De Novo Demonstration and Co-localization of Free-Radical Production and Apoptosis Formation in Rat Kidney Subjected to Ischemia/Reperfusion

CHIANG-TING CHIEN,* PO-HUANG LEE,*† CHAU-FONG CHEN,‡ MING-CHIEH MA,‡ MING-KUEN LAI,§ and SU-MING HSU‖

**Office for Medical Research Administration, National Taiwan University Hospital, Departments of †Surgery, ‡Physiology, §Urology, and ‖Pathology, National Taiwan University College of Medicine, Taipei, Taiwan.*

Abstract. Ischemia-induced oxidative damage to the reperfused kidney was examined. A modified chemiluminescence method, an *in situ* nitro blue tetrazolium perfusion technique, and a DNA fragmentation/apoptosis-related protein assay were adapted for demonstration *de novo* and co-localization of reactive oxygen species (ROS) production and apoptosis formation in rat kidneys subjected to ischemia/reperfusion injury. The results showed that prolonged ischemia potentiated pro-apoptotic mechanisms, including increases in the Bax/Bcl-2 ratio, CPP32 expression, and poly-(ADP-ribose)-polymerase fragments, and subsequently resulted in severe apoptosis, including increases in DNA fragmentation and apoptotic cell number in renal proximal tubules (PT) and distal tubules (DT) in a time-dependent manner. The increased level of ROS detected on the renal surface was correlated with that in blood and was intensified by a prolonged interval of ischemia. The main source of ROS synthesis was the PT epithelial cells. The ROS and apoptotic nuclei detected in the PT cells can be ameliorated by superoxide dismutase (SOD) treatment before reperfusion. However, the apoptotic nuclei remained in DT in the SOD-treated rats, indicating that formation of apoptosis in DT was not influenced by the small amounts of ROS produced. In PT and DT cell cultures, significant increases in apoptotic cells and ROS were evident in PT cells after hypoxia/reoxygenation insult. Furthermore, the oxidative damage in PT, but not in DT, can be alleviated by ROS scavengers SOD and hexa(sulfobutyl)fullerene, confirming that PT are vulnerable to ROS. These results lead us to conclude that ROS produced in significant amounts in PT epithelium under ischemia/reperfusion or hypoxia/reoxygenation conditions may be responsible for the apoptotic death of these cells.

Complete or partial cessation (ischemia) followed by restoration of blood flow (reperfusion) is a serious event that affects many organs, such as the heart, brain, liver, and kidney (1–7). Ischemia/reperfusion (I/R) contributes to abnormal signal transduction or cellular dysfunction (8,9) and initiates the cascade of apoptosis/necrosis, with subsequent inflammatory infiltration (6,7,10).

Apoptosis, or programmed cell death, which can be distinguished on the basis of morphologic and biochemical criteria, is different from necrosis (11–13). The mechanism responsible for post-I/R apoptosis is attributed to the increasing activity of endonuclease by elevation of calcium entry into cells (12), or the release of reactive oxygen species (ROS) (1–3,14–16). ROS induce apoptosis by causing DNA damage, oxidation of lipid membranes, and/or direct activation and expression of the genes/proteins responsible for apoptosis (17). For example, increases in the Bax/Bcl-2 ratio (18), expression of caspase and its activity (19,20), and caspase-mediated cleavage of poly-(ADP-ribose)-polymerase (PARP) (21) have been found in organs subjected to I/R injury or in cells after a cytotoxic insult.

The sources of ROS generated after I/R may be circulatory macrophages/neutrophils (22) or the resident cells (23). In the reperfused heart, infiltrating neutrophils are the primary source of ROS (1). In brains subjected to I/R, superoxide detected in the meninges was located primarily in the extracellular space and occasionally in endothelial cells and vascular smooth muscle cells (2). In the liver, the sources of ROS seem to be Kupffer cells, sinusoidal cells, and infiltrating leukocytes (24). In the kidney, demonstration of increased renal venous ROS after I/R by electron spin resonance and the efficacy of antioxidants or free-radical scavengers in minimizing I/R injury (7,14,16) suggest a role of ROS in I/R injury. However, the precise origin of ROS in the I/R kidney has yet to be defined.

In this study, we adapted a modified chemiluminescence (CL) method (25–27) for direct measurement of the amount of ROS produced from the kidney surface and from renal veins in I/R kidneys. In addition, we determined the cellular location of *de novo* ROS synthesis in the I/R kidney by using an *in situ* vascular

Received June 22, 2000. Accepted October 19, 2000.

Correspondence to Dr. Su-Ming Hsu, Department of Pathology, National Taiwan University College of Medicine, 1-1 Jen-Ai Road, Taipei, Taiwan. Phone: 886-2-23123456 ext. 2980; Fax: 886-2-2394-7927; E-mail: smhsu@ha.mc.ntu.edu.tw or Dr. Ming-Kuen Lai, Department of Urology, National Taiwan University College of Medicine, 1-1 Jen-Ai Road, Taipei, Taiwan. Phone: 886-2-23123456 ext. 2135; Fax: 886-2-2321-9145; E-mail: mingkuen@ha.mc.ntu.edu.tw

1046-6673/1205-0973
Journal of the American Society of Nephrology

perfusion technique, whereby nitro blue tetrazolium (NBT) was perfused through the kidney for depicting cells in which superoxide was formed (28). The onset of apoptosis and its severity in the I/R kidney were determined by examination of the presence of DNA fragmentation and an increase in cellular proteins associated with apoptosis. Our study showed that prolonged ischemia resulted in severe apoptosis in the reperfused kidney in a time-dependent manner. Cells in both proximal tubules (PT) and distal tubules (DT) were subjected to I/R injury, whereas only PT cells produced measurable ROS by the NBT method. Not unexpected, the apoptosis *in vivo* and *in vitro* induced by I/R and hypoxia/reoxygenation (H/R) in PT cells, but not in DT cells, can be prevented by superoxide dismutase (SOD) or a novel free-radical scavenger, hexa(sulfobutyl)fullerene (FC_4S) (14).

Materials and Methods

Surgery

Female Wistar rats (200 to 250 g) were housed at the Experimental Animal Center, National Taiwan University, at a constant temperature and with a consistent light cycle (light from 07:00 to 18:00 h). The animal care and experimental protocol was in accordance with the guidelines of the National Science Council of the Republic of China (NSC 1997). On the day of the experiments, all rats were anesthetized with sodium pentobarbital (50 mg/kg, intraperitoneally) and were tracheotomized. Catheters were placed in the left carotid artery for blood sampling and in the left femoral vein for anesthetic supplement and blood administration. After surgery, the left renal artery was cannulated by introduction of a length of stretched PE10 tubing from the left femoral artery via the aorta. A catheter was placed in the left renal vein for blood sampling. The rat was then placed on its right side, and the left kidney was exposed via a flank incision and dissection from the surrounding tissue.

