

Activation of Sensory Neurons Reduces Ischemia/Reperfusion-induced Acute Renal Injury in Rats

Akio Mizutani, M.D.,* Kenji Okajima, M.D.,† Kazunori Murakami, M.D.,‡ Sachiko Mizutani, M.S.N.,§ Kyosuke Kudo, M.D.,|| Tetsuya Uchino, M.D.,|| Yuji Kadoi, M.D.,# Takayuki Noguchi, M.D.**

Background: Prostaglandin I₂ (PGI₂) produced by endothelial cells improves ischemia/reperfusion-induced acute renal injury by inhibiting leukocyte activation in rats. However, the underlying mechanism(s) of increased PGI₂ production is not fully understood. Activation of sensory neurons increases endothelial PGI₂ production by releasing calcitonin gene-related peptide (CGRP) in rats with hepatic ischemia or reperfusion. We examined here whether activation of sensory neurons increases PGI₂ endothelial production, thereby reducing ischemia/reperfusion-induced acute renal injury.

Methods: Anesthetized rats were subjected to 45 min of renal ischemia/reperfusion. Rats were pretreated with CGRP, capsazepine (a vanilloid receptor-1 antagonist), CGRP(8-37) (a CGRP receptor antagonist), or indomethacin (a cyclooxygenase inhibitor), or subjected to denervation of primary sensory nerves before ischemia/reperfusion.

Results: Renal tissue levels of CGRP and 6-keto-prostaglandin F_{1α}, a stable metabolite of PGI₂, increased after renal ischemia/reperfusion, peaking at 1 h after reperfusion. Overexpression of CGRP was also noted at 1 h after reperfusion. Increases in renal tissue levels of 6-keto-prostaglandin F_{1α} at 1 h after reperfusion were significantly inhibited by pretreatment with capsazepine, CGRP(8-37), and indomethacin. Pretreatment with capsazepine, CGRP(8-37), indomethacin, and denervation of primary sensory nerves significantly increased blood urea nitrogen and serum creatinine levels, renal vascular permeability, renal tissue levels of myeloperoxidase activity, cytokine-induced neutrophil chemoattractant, and tumor necrosis factor-α, and decreased renal tissue blood flow. However, pretreatment with CGRP significantly improved these changes.

Conclusions: Our results suggest activation of sensory neurons in the pathologic process of ischemia/reperfusion-induced acute renal injury. Such activation reduces acute renal injury by attenuating inflammatory responses through enhanced endothelial PGI₂ production.

ISCHEMIA/reperfusion can cause acute renal injury in association with major vascular surgery, cardiopulmonary bypass, renal transplantation, cardiac failure, and septic shock.^{1,2} Tumor necrosis factor-α (TNF-α) plays an important role in the development of ischemia/reperfusion-in-

duced acute renal injury through the activation of neutrophils.³ Activated neutrophils adhere to vascular endothelial cells in the vasa recta of the outer medulla^{2,4,5} and damage endothelial cells by releasing various inflammatory mediators, thereby increasing renal vascular permeability.^{2,4,5} These pathologic events result in reduction of renal tissue blood flow.^{1,6,7} These changes contribute to the development of renal dysfunction, reflected by increases in serum levels of blood urea nitrogen (BUN) and creatinine.^{8,9}

Prostaglandin I₂ (PGI₂) is a well-known cytoprotective agent known to increase tissue blood flow and inhibit leukocyte activation.¹⁰ We have reported that enhanced endothelial release of PGI₂ reduces ischemia/reperfusion-induced acute renal injury by inhibiting TNF-α production and leukocyte activation in rats.⁹ Capsaicin-sensitive sensory neurons are nociceptive neurons that are activated by a wide variety of noxious physical and chemical stimuli.¹¹ Inhibition of sensory neuron activation results in a marked increase in the severity of inflammation; therefore, sensory neurons might be involved in the maintenance of tissue integrity by regulating local inflammatory responses.¹² Neuropeptides play a pivotal role in mediating communication between the nervous, endocrine, and immune systems.¹³ Calcitonin gene-related peptide (CGRP), a 37-amino acid neuropeptide,¹⁴ is localized in both the central and peripheral nervous systems, mainly in nociceptive sensory nerves, including capsaicin-sensitive sensory neurons,¹⁵ as well as in many other tissues, including the kidney.¹⁶ CGRP is released from sensory neurons on activation of vanilloid receptor-1 by a wide variety of noxious physical and chemical stimuli.¹¹ On activation, sensory neurons release CGRP, which is reported to increase endothelial production of PGI₂ *in vitro*.¹⁷ Therefore, various noxious stimuli that activate sensory neurons to release CGRP are capable of reducing tissue damage.¹² Consistent with these observations, we previously showed that, in hepatic ischemia/reperfusion rats, activation of sensory neurons contributed to the ischemia/reperfusion-induced liver injury by attenuating inflammatory responses.¹⁸ These observations suggest that CGRP released from sensory neurons could reduce ischemia/reperfusion-induced acute renal injury by inhibiting leukocyte activation through enhanced endothelial PGI₂ production in the kidney.

The above background suggests that sensory neurons might be activated in the pathologic process leading to ischemia/reperfusion-induced acute renal injury. In the present study, we examined this possibility by analyzing the effects of CGRP; capsazepine, a vanilloid receptor-1

* Associate Professor, || Assistant Professor, ** Professor, Department of Anesthesiology and Critical Care Medicine, and § Associate Professor, Department of Maternal Nursing, Oita University Faculty of Medicine, Yufu, Oita, Japan. † Professor, Department of Biodefense Medicine, Nagoya City University, Graduate School of Medical Sciences, Nagoya, Aichi, Japan. ‡ Chief, Division of Internal Medicine, Touyoukai Azuma Hospital, Kumamoto, Kumamoto, Japan. # Associate Professor, Department of Anesthesiology, Gumma University Hospital, Maebashi, Gumma, Japan.

