/R had significantly higher MPO activity [147 ± 13 , 163 ± 16 , and $147 \pm 11\%$ of sham-operated rats for lidocaine ($n = 4$)-, bupivacaine ($n = 4$)-, and tetracaine ($n = 4$)-treated rats], respectively, compared with rats receiving saline vehicle (Fig. 3).

Local Anesthetic-Treated Rats Show Increased Markers of Apoptosis

DNA laddering. Figure 4 shows representative DNA laddering after agarose gel electrophoresis (representative of 4 experiments). Sham-operated and rats subjected to I/R plus vehicle treatment did not display DNA laddering. In contrast, rats subjected to I/R and local anesthetic treatments showed easily visible DNA laddering composed of DNA fragments in multiples of ~ 200 bp.

TUNEL assay. We failed to detect TUNEL-positive cells in kidney sections (corticomedullary junction) from sham-operated rats (Fig. 5A). Rats receiving saline vehicle infusion and subjected to 30 min of renal ischemia and 24 h of reperfusion (Fig. 5B) showed very few TUNEL-positive cells in the corticomedullary junction. In contrast, rats treated with local anesthetics and subjected to renal I/R showed extensive TUNEL-positive staining in the corticomedullary junction (Fig. 5, C–E;

representative figure of 4 experiments for each treatment group).

Assessment of renal tubular apoptotic bodies. Because TUNEL staining can be nonspecific, we examined H&E-stained rat kidney sections under light microscopy for apoptotic bodies. The degree of renal tubular apoptosis was quantified by counting the number of apoptotic bodies in proximal tubules in the corticomedullary area of the kidney. Twenty-five to fifty tubules were counted per field from each treatment group, and kidneys from four experiments were examined. Sham-operated rats and rats infused with 1% bupivacaine without renal I/R injury had no morphological evidence of apoptosis. Rats infused with 5% lidocaine ($n = 6$), 1% bupivacaine ($n = 4$), or 2.5% tetracaine ($n = 4$) and subjected to renal I/R injury had a significantly higher number of tubules containing apoptotic bodies compared with saline-infused rats subjected to I/R ($n = 4$; 0.42 ± 0.08 , 0.56 ± 0.14 , 0.92 ± 0.08 , and 0.25 ± 0.07 apoptotic bodies \cdot tubule $^{-1}$ \cdot field $^{-1}$ for lidocaine-, bupivacaine-, tetracaine-, and saline-infused rats, respectively, Fig. 6).

Immunoblotting for ICAM-1

ICAM-1 has been shown to be upregulated after I/R injury in many organ systems and is an important contributor of leukocyte infiltration. Increased ICAM-1 protein expression was detected by immunoblotting in rat kidney cortex after I/R injury and local anesthetic treatment compared with saline-infused rats subjected to I/R (Fig. 7; representative of 5 independent experiments).

RT-PCR of Proinflammatory Genes

We performed semiquantitative RT-PCR to detect induction of mRNA encoding proinflammatory proteins (ICAM-1, TNF- α , and IL-1 β). Total RNA was isolated from renal cortices, and the quantitative accuracy of our RT-PCR technique was first established (see MATERIALS AND METHODS). Local an-

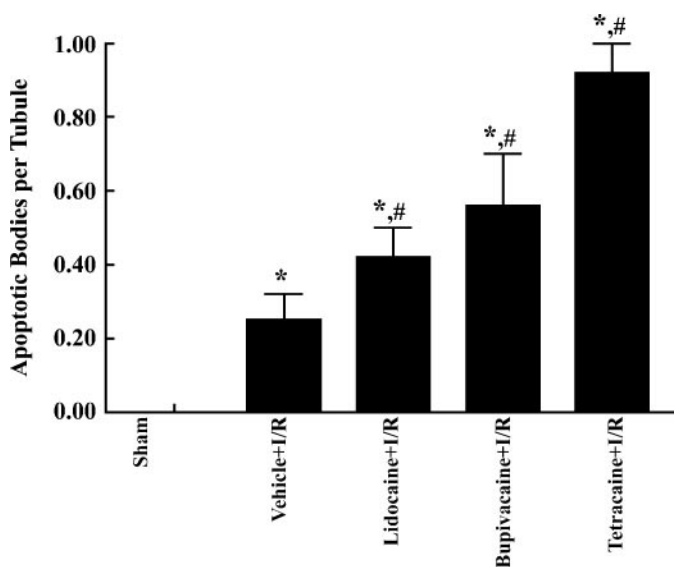


Fig. 6. Quantification of apoptotic bodies in the corticomedullary area of the kidney after sham operation or I/R injury in rats receiving saline vehicle ($n = 4$), lidocaine ($n = 6$), bupivacaine ($n = 4$), or tetracaine ($n = 4$). Error bars, SE. * $P < 0.05$ vs. sham. # $P < 0.05$ vs. vehicle+I/R.