Induction of Unilateral Renal Ischemia

For induction of total ischemia in the kidney, the left renal artery was clamped with a small vascular clamp. All rats were randomly divided into four groups, depending on the duration of ischemia: 0 min (control), 15 min (I15), 45 min (I45), and 60 min (I60). Sham-operated (control) animals underwent similar operative procedures without occlusion of the renal artery. Reperfusion was initiated by removal of the clamp. The rats in each group were divided further into subgroups and were subjected to the following time frames of reperfusion: for 5 min (R5m), 30 min (R30m), 1 h (R1h), 2 h (R2h), 4 h (R4h), 10 h (R10h), or 24 h (R24 h).

SOD Treatment

A set experimental rats ($n = 4$ for each ischemic group) was treated with intravenous SOD (500 U per rat; Sigma, St. Louis, MO) after the ischemic period (before reperfusion). In this experiment, all ischemic kidneys were reperfused for 4 h. The experiment was intended for confirmation of the role of ROS in apoptosis formation in the I/R kidney.

Tissue Preparation

For evaluation of the effect of ischemic intervals on apoptosis formation and apoptosis-related protein expression, 12 rats in each ischemic group (I15, I45, and I60) were used. Four rats were killed with an overdose of anesthetics at the end of ischemia (*i.e.*, no reperfusion), and at 1 h or 4 h after reperfusion. For comparison, three control rats were used. The kidney was resected and divided into three parts. One part was stored in 10% neutral buffered formalin for routine histology and *in situ* apoptotic assay, another was prepared for DNA fragmentation electrophoresis, and the third was quickly frozen in liquid nitrogen and stored at −70°C for protein isolation.

In Situ Apoptotic Assay

The method for *in situ* apoptotic assay (*i.e.*, terminal deoxynucleotidyl transferase-mediated nick-end labeling [TUNEL] method) was performed according to the method of Gavrieli *et al.* (29) with minor modifications (30). Briefly, 4- to 6-μm-thick sections of the kidney were prepared, deparaffinized, and stained by the TUNEL–avidin-biotin-peroxidase complex method (10,30). Twenty high-power (×400) fields of the outer medulla were randomly selected in each section, and the number of apoptotic cells was counted. PT were distinguished from other segments by their characteristic brush border and their relatively large epithelial cell bodies. DT were recognized by their smaller (the nuclei are close together) and more regular cells (31). The number of apoptotic cells was expressed per 100 of the PT or DT cells in each section.

DNA Extraction and Electrophoresis

The kidneys were homogenized and lysed with 20 ml of buffer containing 10 mM Tris-HCl (pH 7.5), 1 mM ethylenediaminetetraacetate, 0.1 M NaCl, and 1 ml of 10% sodium dodecyl sulfate (SDS). The lysates were incubated with 200 μl of 10 mg/ml proteinase K at 50°C overnight. DNA was extracted twice with phenol and twice with phenol/chloroform (1/1); the upper layers were collected, and 5 M NaCl was added in a 1:50 (vol/vol) ratio. The sample was then precipitated overnight at −20°C in absolute ethanol. DNA was pelleted by centrifugation at 3000 × *g* at 4°C for 10 min, washed with 70% ethanol, and dried. The DNA obtained was resuspended in TE (10 mM Tris-HCl, 1 mM ethylenediaminetetraacetate) at 1 μg/ml and incubated with 0.1 mg/ml DNAse-free RNAse at 37°C for 30 min. Four μl of 0.25% bromophenol blue and 0.25% xylene cyanol in 40% sucrose were added to DNA samples at a 1:5 (vol/vol) ratio. Electrophoresis was performed at 50 V in 1.6% agarose gels. DNA was visualized with ethidium bromide.

In Vivo CL Recording

The method for detection of CL from the organ surface after intrarenal arterial 2-Methyl-6-[4-methoxyphenyl]-3,7-dihydroimidazo-[1,2-a]-pyrazin-3-one hydrochloride (MCLA) (TCI-Ace; Tokyo Kasei Kogyo Co. Ltd., Tokyo, Japan) injection (2,26) was adapted for demonstration of ROS production in the I/R kidney. The rat was maintained on a respirator (tidal volume, 1.0 to 1.5 ml; rate, 80 to 90 cycles/min; inspiratory pressure, 20 to 30 cm H_2O) and a circulating water pad at 37°C during photon detection. For excluding photon emission from sources other than the kidney, the animal was housed in a dark box with a shielded plate. Only the renal window was left unshielded and was positioned under a reflector, which reflected the photons from the exposed kidney surface onto the detector area.

A single dose (1 mM in 0.1 ml) of *N,N′*-dimethyldiacridium (lucigenin; Sigma) or a continuous infusion (0.2 mg/ml per h) of MCLA was administered into the control or I/R kidney via an intrarenal arterial catheter. The lucigenin- or MCLA-enhanced CL signal from the kidney surface was measured continuously during administration by use of a Chemiluminescence Analyzing System (CLD-110, Tohoku Electronic Industrial Co., Sendai, Japan). Eight rats (five for lucigenin and three for MCLA treatment) in each ischemic group (I15, I45, and I60) were used in this part of the experiment.

A separate experiment on I45 rats ($n = 3$) treated with intravenous SOD (cumulative dose to 500 U per rat) before lucigenin injection at 4 h reperfusion was performed. This experiment was intended for confirmation of the enhanced CL induced by I/R that originated from ROS.

Preparation of Whole-Blood Sample for Lucigenin-Enhanced CL Determination

Four groups of rats (I15, I45, I60, and control; five rats in each group) were used for measurement of ROS in whole-blood samples. A series of blood samples (0.2 ml) from the left renal vein or carotid artery were obtained immediately after reperfusion for various times. The blood samples were immediately wrapped in aluminum foil and kept on ice until CL measurement, usually done within 2 h (32).

Immediately before CL measurement, 0.1 ml of phosphate-buffered saline (pH 7.4) was added to 0.2 ml of blood sample. The CL was measured in a completely dark chamber of the Chemiluminescence Analyzing System. After 100-s background level determination, 1.0 ml of 0.1 mM lucigenin in phosphate-buffered saline (pH 7.4) was injected into the sample. The CL was monitored continuously for an additional 600 s. The total amount of CL was calculated by integrating of the area under the curve and subtracting it from the background level. The assay was performed in duplicate for each sample and was expressed as CL counts/10 s for blood CL. The mean ± SEM of the CL level of each sample was calculated.