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Address correspondence to Dr. A. Mizutani: Department of Anesthesiology, Oita University Faculty of Medicine, Yufu, Oita 879-5593, Japan. mizutani@med.oita-u.ac.jp. Information on purchasing reprints may be found at www.anesthesiology.org or on the masthead page at the beginning of this issue. ANESTHESIOLOGY's articles are made freely accessible to all readers, for personal use only, 6 months from the cover date of the issue.

antagonist; CGRP(8-37), a CGRP receptor antagonist; denervation of primary sensory nerves; and indomethacin, a cyclooxygenase inhibitor, on a series of renal responses leading to acute renal injury in rats subjected to renal ischemia/reperfusion.

Materials and Methods

Materials

Capsazepine, capsaicin, and indomethacin were purchased from Sigma Chemical (St. Louis, MO), rat α -CGRP and CGRP(8-37) were purchased from the Peptide Institute (Osaka, Japan), and iloprost was kindly supplied by Eizai Co. (Tokyo, Japan). Evans blue dye and myeloperoxidase were purchased from Sigma Chemical. All other reagents were of analytical grade.

Animal Model of Renal Ischemia/Reperfusion

All experimental procedures were approved by the Oita University Animal Care and Use Committee (Yufu, Oita, Japan). The care and handling of the animals used in the present study were in accordance with the guidelines of the National Institutes of Health. Adult male Wistar rats (Kyudo; Kumamoto, Japan) weighing 200–220 g ($n = 492$) were used throughout the experiments. The rats were anesthetized with an intraperitoneal injection of 50 mg/kg pentobarbital sodium (Abbott Laboratories, North Chicago, IL), followed by 20 mg/kg (intraperitoneal) every hour for 4 h, and were supplemented with an intraperitoneal injection of 2 μ g/kg buprenorphine for postoperative analgesia. Renal ischemia/reperfusion injury was induced as described previously.⁹ In brief, to induce ischemia, the right pedicle was clamped with a noncrushing microvascular clamp for 45 min. Ischemia was confirmed visually by blanching of the kidney. Sham-operated animals underwent the same operation, but without clamping. No animal died until sacrifice after renal reperfusion. Renal dysfunction was evaluated by measuring serum levels of BUN and creatinine by standard assays and picric acid reaction.⁸ Blood samples were taken from the abdominal aorta and collected in tubes, and centrifuged at 2,000g for 10 min for examination of BUN and serum creatinine. The kidneys were harvested for histopathological examination. We also measured renal vascular permeability, renal myeloperoxidase activity, renal tissue content of cytokines, renal tissue levels of 6-keto-prostaglandin $F_{1\alpha}$ (6-keto-PGF $_{1\alpha}$) and CGRP (see *Evaluation of Renal Vascular Permeability; Assay of Renal Myeloperoxidase Activity, Cytokine-induced Neutrophil Chemoattractant, and TNF- α ; Measurement of Renal Tissue Levels of 6-keto-PGF $_{1\alpha}$; and Immunohistochemical Staining for CGRP and Plasma and Renal CGRP Levels*). When measurements were completed, animals were anesthetized with an overdose of pentobarbital sodium and sacrificed.

To elucidate whether activation of sensory neurons with subsequent CGRP release promotes endothelial PGI $_2$ production in rats with renal ischemia/reperfusion injury, we investigated the effects of subcutaneously administered 0.01 mg/kg CGRP, subcutaneously administered 20 mg/kg capsazepine (a vanilloid receptor-1 antagonist), intravenously administered 0.1 mg/kg CGRP(8-37) (a CGRP receptor antagonist), and subcutaneously administered 5 mg/kg indomethacin (a cyclooxygenase inhibitor), injected 30 min before renal ischemia. Iloprost (a stable derivative of PGI $_2$) was dissolved in saline and infused continuously (100 ng \cdot kg $^{-1}$ \cdot min $^{-1}$) via the right jugular vein for 3 h after the onset of renal reperfusion, as described previously.⁹ The same volume of saline was administered continuously in control rats. Furthermore, to elucidate effects of CGRP, capsazepine, CGRP(8-37), and indomethacin on the sham-operated animals, we examined the effects of the agents on the sham-operated animals as compared with sham (saline-administered instead of each agent) at specified time points.

Denervation of Primary Sensory Nerves by Capsaicin

The rats received a total dose of 125 mg/kg capsaicin, administered subcutaneously in divided doses over 2 days.¹⁹ Two weeks after treatment with high-dose capsaicin, the animals were subjected to stress. To determine the effectiveness of the sensory afferent nerve denervation procedure, a drop of 0.001% capsaicin in saline was instilled into either eye of the rats, and their protective wiping movements were observed. Capsaicin-treated rats that showed any wiping movement were excluded from the study. Control animals were injected subcutaneously 1 ml of 10% Tween 20/10% ethanol (10%) with normal saline.

Immunohistochemical Staining for CGRP and Plasma and Renal CGRP Levels

The peroxide-antiperoxide technique was used for immunohistochemical staining of the kidney with anti-CGRP antibody according to the method described previously.¹⁹ In brief, the unfixed tissue blocks of rat kidneys were frozen in dry ice-cooled optimum cutting temperature compound (Tissue-Tek; Miles Inc., Elkhart, IN). Sections (3–4 μ m thick) were mounted on glass slides, immersed in absolute acetone at -20°C for 5 min, rinsed in phosphate-buffered saline 5 times for 5 min each, and then incubated for 20 min with 10% porcine serum in phosphate-buffered saline at room temperature. They were incubated for 1 h at 37°C with rabbit anti-CGRP polyclonal antibody at 1:100 dilution. After five rinses in phosphate-buffered saline, the sections were treated with horseradish peroxidase-conjugated antirabbit immunoglobulin G (MBL, Nagoya, Japan) at 1:2,000 dilution for 1 h at 37°C. Reaction products were

developed by immersing the sections in 3',3'-diaminobenzidine tetrahydrochloride solution containing 0.03% hydrogen peroxide. The control for immunostaining was performed by nonimmune rabbit serum as the first step in place of primary antiserum, and omission of the first step or use of the first antiserum preabsorbed with an excess of the homologous antigen. Samples were mounted with Entellan onto glass slides, examined, and photographed under a light microscope. Samples were analyzed by investigators who were blinded to the experimental groups.