Fig. 7. Representative immunoblot of ICAM-1. A single protein band of an apparent 60-kDa molecular mass was detected using monoclonal ICAM-1 antibody. Infusion of local anesthetics during reperfusion increased ICAM-1 protein expression [5% lidocaine (L), 1% bupivacaine (B), and 2.5% tetracaine (T)] compared with sham-operated (S) or vehicle-treated rats subjected to I/R (V). Data are representative of 5 independent experiments.

esthetic-treated rats subjected to I/R injury showed significantly increased expression of mRNA encoding ICAM-1 and TNF- α (Fig. 8) compared with either sham-operated or vehicle-treated rats subjected to I/R. There was no significant difference in the expression of IL-1 β (data not shown).

DISCUSSION

The major findings of this study are that infusion of clinically utilized local anesthetics after renal I/R injury significantly worsened renal function in rats. Exacerbation of renal function with local anesthetic treatment was associated with increased necrosis, apoptosis, and inflammation.

Our study is the first report to describe that local anesthetics worsen renal injury in rats after I/R *in vivo*. Renal I/R alone doubled Cr values 24 h after injury, with a complete return to baseline Cr values at 48 h after injury. However, chronic infusion with 5% lidocaine, 1% bupivacaine, or 2.5% tetracaine during the period of I/R caused a further doubling of Cr values at 24 h above those increases seen with I/R alone.

Moreover, increases in Cr values persisted to 48 h. Thus chronic local anesthetic infusions not only augmented the initial rise in Cr values but added to the duration of renal insufficiency.

We next sought to illustrate the mechanism (necrosis, apoptosis, and/or inflammation) by which local anesthetics potentiated renal injury after I/R. Our histological analyses focused on the proximal tubule cells because these cells have been shown to be the most susceptible to I/R injury (22, 28). The exacerbation of necrotic injury after local anesthetic infusions with I/R was demonstrated qualitatively by severe tubular necrosis, medullary congestion and hemorrhage, and the development of proteinaceous casts seen on H&E-stained corticomedullary junctions. Local anesthetic-induced increase in necrosis was confirmed by the blinded scoring of H&E-stained corticomedullary junctions by the method of Jablonski et al. (13).

We next sought to determine whether local anesthetic-induced potentiation of renal dysfunction after I/R injury was associated with apoptosis. As no single indicator is considered pathognomonic for apoptosis, we utilized three independent methods to identify apoptosis, including TUNEL staining, DNA laddering, and quantification of apoptotic bodies (condensed nuclei). All three approaches revealed that chronic infusion of 5% lidocaine, 1% bupivacaine, or 2.5% tetracaine during ischemia and for 24 h of reperfusion greatly accentuated subtle apoptotic changes present with I/R alone. Thus both necrosis and apoptosis increased with local anesthetic treatment.

We next sought to determine whether local anesthetic-induced potentiation of renal dysfunction after I/R injury was associated with increased evidence of inflammation. The infu-