In Situ Demonstration of Superoxide Formation by NBT

Three rats in each ischemic group were killed at the end of ischemia, at 1 h and 4 h after reperfusion. An NBT perfusion method was used for localizing *de novo* ROS generation in the I/R kidney (28). An 18-gauge needle connected to an infusion pump (Infors AG, CH-4103, Bottmingen, Switzerland) was inserted in the upper abdominal aorta just above both kidneys, whereas the lower abdominal aorta below both kidneys was ligated. After the bilateral renal veins were cut, the kidneys were perfused with 37°C Hanks' balanced salt solution (HBSS; flow rate, 10 ml/min [pH 7.4]). Once blood had been removed, NBT (1 mg/ml) was added to the HBSS, and the kidneys were perfused for an additional 10 min at a flow rate of 5 ml/min. All unreacted NBT was removed from the kidneys by postperfusion with HBSS. The NBT-perfused kidneys were cut and fixed in zinc/formalin for histologic examination for formazan deposits, and some samples were prepared for localization of apoptotic cells. In separate experiments, SOD was included in the perfusate (500 μg/ml) for confirmation of the specificity of the NBT deposition as the product of ROS.

Immunoblot Analysis for Bax, Bcl-2, CPP32, and PARP

We measured the amounts of Bax/Bcl-2, caspase 3, and PARP (18,19,21) in renal tissues of I/R rats. For protein analysis, left kidney samples were homogenized with a prechilled mortar and pestle in extraction buffer, which consisted of 10 mM Tris-HCl (pH 7.6), 140 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 1% NP-40, 0.5% deoxycholate, 2% β-mercaptoethanol, 10 μg/ml pepstatin A, and 10 μg/ml aprotinin. The mixtures were homogenized completely by vortexing and kept at 4°C for 30 min. The homogenate was centrifuged at 12,000 × *g* for 12 min at 4°C, the supernatant was collected, and the protein concentrations were determined by BioRad Protein Assay (BioRad Laboratories, Hercules, CA). Antibodies raised against Bax (Catalog # AB2916, human Bax synthetic peptide, amino acids 44 to 62; Chemicon, Temecula, CA), Bcl-2 (Catalog # B46620, human Bcl-2 peptide, amino acids 49 to 179; Transduction, Bluegrass-Lexington, KY), the activation fragments (32 kD of proenzyme and 17 kD of cleaved product) of caspase 3 (CPP32/Yama/Apopain; Catalog # 06-735, human full-length caspase 3 fusion protein containing a histidine-6 tag; Upstate Biotechnology, Lake Placid, NY), PARP (Catalog # G3741, N-terminal peptide from the p85 fragment; Promega, Madison, WI), and β-actin (Catalog # A5316, Clone AC-74; Sigma) were used. All of these antibodies cross-react with respective rat antigens.

SDS-polyacrylamide gel electrophoresis (PAGE) was performed on 12.5% separation gels in the absence of urea and was stained with Coomassie brilliant blue. Proteins on the SDS-PAGE gels, each lane containing 30 μg of total protein, were transferred to nitrocellulose filters. The immunoreactive bands were detected by incubation with the antibody described above, followed by secondary antibody-alkaline phosphatase, and finally with NBT and 5-bromo-4chloro-3-indolyl phosphate, toluidine salt (Roche Diagnostic GmbH, Mannheim, Germany) stock solution for 30 min at room temperature.

Isolation of Renal PT and DT

We also designed experiments to study the role of ROS in the formation of apoptosis in different portions of the renal tubules. Female Wistar rats ($n = 4$) were used. Animals were anesthetized with sodium pentobarbital (60 mg/kg, intraperitoneally). Kidneys were flushed with 20 ml of ice-cold Krebs-Henseleit-saline buffer (KHS) via an aortal catheter. Specific isolation of PT and DT was performed as described previously (33). After the kidneys were flushed with KHS, they were perfused with 10 ml of 0.1% Type IV collagenase in KHS (Sigma). Minced renal cortices were incubated for 25 min at 37°C in 30 ml of 0.1% collagenase-KHS, continuously gassed with 95% O_2/5% CO_2, and gently agitated. PT and DT cells were isolated in pregassed 45% Percoll-KHS solution (Sigma) by centrifugation at 20,000 × *g* in a fixed-angle rotor (SS34 rotor of Sorvall RC5C centrifuge, Newtown, CT) for 30 min at 4°C. The band enriched in PT or DT was withdrawn and washed two times with ice-cold KHS. Each of the two types of cells was cultured. Alkaline phosphatase activity as a marker enzyme for PT and hexokinase activity as a marker enzyme for DT were measured spectrophotometrically with *p*-nitrophenylphosphate and a pyridine nucleotide-linked assay, respectively (34). The PT and DT cultures were also processed for cytologic examination after formal fixation and coloration with periodic acid-Schiff. Identification of PT by their granular yellow cytoplasm and pink brush border membrane and DT by their clear cytoplasm and the absence of a brush border membrane is made possible by this procedure (34).

Induction of H/R of the renal tubule cells was performed as described previously (10). The cultures first were placed in an atmosphere of 95% O_2/5% CO_2 at 37°C for 30 min. Hypoxia was achieved by gassing with 95% N_2/5% CO_2 for 15 min, whereas reoxygenation was performed by reintroduction of 95% O_2/5% CO_2 for 30 min. To study the role of ROS in the formation of apoptosis, we added 100 U of SOD and 50 μM FC_4S (provided by Dr. L. Y. Chiang, Center for Condensed Matter Sciences, National Taiwan University) (14), a novel water-soluble fullerene with free-radical scavenging activity, to the cell medium. For determination of the number of apoptotic cells in culture, the cells were fixed with 70% ethanol, stained with propidium, and counted with FACSCalibur (Becton Dickinson, San Jose, CA). Cell viability was counted with a Trypan blue dye exclusion test (33). The amounts of ROS in PT and DT cell cultures (10^6 cells/ml) were detected by the lucigenin-enhanced CL test described above.

For immunoblotting analysis of PT and DT, 1×10^6 cells were used per sample; these were centrifuged and washed in KHS. Next, 40 μl

of sample buffer (containing 50 mM Tris [pH 6.8], 6 M urea, 6% β-mercaptoethanol, 0.03% bromophenol blue, and 3% SDS) was added, and the samples were sonicated. These were then heated at 80°C for 5 min, and samples were subsequently loaded and run on a 10% SDS-PAGE gel for 1 h at 140 V. After electrophoretic transfer of the separated polypeptides to nitrocellulose, the membrane was probed with the apoptosis-related antibodies as described above.

Statistical Analyses

All values were expressed as mean ± SEM. Differences within groups were evaluated by paired *t* test. One-way ANOVA was used for establishing differences among groups. Intergroup comparisons were made by Duncan's multiple-range test. Differences were regarded as significant if $P < 0.05$ was attained.