Renal tissue and plasma CGRP levels were determined as described previously.²⁰ In brief, the tissue samples were weighed and then homogenized in 3 ml of 2N acetic acid. The homogenates were placed in a 90°C water bath for 20 min and then centrifuged (4,500g for 10 min at 4°C). CGRP was extracted from the supernatant by using reverse phase C18 columns (Amersham Biosciences, Little Chalfont, England). Columns were prepared by washing with 5 ml of methanol, followed by 10 ml of water before use. The supernatant was applied onto the column, followed by washing with 20 ml of 0.1% trifluoroacetic acid. CGRP was eluted with 3 ml of 60% acetonitrile in 0.1% trifluoroacetic acid, and the solvent was evaporated under a stream of nitrogen gas. The concentration of CGRP was assayed by using a specific enzyme immunoassay kit (SPI-BIO; Massey Cedex, France). The antiserum cross-reacts 100% of rat α - and β -CGRP, based on the data sheet supplied by the manufacturer.

Measurement of Renal Tissue Levels of 6-keto-PGF_{1 α}

Endothelial production of PGI₂ in kidneys was assessed by measuring renal tissue levels of 6-keto-PGF_{1 α} , a stable metabolite of PGI₂, using the method described previously.⁹ The PGI₂ concentration was assayed using a rat-specific enzyme immunoassay system (Amersham).

Measurement of Renal Tissue Blood Flow

Renal tissue blood flow was evaluated by measuring renal cortical blood flow monitored by a laser-Doppler flow meter (ALF21N; Advance, Tokyo, Japan) as described previously.^{8,9} The Doppler probe was directed towards the cortex. Renal cortical blood flow was measured from 30 min before ischemia until 3 h after reperfusion.

Evaluation of Renal Vascular Permeability

Renal vascular permeability was assessed by measuring extravasation of Evans blue dye, as described previously.^{8,9} In brief, the dye (25 μ g/kg) was injected intravenously 10 min before sacrifice by exsanguination from the abdominal aorta. The kidneys were removed, weighed, and placed in 5 ml of dimethylformamide (Wako, Osaka, Japan) for 7 days. After centrifugation (2,000g for 10 min), the concentration of Evans blue dye extracted in the dimethylformamide was measured by a

spectrophotometer (DU-54; Beckman, Irvine, CA) at a wavelength of 610 nm, and compared with the results obtained with standards.

Assay of Renal Myeloperoxidase Activity, Cytokine-induced Neutrophil Chemoattractant, and TNF- α

Accumulation of neutrophils in the kidney was evaluated by measuring renal myeloperoxidase activity, as described previously.^{8,9} The rats were sacrificed at various time points after reperfusion. The kidneys were removed, weighed, and homogenized (Physcotron; Niton, Tokyo, Japan) in 10% (weight/volume) 0.05 M phosphate buffer (pH 6.0) containing 0.5% hexadecyltrimethylammonium bromide and sonicated for 20 s. After centrifugation (4,500g for 20 min at 4°C), 0.1 ml of the supernatant was added to 0.55 ml of 0.1 M phosphate buffer (pH 6.0) containing 1.25 mg/ml o-dianisidine and 0.05% hydrogen peroxide. After 5 min, changes in absorbance at 460 nm were measured using a spectrophotometer. The activity of purified myeloperoxidase was used as the standard. Results are expressed as units of myeloperoxidase activity per gram of tissue.

Renal tissue concentrations of cytokine-induced neutrophil chemoattractant (CINC), the rat equivalent of human interleukin 8,²¹ and TNF- α were measured using enzyme-linked immunosorbent assay kits for rat CINC (Amersham) and TNF- α (Genzyme Corp., Cambridge, MA) according to the instructions supplied by the manufacturer.

Statistical Analysis

Data are expressed as mean \pm SD. Statistical analysis was performed on a Macintosh computer system with Statview 4.5 statistical analysis software (Abacus Concepts, Inc., Berkeley, CA). Renal CGRP time course data were analyzed with a Student *t* test to compare the differences between a baseline (Pre) and responses after the injury, and those between the sham and ischemia/reperfusion groups. Renal CGRP, 6-keto-PGF_{1 α} , BUN, creatinine, Evans blue leakage, myeloperoxidase, CINC, and TNF- α data were analyzed for statistical significance by means of one-way ANOVA, followed by the Bonferroni-Dunn method for multiple comparison. Renal cortical blood flow data were analyzed with a Student *t* test for comparisons among five groups at each time point. A *P* value of less than 0.05 was considered statistically significant.