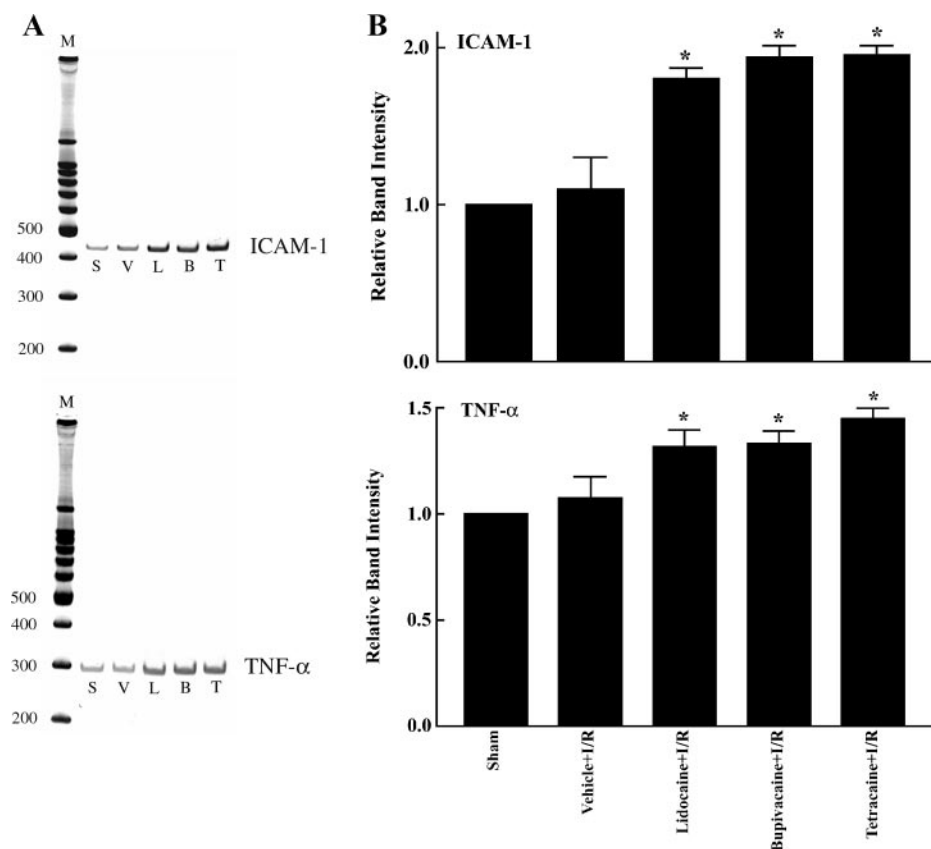


Fig. 8. *A*: representative ICAM-1 (*top*) and TNF- α (*bottom*) mRNA expression measured by semiquantitative RT-PCR in rat kidney cortex. Rats were subjected to sham operation (S), vehicle treatment and I/R (V), or local anesthetic infusions (as in Fig. 7) and I/R. Data are representative of 5 independent experiments. For each gel, a 100-bp RNA size marker (M) is shown. *B*: densitometric quantifications of band intensities from RT-PCR reactions for ICAM-1 and TNF- α ($n = 5$). Data in bar graphs are means \pm SE. * $P < 0.05$ vs. vehicle-treated rats subjected to I/R.

sion of 5% lidocaine, 1% bupivacaine, or 2.5% tetracaine during ischemia and 24 h of reperfusion significantly increased the expression of mRNA encoding ICAM-1 and TNF- α and the protein expression of ICAM-1 in renal cortex. Donnahoo et al. (6) demonstrated that early expression of tumor necrosis factor- α (TNF- α) in renal tissue contributes to subsequent neutrophil infiltration in I/R injury and TNF- α plays a key role in increasing adhesion molecule (e.g., ICAM-1) expression after injury. Consistent with the increases in proinflammatory molecules with local anesthetic treatment, we demonstrate increased MPO activities in local anesthetic-treated rat kidney cortex. MPO is an enzyme present in leukocytes and is an index of leukocyte infiltration into renal parenchyma (6). Several recent studies demonstrated that leukocyte infiltration into the kidney after I/R injury contributes significantly to the pathogenesis of ARF (10, 14, 15). The influx of leukocytes, including polymorphonuclear neutrophils, macrophages, and lymphocytes (30), during reperfusion initiates a cascade of proinflammatory events, including cytokine/chemokine liberation and free radical-mediated tubular damage. Sublethally damaged proximal tubular epithelial cells release chemokines and cytokines and have the capacity to upregulate adhesion molecules (e.g., ICAM-1) to facilitate the leukocyte infiltration and adhesion (6, 7).

Increased renal tubular apoptosis with local anesthetic treatment and I/R injury is in agreement with our previous in vitro study demonstrating the induction of apoptosis in cultured human proximal tubules in a concentration- and potency-dependent (lidocaine < bupivacaine < tetracaine) manner (20). Furthermore, our study is consistent with the study of Daemen et al. (3–5), who demonstrated that increases in apoptosis after renal I/R injury were directly associated with increased renal inflammation. They also demonstrated that blocking apoptosis prevented renal inflammation after I/R. They hypothesized that a significant component of renal inflammation is induced by apoptosis. Insufficient local phagocytotic capacity to clear apoptotic cells has been associated with subsequent necrosis, a process termed “secondary necrosis” (3). It is difficult to estimate the contribution of secondary necrosis to I/R-induced inflammation in the in vivo model used in the present study. However, our previous in vitro study demonstrated that local anesthetics induced apoptosis, at least in part, by inhibiting prosurvival kinases (PKB and ERK MAPK) and activating caspases (20).

We chose to infuse doses of lidocaine (2 mg·kg⁻¹·h⁻¹) and bupivacaine (0.4 mg·kg⁻¹·h⁻¹) to mimic a clinically applicable epidural infusion regimen after major abdominal or vascular surgical procedures for a 70-kg person. Plasma concentrations of local anesthetics range from 5 to 20 μ M after intravenous (8, 9) and epidural (11, 23, 27, 29) injections to the 1–10 mM range (12) after local nerve plexus infiltrations (e.g., interscalene or axillary block). Our regimen of local anesthetic treatment achieved plasma concentrations of 3.1 and 0.9 μ M for lidocaine and bupivacaine, respectively, which are similar to plasma levels achieved with chronic epidural infusion of lidocaine (11, 29) and bupivacaine (23, 27). Infusion of less concentrated bupivacaine (0.5%) resulted in a plasma concentration of 0.2 μ M, which failed to exacerbate renal dysfunction after I/R injury. It is unlikely that our findings are attributable to hemodynamic changes induced by local anesthetics because

bupivacaine-infused and control rats had similar blood pressures and HR before, during, and after renal I/R.

In conclusion, we demonstrated that 48 h of local anesthesia administration after renal ischemia significantly worsened renal function and increased both necrotic and apoptotic cell death in the kidney. As local anesthetics are widely used drugs in the perioperative setting, understanding the influence of local anesthetics on perioperative renal dysfunction is of great clinical importance.

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