Results

Histologic Detection of Apoptotic Cells in Reperfused Renal Tissue

The apoptotic cells were distinguished by their dark-stained nuclei. Apoptotic cells were not detected or were only rarely present in sections from control rat kidney and from all kidneys subjected to ischemia without reperfusion.

In the I15 kidneys, a few apoptotic cells were observed in the specimens at 1 to 4 h after reperfusion (Figure 1, Table 1), and the apoptotic cells occurred mainly in the PT. In I45 and I60 kidneys, increased numbers of apoptotic cells were detected in both PT and DT after reperfusion. The numbers of apoptotic cells reached a plateau when the kidneys were reperfused for 4 to 6 h. There were no apparent apoptotic cells detected in the kidneys subjected to 10 h and 24 h of reperfu-

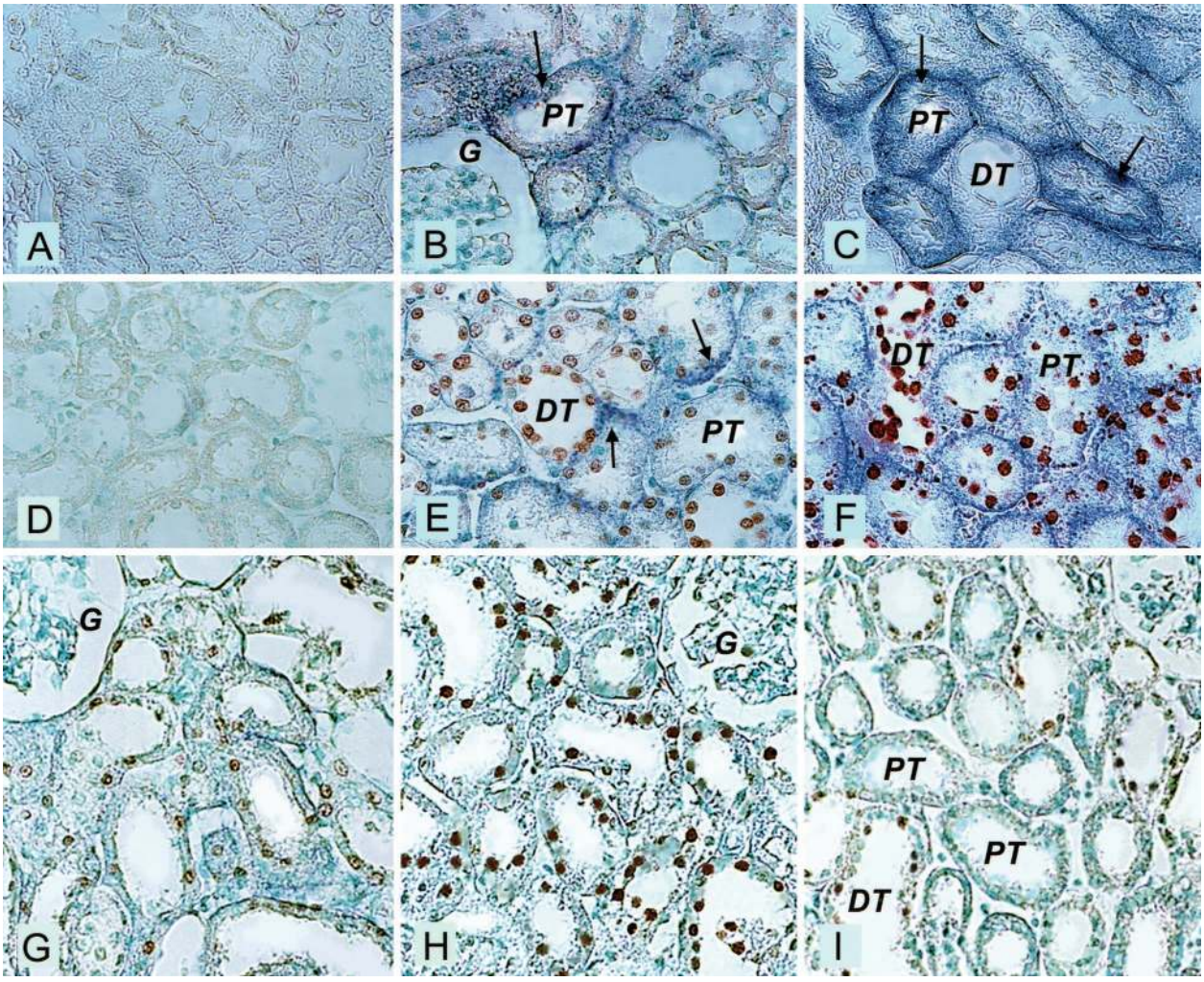

Figure 1. Nitro blue tetrazolium (NBT) deposits (blue precipitate) were used to demonstrate *de novo* production of reactive oxygen species (ROS) (A through F). TUNEL staining was used to locate the apoptotic cells (with brownish-colored nuclei) (D through I). Both methods were used in combination (D through F). NBT deposits and apoptotic cells were absent in control kidney (A, D) but clearly visible in proximal tubules (PT) in I15/R4h (B, E) and I45/R4h (C, F) kidneys. Distal tubule (DT) cells and glomerular (G) cells showed no or very weak NBT deposits. TUNEL-positive cells occurred in both PT and DT cells. A study also was conducted to show the effect of superoxide dismutase (SOD) for prevention of apoptosis. Prolonged ischemia seemed to increase the number of apoptotic cells in I45/R4h kidneys (H) *versus* I15/R4h kidneys (G). SOD treatment (500 U) clearly diminished TUNEL-positive cells in PT in I45/R4h kidneys (I).

Table 1. Percentages of renal proximal and distal tubular apoptosis in I15, I45, and I60 kidneys subjected to different time frames of reperfusion[a]

Group	Reperfusion			
	1 h ($n = 5$)	4 h ($n = 5$)	10 h ($n = 5$)	24 h ($n = 5$)
I15				
proximal	2.4 ± 0.3	3.0 ± 0.6	0.3 ± 0.1	0.6 ± 0.1
distal	0.1 ± 0.1	0.4 ± 0.2	0.1 ± 0.1	0.2 ± 0.1
total	2.5 ± 0.4	3.4 ± 0.8	0.4 ± 0.2	0.8 ± 0.2
I45				
proximal	5.1 ± 1.1[b]	8.9 ± 1.9[b]	0.5 ± 0.1	0.7 ± 0.2
distal	1.4 ± 0.3	4.1 ± 1.2[b]	0.4 ± 0.1	0.3 ± 0.1
total	6.5 ± 1.4[b]	14 ± 3.1[b]	0.9 ± 0.2	1.0 ± 0.3
I60				
proximal	9.4 ± 1.6[b]	13.8 ± 2.7[b]	0.6 ± 0.1	0.9 ± 0.2
distal	3.5 ± 0.9	7.2 ± 1.2[b]	0.3 ± 0.1	0.3 ± 0.1
total	13 ± 2.5[b]	21 ± 3.9[b]	0.9 ± 0.2	1.2 ± 0.3

[a] Values are means ± SEM. The percentage of renal apoptotic cells is expressed per 100 proximal tubule or distal tubule cells. I15, renal ischemia for 15 min; I45, renal ischemia for 45 min; I60, renal ischemia for 60 min.