Results

Changes in CGRP Levels in Kidneys and Plasma, and Renal Immunohistochemical Expression of CGRP in the Kidney of Rats Subjected to Renal Ischemia/Reperfusion

Renal tissue levels of CGRP were significantly increased 1 h after reperfusion, as compared with the

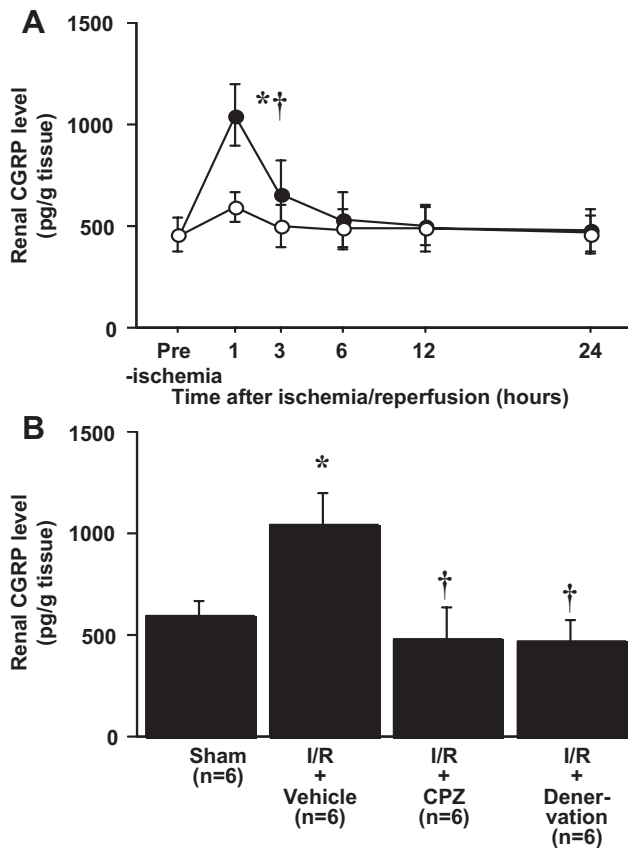


Fig. 1. Changes in renal tissue levels of calcitonin gene-related peptide (CGRP) in rats subjected to renal ischemia/reperfusion (I/R), and effect of capsazepine (CPZ) and denervation of primary sensory nerves on renal I/R-induced increases in renal tissue CGRP levels at 1 h after I/R. (A) CGRP were determined at the indicated time points after 45 min of I/R (solid circles), or sham-operated animals (without I/R, open circles). Renal levels of CGRP began to increase and reached a peak level at 1 h after I/R. The values observed in rats subjected to I/R were higher than those in sham-operated rats. Data are expressed as mean \pm SD values of six animals. * $P < 0.05$ versus preischemia level; † $P < 0.05$ versus sham-operated group. (B) CPZ was administered subcutaneously 30 min before renal ischemia. Denervation of primary sensory nerves was accomplished by high-dose capsaicin. Data are expressed as mean \pm SD values of six animals. * $P < 0.05$ versus sham-operated group; † $P < 0.05$ versus vehicle-treated group.

preischemia levels (fig. 1A). However, the levels decreased rapidly to the preischemia levels 3 h after reperfusion, and remained unchanged during 3 to 24 h (fig. 1A). Renal tissue levels of CGRP at 3, 6, 12, and 24 h after reperfusion were not significantly different from those in sham-operated animals (fig. 1A). Plasma CGRP levels were below the detection limit before and after renal ischemia/reperfusion (data not shown). Both pretreatment with capsazepine (a vanilloid receptor-1 antagonist) and denervation of primary sensory nerves by capsaicin significantly inhibited ischemia/reperfusion-induced increases in renal tissue levels of CGRP (fig. 1B). Rats subjected to renal ischemia/reperfusion showed overexpression of CGRP in the outer renal medulla 1 h after

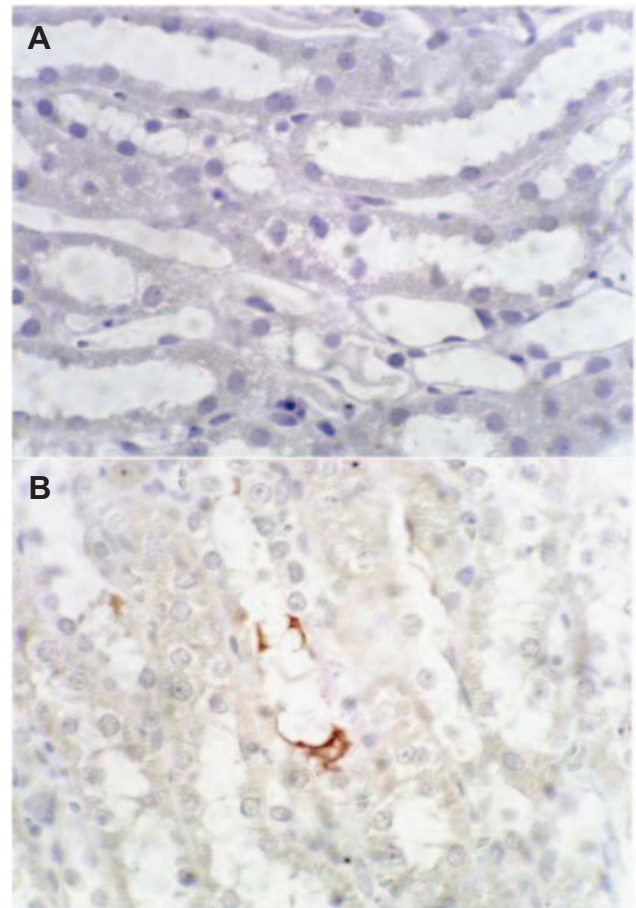


Fig. 2. Immunohistochemical staining of calcitonin gene-related peptide (CGRP) in the kidneys of rats subjected to ischemia/reperfusion (I/R). Immunohistochemical staining for CGRP in frozen sections of the kidney using a rat anti-CGRP antibody in sham-operated rats (A), or rats 1 h after I/R (B). Original magnification $\times 400$.

reperfusion, as compared with the sham-operated rats (fig. 2, A and B).

Effects of CGRP, Capsazepine, CGRP(8-37), Denervation of Primary Sensory Nerves, and Indomethacin on Ischemia/Reperfusion-induced Increases in Renal Tissue Levels of 6-keto-PGF_{1α}

Renal tissue levels of 6-keto-PGF_{1α} were increased after reperfusion, reaching a peak level 1 h after reperfusion.⁹ To examine whether activation of sensory neurons with subsequent CGRP release contributes to the observed increase in endothelial PGI₂ production, we tested the effects of CGRP, capsazepine, CGRP(8-37), denervation of primary sensory nerves, and indomethacin on renal tissue levels of 6-keto-PGF_{1α} 1 h after reperfusion. Renal tissue levels of 6-keto-PGF_{1α} in rats subjected to renal ischemia/reperfusion were significantly higher 1 h after reperfusion than those in sham-operated rats (fig. 3). Increases in renal tissue levels of 6-keto-PGF_{1α} 1 h after reperfusion were significantly inhibited by both pretreatment with capsazepine, CGRP(8-37), and indomethacin and dener-

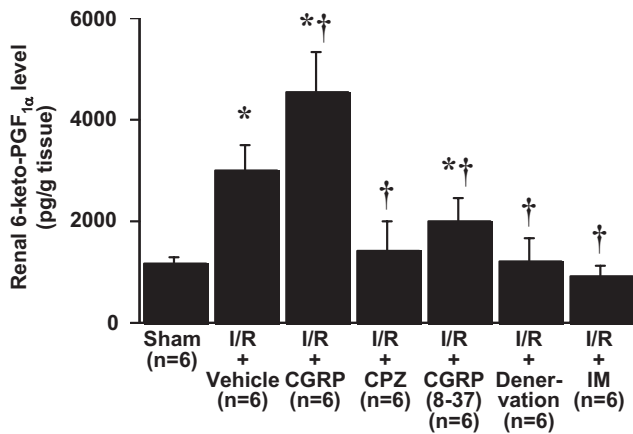


Fig. 3. Effects of calcitonin gene-related peptide (CGRP), capsazepine (CPZ), CGRP(8-37), denervation of primary sensory nerves, and indomethacin (intramuscular) on renal ischemia/reperfusion (I/R)-induced increases in renal 6-keto-prostaglandin F_{1α} (6-keto-PGF_{1α}) levels 1 h after I/R. CPZ (20 mg/kg) was administered subcutaneously 30 min before renal ischemia, CGRP(8-37) (0.1 mg/kg) was administered intravenously 30 min before renal ischemia, denervation of primary sensory nerves was accomplished by high-dose capsaicin, and intramuscular (5 mg/kg) was administered subcutaneously 30 min before renal ischemia. Data are expressed as mean ± SD values of six animals. * *P* < 0.05 versus sham-operated group; † *P* < 0.05 versus vehicle-treated group.

vation of primary sensory nerves (fig. 3). However, these levels in rats pretreated with CGRP were significantly higher than those in vehicle rats.

Effects of CGRP, Capsazepine, CGRP(8-37), Denervation of Primary Sensory Nerves, and Indomethacin on Renal Dysfunction, Renal Cortical Blood Flow, and Renal Vascular Permeability

Serum levels of BUN and creatinine were significantly increased after renal ischemia/reperfusion, reaching peak levels 24 h after reperfusion.⁹ Renal cortical blood flow decreased to approximately 40% of the preischemic level 1 to 3 h after reperfusion.⁹ Renal vascular permeability, as assessed by Evans blue dye leakage, increased significantly after renal ischemia/reperfusion, reaching a maximum level 6 h after reperfusion.⁹ To examine whether CGRP released from sensory neurons reduces various pathologic events by increasing the endothelial production of PGI₂, we investigated the effects of CGRP, capsazepine, CGRP(8-37), denervation of primary sensory nerves, and indomethacin on BUN and serum creatinine 24 h after reperfusion, renal cortical blood flow after reperfusion, and vascular permeability 6 h after reperfusion. Pretreatment with capsazepine, CGRP(8-37), and indomethacin, and denervation of primary sensory nerves exacerbated these pathologic events in rats subjected to renal ischemia/reperfusion (figs. 4A, 4B, 5, and 6). However, pretreatment with CGRP significantly improved ischemia/reperfusion-induced changes.

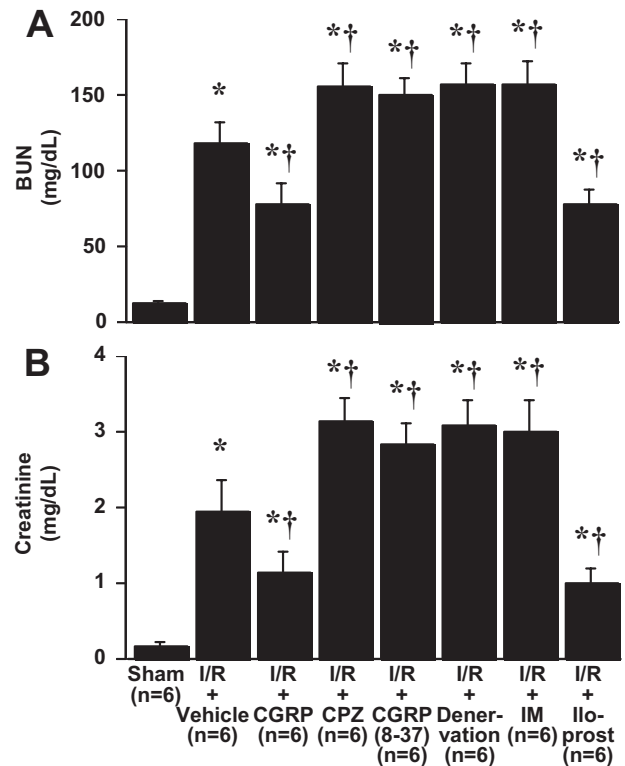


Fig. 4. Effects of calcitonin gene-related peptide (CGRP), capsazepine (CPZ), CGRP(8-37), denervation of primary sensory nerves, indomethacin (intramuscular), and iloprost on renal ischemia/reperfusion (I/R)-induced increases in blood urea nitrogen (BUN) (A) and serum creatinine (B) 24 h after the injury. CPZ (20 mg/kg) was administered subcutaneously 30 min before renal ischemia, CGRP(8-37) (0.1 mg/kg) was administered intravenously 30 min before renal ischemia, denervation of primary sensory nerves was accomplished by high-dose capsaicin, and intramuscular (5 mg/kg) was administered subcutaneously 30 min before renal ischemia. Iloprost (100 ng · kg⁻¹ · min⁻¹) was continuously infused for 3 h after the onset of renal reperfusion. Data are expressed as mean ± SD values of six animals. * *P* < 0.05 versus sham-operated group; † *P* < 0.05 versus vehicle-treated group.