[b] $P < 0.05$ compared with control group (0 ± 0).

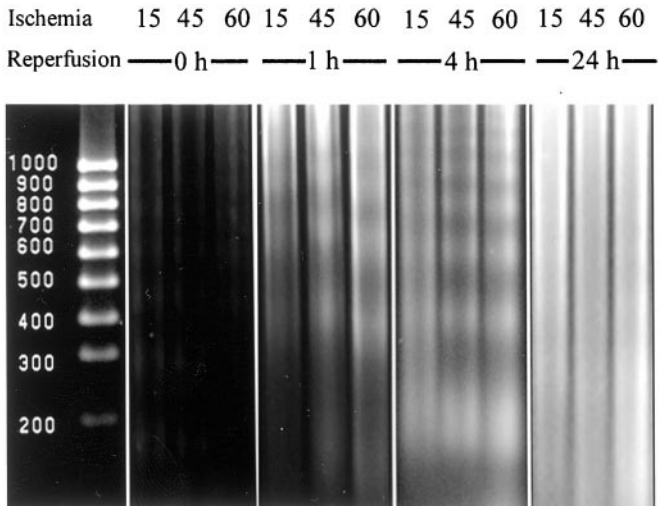

Figure 2. Agarose gel electrophoresis of DNA from kidney with ischemia alone (reperfusion 0 h) and ischemia/reperfusion (I/R; reperfusion from 1 h to 24 h). DNA "ladder" pattern was visible during early reperfusion (1 h), grew progressively by 4 h, and diminished slowly thereafter. A smear pattern was noted in kidney subjected to 24 h of reperfusion.

sion, probably because of the removal of apoptotic cells by a tissue repair mechanism.

SOD treatment (500 U/rat) before reperfusion reduced the number of apoptotic cells in renal tubules, especially in the PT (Figure 1). The number of apoptotic cells in PT in I15/R4 h kidneys was 3.0 ± 0.6% and was reduced to 0.2 ± 0.1% with SOD treatment. Similar results were obtained in I45/R4 h and I60/R4 h kidneys; the percentages of apoptotic cells were 8.9 ± 1.9% and 13.8 ± 2.7%, respectively, without SOD treatment and were reduced to 0.3 ± 0.1% and 3.2 ± 0.7% with SOD treatment. However, the number of apoptotic cells remained unchanged in DT in both nontreated and SOD-treated rats (4.1 ± 1.2% *versus* 3.8 ± 0.9% in I45 kidneys and 7.2 ± 1.2% *versus* 6.5 ± 1.4% in I60 kidneys; $P > 0.01$).

Effect of Ischemic Interval or I/R on Renal DNA Fragmentation Patterns

By gel electrophoresis, no apparent DNA fragmentation was detected from ischemic kidneys without reperfusion. An apoptotic DNA laddering pattern was clearly evident when ischemic kidneys were subjected to 1 to 4 h of reperfusion. In all three ischemic groups of animals, there was an increasing "smear" DNA pattern after prolonged reperfusion, *i.e.*, >10 to 24 h (Figure 2).

Kidney CL In Vivo

Intrarenal arterial injection of lucigenin into the I/R kidney led to a rapid rise in CL counts to a peak at 2 min postinjection (Figure 3). The peak value of lucigenin-enhanced CL from the I/R kidney surface was then rapidly decreased to a basal level as the renal circulation resumed (this usually took 4 min). The basal CL level detected from the normal kidney surface was approximately 1437 to 1566 counts/10 s. The sham-operated kidneys and the ischemic kidney without reperfusion did not show increased CL after administration of lucigenin (Table 2), suggesting that the enhanced CL resulted from reperfusion injury.

A minimal 5-min reperfusion was required for detection of increases in CL, and the observed peak level (maximal value) of CL, *i.e.*, the amount of ROS product, seemed to be correlated with the duration of ischemia. The peak levels of CL from the kidney surface were increased 3.5-fold (5811 counts/10 s), 7-fold (9666 counts/10 s), and 9-fold (13,924 counts/10 s) in the I15, I45, and I60 groups followed by 5-min reperfusion, respectively. Lucigenin-enhanced CL was not demonstrated in kidneys after 10 to 24 h of reperfusion (Table 2).

The CL detected from a single dose of lucigenin and that from continuous infusion of MCLA were similar. Continuous infusion of MCLA into a kidney without I/R insult displayed a basal level of CL at approximately 1150 to 1500 counts/10 s. In I/R kidneys, an abrupt increase in MCLA-enhanced CL was observed and was maintained at the increased level for 4 to 5 h (Figure 3B).

In I45 rats treated with SOD before lucigenin injection at 4 h of reperfusion, the peak value of CL from the kidneys was depressed significantly (Figure 3C), suggesting that the increased CL counts were derived from ROS after I/R insult. When we correlated the apoptotic cell number with the renal CL in the sham-operated rats and the three ischemic groups, a positive linear regression curve ($r^2 = 0.9758$, $P < 0.01$) was obtained (Figure 4).