Effects of CGRP, Capsazepine, CGRP(8-37), Denervation of Primary Sensory Nerves, and Indomethacin on Renal Tissue Levels of Myeloperoxidase Activity, CINC, and TNF-α

Renal tissue levels of myeloperoxidase activity, CINC, and TNF-α were markedly increased after ischemia/reperfusion, reaching peak levels 6 h, 3 h, and 3 h after reperfusion, respectively.⁹ Such increases were significantly enhanced by pretreatment with capsazepine, CGRP(8-37), and indomethacin, and denervation of primary sensory nerves (figs. 7A, 7B, and 7C). However, pretreatment with CGRP significantly improved these increases.

Effects of Iloprost on Ischemia/Reperfusion-induced Acute Renal Injury

Iloprost, a stable derivative of PGI₂, significantly improved ischemia/reperfusion-induced increases in BUN, creatinine, Evans blue leakage, renal tissue levels of myeloperoxidase, CINC, and TNF-α (fig. 4, A and B; 6; and 7, A-C), and decrease in renal cortical blood flow (fig. 5).

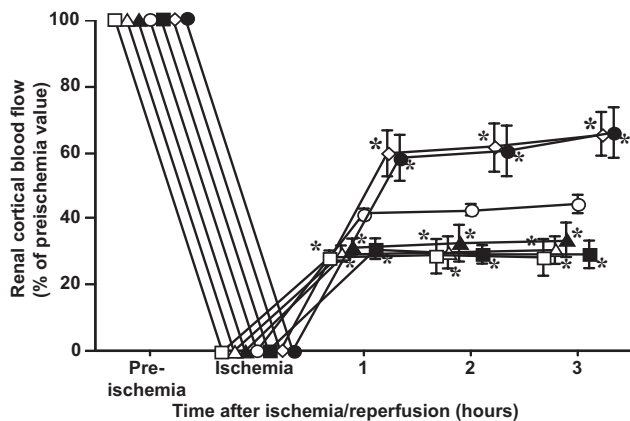


Fig. 5. Effects of calcitonin gene-related peptide (CGRP), capsazepine (CPZ), CGRP(8-37), denervation of primary sensory nerves, indomethacin (intramuscular), and iloprost on renal cortical blood flow during renal ischemia/reperfusion (I/R). Renal cortical blood flow was measured continuously from 30 min before ischemia (Pre) until 3 h after reperfusion. Data are expressed as mean \pm SD of six experiments. *Open circles:* Vehicle-treated rats. *Solid circles:* CGRP-treated rats. *Open triangles:* CPZ-treated rats. *Solid triangles:* CGRP(8-37)-treated rats. *Open squares:* Denervation of primary sensory nerves-treated rats. *Solid squares:* Intramuscular-treated rats. *Open diamonds:* Iloprost-treated rats. * $P < 0.05$ versus vehicle-treated group.

Effects of CGRP, Capsazepine, CGRP(8-37), Denervation of Primary Sensory Nerves and Indomethacin in Sham-Operated Rats

Pretreatment with CGRP, capsazepine, CGRP(8-37) or indomethacin, denervation of primary sensory nerves,

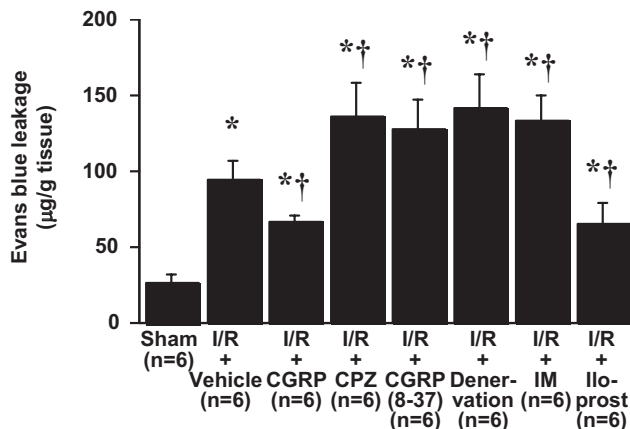


Fig. 6. Effects of calcitonin gene-related peptide (CGRP), capsazepine (CPZ), CGRP(8-37), denervation of primary sensory nerves, indomethacin (intramuscular), and iloprost on renal ischemia/reperfusion (I/R)-induced increase in renal vascular permeability 6 h after the injury. Renal vascular permeability was assessed by measuring extravasation of Evans blue dye. CPZ (20 mg/kg) was administered subcutaneously 30 min before renal ischemia, CGRP(8-37) (0.1 mg/kg) was administered intravenously 30 min before renal ischemia, denervation of primary sensory nerves was accomplished by high-dose capsaicin, and intramuscular (5 mg/kg) was administered subcutaneously 30 min before renal ischemia. Iloprost (100 ng \cdot kg⁻¹ \cdot min⁻¹) was infused continuously for 3 h after the onset of renal reperfusion. Data are expressed as mean \pm SD values of six animals. * $P < 0.05$ versus sham-operated group; † $P < 0.05$ versus vehicle-treated group.

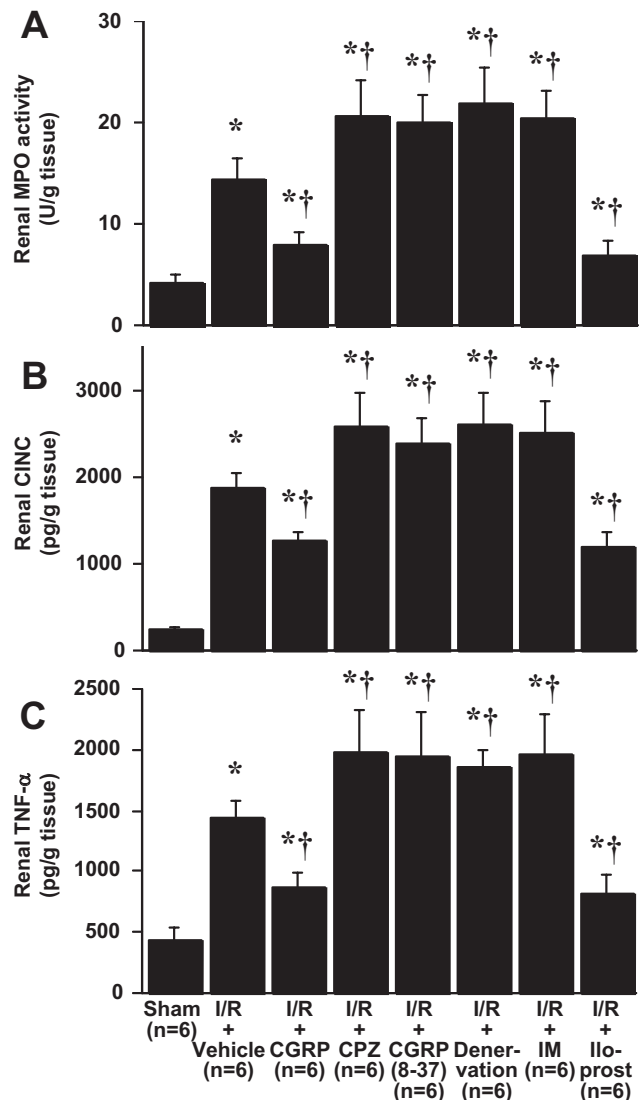


Fig. 7. Effects of calcitonin gene-related peptide (CGRP), capsazepine (CPZ), CGRP(8-37), denervation of primary sensory nerves, indomethacin (intramuscular), and iloprost on renal ischemia/reperfusion (I/R)-induced increases in renal tissue levels of myeloperoxidase (MPO) activity (A), cytokine-induced neutrophil chemoattractant (CINC) (B), and tumor necrosis factor (TNF)- α (C). Renal tissue levels of MPO activity were determined 6 h after renal I/R; CINC and TNF- α were determined 3 h after renal I/R. CPZ (20 mg/kg) was administered subcutaneously 30 min before renal ischemia, CGRP(8-37) (0.1 mg/kg) was administered intravenously 30 min before renal ischemia, denervation of primary sensory nerves was accomplished by high-dose capsaicin, and intramuscular (5 mg/kg) was administered subcutaneously 30 min before renal ischemia. Iloprost (100 ng \cdot kg⁻¹ \cdot min⁻¹) was infused continuously for 3 h after the onset of renal reperfusion. Data are expressed as mean \pm SD values of six animals. * $P < 0.05$ versus sham-operated group; † $P < 0.05$ versus vehicle-treated group.

and vehicle (saline) did not alter BUN, serum creatinine, Evans blue leakage, renal tissue levels of myeloperoxidase, CINC, and TNF- α at 24 h, 24 h, 6 h, 6 h, 3 h, and 3 h after administration of the agents, respectively (data not shown).

Discussion

In the present study, we showed that ischemia/reperfusion-induced increases in renal tissue levels of 6-keto-PGF_{1α} were significantly promoted by pretreatment with CGRP and reduced by both pretreatment with capsazepine (a vanilloid receptor-1 antagonist) and CGRP(8-37) (a CGRP receptor antagonist), and denervation of primary sensory nerves in rats. These suggest that such increases in renal tissue levels of 6-keto-PGF_{1α} are a consequence of the sensory neuron activation. Consistent with these observations are our previous reports demonstrating that inhibition of sensory neuron activation inhibits reperfusion-induced increases in endothelial production of PGI₂ in rats subjected to hepatic ischemia/reperfusion, water-immersion restraint stress, and endotoxin-induced hypotension.^{18,22,23}

TNF-α and histamine stimulate sensory neurons,^{24,25} and renal production of these substances is increased after renal ischemia/reperfusion in rats.²⁶ Therefore, it is possible that sensory neurons are activated by these substances under renal ischemia/reperfusion injury in the present study.

Activated leukocytes are critically involved in the development of ischemia/reperfusion-induced acute renal injury.⁸ This notion was confirmed in the present study by the findings of significantly increased renal tissue levels of TNF-α, CINC, and myeloperoxidase, which reflect leukocyte activation, after renal ischemia/reperfusion injury. TNF-α excessively produced by monocytes plays an important role in the development of ischemia/reperfusion-induced acute renal injury by activating neutrophils.³ Activated neutrophils damage endothelial cells by releasing various inflammatory mediators such as neutrophil elastase and oxygen radicals, leading to an increase in renal vascular permeability.^{2,4,5} These pathologic events result in reduction of blood flow to the renal medulla and cortex,^{1,6,7} thereby contributing to the development of acute tubular necrosis and reduction of glomerular filtration rate.²