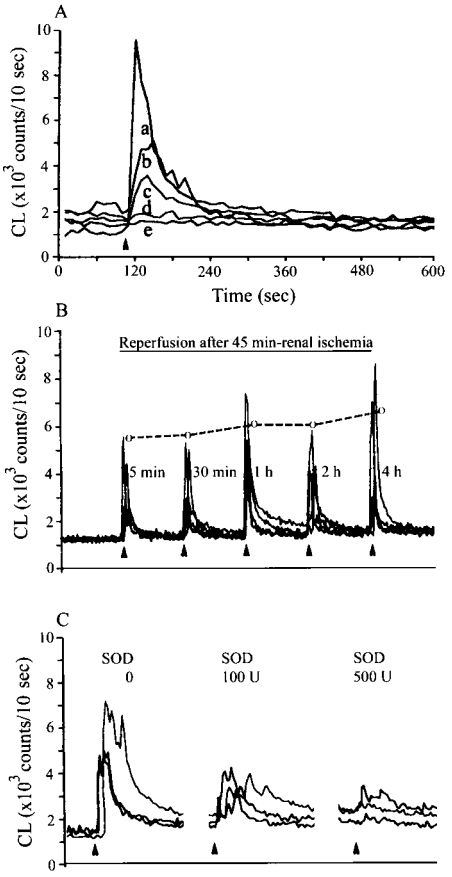

Figure 3. Chemiluminescence (CL) detection from rat kidney surface *in vivo*. (A) Typical recordings of lucigenin (1.0 mM)-enhanced CL from the ischemic kidney surface in I60 (a), I45 (b), and I15 (c) after 5 min of reperfusion and in two control rats (d and e). The basal CL level before I/R was approximately 1000 to 2000 counts/10 s. An abrupt increase in CL was noted in the kidney subjected to I/R injury, and the amounts were related directly to the duration of ischemia. The CL returned to the control value within 3 to 5 min. (B) Similar levels of lucigenin-enhanced CL (solid lines) from the kidney surface were obtained after various durations of reperfusion. Continuous infusion of 2-Methyl-6-[4-methoxyphenyl]-3,7-dihydroimidazo-[1,2-a]-pyrazin-3-one hydrochloride yielded a CL (dashed line) level similar to that obtained with repeated single injections of lucigenin. (C) SOD decreased kidney CL in I45/R1h kidneys in a dose-dependent manner. Arrowheads indicate the times of lucigenin injection.

Effect of Ischemic Interval on Renal Venous and Systemic Arterial-Blood CL

We also adapted the CL method to examine the amounts of ROS in the renal venous and carotid arterial blood samples. The renal venous CL was increased 45-fold (47,864 counts/10 s), 85-fold (85,230 counts/10 s), and 110-fold (117,410 counts/10 s) in I15, I45, and I60 rats (with 5-min reperfusion), respectively (Figure 5, Table 1). A similar result was obtained with the carotid arterial CL, with 40-fold, 75-fold, and 95-fold increases in I15/R5m, I45/R5m, and I60/R5m rats, respectively. The increases in renal venous and carotid arterial CL persisted for 4 h after reperfusion and returned nearly to basal levels after 10 to 24 h of reperfusion.

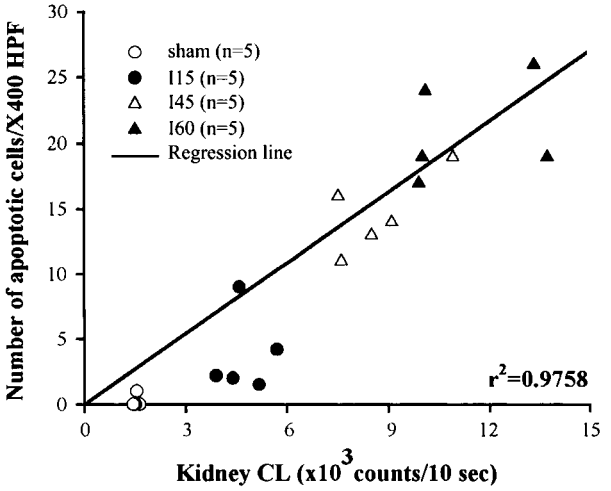

Figure 4. A positive linear correlation between the number of apoptotic cells and kidney CL in sham-operated kidneys and kidneys subjected to 15, 45, and 60 min of ischemia followed by 4 h of reperfusion.

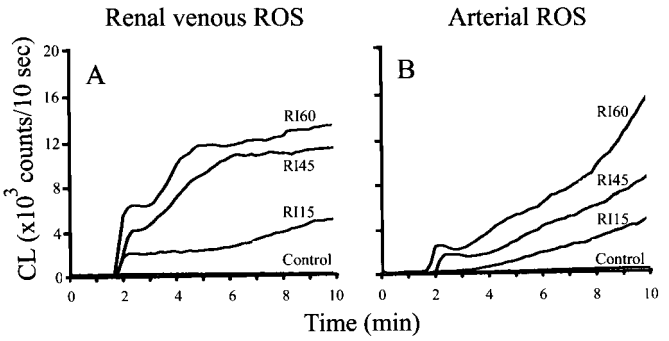

Figure 5. Representative examples of renal venous (A) and carotid arterial (B) CL-ROS after renal ischemia (reperfused for 5 min). Each curve is representative of one rat. A prolonged ischemic interval produced increased amounts of CL-ROS.

In Situ Localization of ROS Formation by NBT Perfusion

We used an NBT staining method for localization of ROS production in renal tissue. NBT is a dye that is reduced to an insoluble formazan derivative upon exposure to superoxide (28). The blue-colored formazan is readily detectable in tissue by light microscopy. Figure 1A shows that superoxide generation was not seen in control kidneys not subjected to ischemia. There was significant superoxide production (blue formazan deposits) in ischemic kidneys after 4 h of reperfusion (Figure 1). An increased ischemic interval in I45 kidneys resulted in a higher density of blue formazan deposits as compared with that in I15 kidneys. The NBT deposits were located mainly in the PT and very sparsely in DT (Figure 1, B and C). In rats treated with SOD, significant decreases in NBT deposits in PT cells were observed.

We performed double staining with the use of the NBT and TUNEL methods for evaluation of the spatial relationship of ROS production and apoptotic nucleus formation in renal tis-

Table 2. Lucigenin-enhanced chemiluminescence of the kidney surface and whole blood from renal venous and carotid arterial flow after 15, 45, and 60 min of renal ischemia[a]

Sample CL (counts/10 s)	Time after Renal Ischemia							
	Control	5 Min	30 Min	60 Min	120 Min	240 Min	10 H	24 H
Kidney CL								
I15 (n = 5)	1552 ± 97	5181 ± 765*	4202 ± 677*	5077 ± 832*	4954 ± 658*	4701 ± 767*	1656 ± 260	1542 ± 65
I45 (n = 5)	1437 ± 99	9666 ± 1012*[b]	7969 ± 895*[b]	6821 ± 1011*	7463 ± 911*[b]	8732 ± 910*[b]	1767 ± 312	1675 ± 98
I60 (n = 5)	1566 ± 89	13,924 ± 2106*	10,099 ± 1893*[c,d]	9769 ± 1675*[c,d]	9441 ± 997*[c]	11,202 ± 2312*[c,d]	2108 ± 432	1599 ± 126
Venous CL								
I15 (n = 6)	1148 ± 46	47,864 ± 6795*	32,421 ± 2665*	31,667 ± 2055*	22,833 ± 1998*	43,032 ± 2080*	2943 ± 879	1446 ± 120
I45 (n = 5)	1130 ± 32	85,230 ± 9839*[b]	67,023 ± 8951*[b]	44,111 ± 3443*[b]	48,474 ± 1729*[b]	74,026 ± 3614*[b]	4345 ± 1654	18,791 ± 66
I60 (n = 4)	1090 ± 29	117,410 ± 16,991*[c,d]	83,162 ± 9548*[c]	67,227 ± 9670*[c,d]	71,118 ± 8216*[c,d]	109,420 ± 17,327*[c,d]	5593 ± 2133	2058 ± 210
Arterial CL								
I15 (n = 5)	1087 ± 11	40,573 ± 5328*	22,072 ± 3391*	20,683 ± 1071*	19,766 ± 5392*	26,710 ± 1744*	2109 ± 1029	1787 ± 199
I45 (n = 6)	1212 ± 47	77,025 ± 4557*[b]	36,867 ± 3149*[b]	41,081 ± 5406*[b]	25,771 ± 3059*	41,508 ± 6815*[b]	2494 ± 1253	1889 ± 98
I60 (n = 5)	1132 ± 25	97,587 ± 14,923*[c]	56,839 ± 9392*[c,d]	52,847 ± 9601*[c,d]	48,294 ± 6767*[c,d]	68,211 ± 4513*[c,d]	2239 ± 797	2356 ± 879

[a] Values are mean ± SEM. Statistical differences ($P < 0.05$); *versus* control value and between groups; [b] compared with I15 and I45 groups; [c] compared with I15 and I60 groups; [d] compared with I45 and I60 groups. CL, chemiluminescence.

sues. Both NBT cellular deposits and apoptotic nuclei could be detected readily in tissue sections. As shown in Figure 1, the apoptotic nuclei appeared in either PT or DT epithelial cells but rarely in glomerular cells. SOD treatment reduced the NBT deposit, as did the formation of apoptotic nuclei in PT. The apoptotic nuclei remained in DT in the SOD-treated rats, indicating that formation of apoptosis in DT was not influenced by the small amounts of ROS produced (see data presented in the Effects of H/R on Tubular Cells in Culture section).

Protracted Ischemic Intervals and Apoptosis-Regulated Gene Expression

The expression of Bax, Bcl-2, CPP32, and PARP in the kidney samples after I/R was assessed by immunoblotting with antibodies against Bax, Bcl-2, CPP32, and PARP (Figure 6). The expression of Bcl-2 was detected in control renal tissues. Bcl-2 was apparently depressed in the ischemic stage, but gradually returned to its pre-ischemic level or higher after reperfusion.

Expression of Bax but not of CPP32 and PARP was detected in control tissues. The expression of all three proteins was increased after 1 h of reperfusion and reached a high level at 4 h of reperfusion. Increased ischemic intervals seemed to potentiate their expression (Figure 6). By densitometry, the Bax/Bcl-2 ratio was increased 1.5-, 1.9-, and 2.1-fold in I15/R4h, I45/R4h, and I60/R4h kidneys, respectively. Expression of CPP32 (32 kD, proenzyme) and its cleaved product (17 kD) was increased 8-, 10-, and 12-fold and 6-, 10-, and 17-fold in I15, I45, and I60 kidneys, respectively. Expression of PARP was increased 3-, 5-, and 8-fold in I15, I45, and I60 kidneys, respectively.

Effects of H/R on Tubular Cells in Culture

PT and DT cells isolated from rat kidney displayed >88% viability by the Trypan blue exclusion test. The characterization of PT and DT was performed by measurement of marker enzyme activity. Alkaline phosphatase activity and hexokinase activity in PT culture (n = 3) were 545 ± 125 nmol/min and 98 ± 27 nmol/min, respectively, whereas these activities in DT culture (n = 3) were 107 ± 24 nmol/min and 866 ± 189 nmol/min, respectively. The purity of PT and DT cell cultures was 90 ± 3% and 86 ± 4%, respectively, as determined by periodic acid-Schiff staining. When these cells were exposed to H/R, a greater decrease in cell viability in PT cells (56 ± 7%) was evident as compared with that of DT cells (69 ± 5%; Figure 7). A 10-fold increase in the number of apoptotic cells was noted in PT culture (from 2.4 ± 0.5% to 23 ± 5%), as compared with a 3-fold increase in DT culture (from 1.9 ± 0.5% to 6.8 ± 2.1%; $P < 0.05$).

Treatment of cultures with SOD or FC_4S, a novel water-soluble fullerene with free-radical scavenging activity (14), protected the PT cells from H/R injury (Figure 7). The viability of PT cells subjected to H/R was 56 ± 7% *versus* 79 ± 5% or 77 ± 5% in cultures treated with SOD or FC_4S. The percentage of apoptotic cells among PT cells subjected to H/R was 23 ± 5% without free-radical scavenger treatment *versus* 13 ± 4% with SOD treatment or 14 ± 4% with FC_4S treatment ($P <$

0.05). However, treatment with SOD or FC_4S did not seem to affect the viability of the reoxygenated DT cells (viability, 69 ± 5% without free-radical scavenger *versus* 74 ± 6% with SOD or 72 ± 6% with FC_4S, and of apoptotic cells from 6.8 ± 2.1% without free-radical scavenger *versus* 6.2 ± 2.0% with SOD or 6.5 ± 1.5% with FC_4S).

The ROS response of PT and DT culture to H/R is displayed in Figure 7. ROS detected in PT and DT culture media were 606 ± 115 counts/10 s and 558 ± 99 counts/10 s, respectively. After H/R, ROS in PT and DT culture media were increased to 2732 ± 488 counts/10 s and 1215 ± 205 counts/10 s, respectively. Addition of SOD or FC_4S to the culture medium significantly decreased H/R-induced ROS to near-basal levels in both PT and DT cultures.

The expression of Bax, Bcl-2, CPP32, and PARP in PT and DT cultures after H/R was assessed by immunoblotting with antibodies against Bax, Bcl-2, CPP32, and PARP (Figure 8). Weak expression of Bcl-2 was detected in control PT and DT cultures, and its expression was apparently enhanced in both cultures after H/R insult. The enhanced Bcl-2 expression could be suppressed by treatment with free-radical scavengers (especially SOD). Expression of Bax, CPP32, and PARP was readily detected in control PT and DT cultures. The expression of all three of proteins was increased after H/R; however, the increased expression was depressed by SOD and FC_4S treatment (Figure 8).

Discussion

During the stage of prolonged ischemia during surgery or organ harvest for renal transplantation, hypoxia and the following reperfusion could initiate the cascade of cellular apoptosis, tissue necrosis, and subsequent inflammatory cell infiltration (6,7,10). In this study, we established an I/R model in rats to examine the effect of the duration of ischemia and/or reperfusion on the formation of apoptosis in the kidneys.