Since PGI₂ is known to inhibit monocyte TNF-α production by inhibiting its transcription through regulation of nuclear factor-kappaB (NF-κB) activation,²⁷ the sensory neuron-mediated increase in the renal endothelial production of PGI₂ might contribute to reduce ischemia/reperfusion-induced acute renal injury by inhibiting leukocyte activation. Consistent with this hypothesis are observations in the present study demonstrating that pretreatment with iloprost, a stable derivative of PGI₂, significantly improved both ischemia/reperfusion-induced increase in TNF-α production and exacerbation of series of pathologic events leading to the ischemia/reperfusion. These observations suggest that CGRP released from sensory neurons reduces ischemia/reperfusion-induced acute renal injury by increasing endothelial PGI₂ production. Consistent with this notion, changes in

renal tissue levels of CGRP during preischemia and after reperfusion appeared to parallel those in renal tissue of 6-keto-PGF_{1α}. Our observations in the present study demonstrated that pretreatment with capsazepine, CGRP(8-37) and indomethacin, and denervation of primary sensory nerves significantly enhanced ischemia/reperfusion-induced decreases in renal cortical blood flow and increases in Evans blue leakage, and renal tissue levels of TNF-α, CINC, and myeloperoxidase and exacerbated a series of pathologic events leading to ischemia/reperfusion-induced renal injury.⁹ Furthermore, pretreatment with CGRP significantly improved these ischemia/reperfusion-induced changes. These observations implicate endogenous CGRP in prevention of ischemia/reperfusion-induced acute renal injury by inhibiting leukocyte activation in the kidney through increased endothelial PGI₂ production. Thus, it is possible that increase in endothelial PGI₂ production induced by sensory neuron activation is a reparative host response to noxious stimuli that evoke tissue injury.

CGRP itself has been shown to inhibit TNF-α production by inhibiting NF-κB activation in macrophages *in vitro*.^{28,29} In this study, pretreatment with indomethacin significantly promoted ischemia/reperfusion-induced increase in renal tissue levels of TNF-α, suggesting that endogenous CGRP inhibits TNF-α production by promoting endothelial PGI₂ production *in vivo*. The limitation of this study was that indomethacin is a nonspecific cyclooxygenase inhibitor. Indomethacin also inhibits activities of cyclooxygenases, thereby inhibiting the production of certain eicosanoids other than PGI₂. Therefore, some other eicosanoids might be involved in the effects of CGRP in the present study. Because PGE₂ as well as PGI₂ have been shown to inhibit leukocyte activation and these prostaglandins are synthesized from PGE₂ in endothelial cells,^{30,31} it seems that PGE₂ mediates the effects of CGRP in the present study. Consistent with this conclusion, we previously showed that CGRP promotes endothelial PGE₂ production as well as PGI₂ in water immersion restraint stress-induced gastric mucosal injury in rats.²² This possibility should be investigated further using specific cyclooxygenase inhibitors in this model. In addition, there are some other materials, which stimulate endothelial PGI₂ production. Thrombin is one of these substances.³² It would be very interesting to investigate the role of thrombin in this animal model.

In the present study, renal tissue levels of CGRP in rats subjected to 45 min of ischemia/reperfusion were significantly increased 1 h after ischemia/reperfusion and then decreased to the preischemia levels. The renal tissue levels of CGRP were significantly inhibited by pretreatment of capsazepine, a vanilloid receptor-1 antagonist, and denervation of primary sensory nerves. Furthermore, immunohistochemical expression of CGRP was also observed in the kidneys of rats with ischemia/reper-

fusion injury, as compared with sham-operated rats. These observations suggest that capsaicin-sensitive sensory neurons might be activated in the pathologic process leading to ischemia/reperfusion-induced acute renal injury. Renal tissue levels of CGRP were increased after renal ischemia/reperfusion, whereas plasma levels of CGRP were not detected after the ischemia/reperfusion. These findings suggest that CGRP locally inhibits the induction of TNF- α production by promoting endothelial PGI₂ production. TNF- α is involved in the development of ischemia/reperfusion-induced acute renal injury by activated neutrophils that adhere to the vascular endothelium, leading to renal vascular endothelial damage, resulting in increased renal vascular permeability.^{2,3} Consistent with this hypothesis are data in this study demonstrating that the effects of renal tissue levels of TNF- α , CINC, and myeloperoxidase appeared to parallel those of renal tissue levels of Evans blue leakage in rats subjected to renal ischemia/reperfusion. These findings suggest that local effects of CGRP in the kidney are critical in preventing ischemia/reperfusion-induced acute renal injury in rats.

Although CGRP plays a pivotal role in preventing ischemia/reperfusion-induced acute renal injury, acute renal injury developed in rats with significantly high levels of endogenous CGRP release. Therefore, CGRP release in response to renal ischemia/reperfusion might not be sufficient to prevent acute renal injury, probably because of depletion of CGRP or inhibition of CGRP release from the nerve ends. In this regard, we showed previously that activation of β_2 -adrenoceptor on the nerve cells inhibits CGRP release from sensory nerve ends and increases renal tissue blood flow in rats.²⁰ In rats with renal ischemia/reperfusion injury, the sympathetic nervous system is activated;³³ therefore, it is possible that stimulation of β_2 -adrenoceptors plays a role in the pathology of renal ischemia/reperfusion injury by inhibiting CGRP release. Consistent with this notion, Kurata *et al.*³³ reported that enhancement of renal sympathetic activity and its consequent effect on norepinephrine overflow from nerve endings are important events in ischemia/reperfusion-induced acute renal failure. They also demonstrated a significant augmentation of renal sympathetic activity during renal ischemia, and that this change is accompanied by suppression of elevated renal venous norepinephrine levels immediately after reperfusion.³³ These findings suggest that pharmacologic intervention to modulate sensory nerve function resulting in an increase of CGRP release might be useful in preventing ischemia/reperfusion-induced acute renal injury.

In conclusion, sensory neurons play a critical role in reducing ischemia/reperfusion-induced acute renal injury in rats by attenuating the inflammatory response through promotion of endothelial production of PGI₂. In addition, we reported previously that antithrombin, a

physiologic serine protease inhibitor, reduced ischemia/reperfusion-induced acute renal injury in rats.⁹ Since antithrombin activates sensory neurons,¹⁹ we are currently investigating the neuronal mechanisms by which antithrombin enhances endothelial production of PGI₂ in the same rat model.

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