Our study showed that prolonged ischemia resulted in severe apoptosis in the reperfused kidney in a time-dependent manner. This was demonstrated by increases in DNA fragmentation, in apoptotic cell number, and in "proapoptotic" proteins such as Bax, CPP32, and PARP. A minimum of 15 min of ischemia is required for the detection of apoptotic nuclei during the reperfusion phase, whereas reperfusion as short as 5 min was sufficient to allow production of ROS by PT cells in the kidneys. Prolonged ischemia seemed to potentiate the proapoptotic mechanisms.

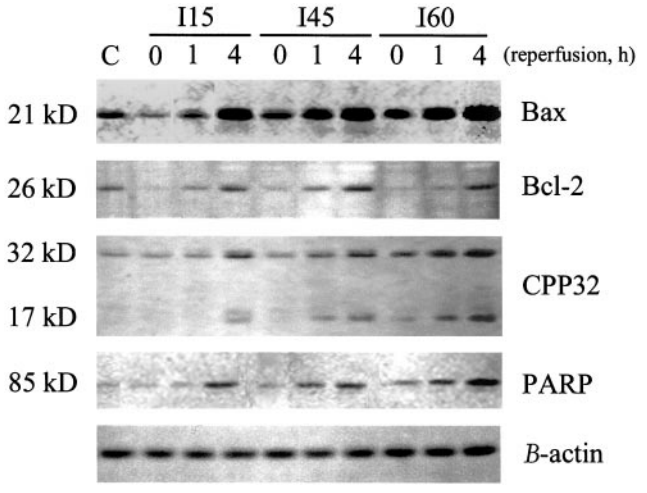

Figure 6. Western blot analysis with specific antibodies to Bax, Bcl-2, CPP32, poly-(ADP-ribose)-polymerase (PARP), and β-actin of homogenates of rat kidney subjected to I/R injury. Note the increased expression of Bax, CPP32 (32-kD proenzyme and 17-kD cleaved product), and PARP after reperfusion. The expression of Bcl-2 was decreased after ischemic insult and returned to its pre-ischemic level after reperfusion. Fifteen min of ischemia plus reperfusion was sufficient to cause an increase in the Bax/Bcl-2 ratio (see text). Equal protein loading was displayed by β-actin.

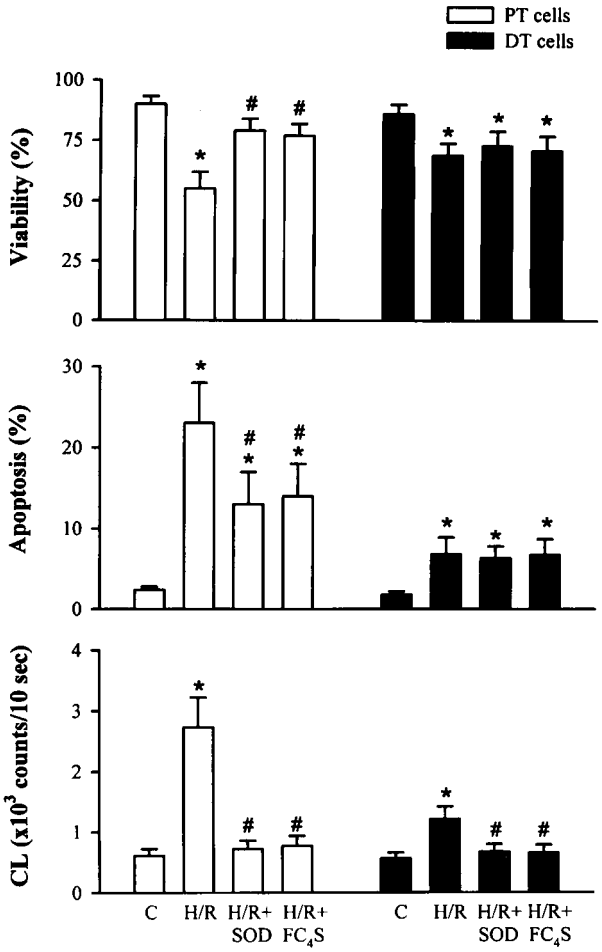

Figure 7. Effect of hypoxia/reoxygenation (H/R) on renal PT and DT cells. Isolated PT and DT cells were subjected to 15 min of hypoxia (95% N_2/5% CO_2) followed by 30 min of reoxygenation (95% O_2/5% CO_2). Cell viability and tubular apoptosis were measured by Trypan blue exclusion and flow cytometry. Results are mean ± SEM for four cell preparations. H/R to PT and DT cells produced a statistically significant ($P < 0.05$) decrease in cell viability and an increase in tubular apoptosis and ROS generation (CL). Addition of SOD or FC_4S to PT and DT cells greatly protected cells from H/R injury in PT but not in DT cells. *, $P < 0.05$ when compared with control stage (C). #, $P < 0.05$ when compared with H/R stage.

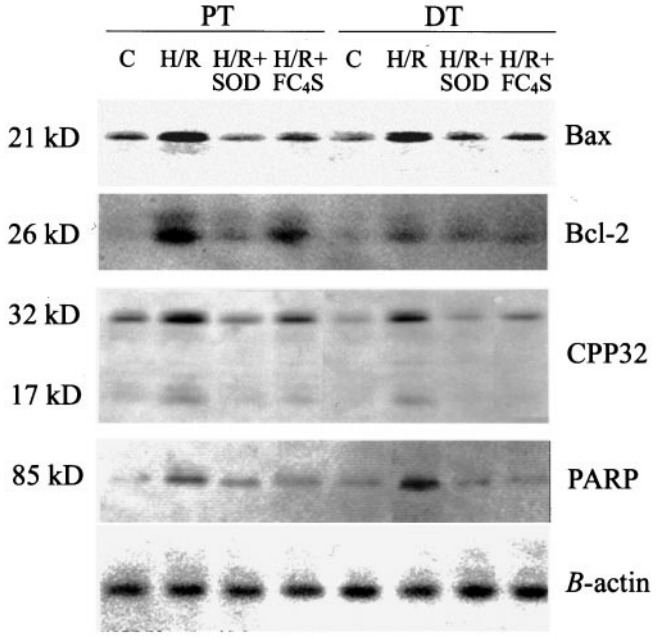

Figure 8. Western blot analysis with specific antibodies to Bax, Bcl-2, CPP32, PARP, and β-actin of PT and DT cultures subjected to H/R injury. Note the increased expression of Bax, CPP32, and PARP after H/R. The expression of Bcl-2 was enhanced in both PT and DT cultures after H/R insult. Significant increases in CPP32 and PARP were also evident after H/R. SOD or FC_4S pretreatment depressed Bax, CPP32, and PARP expression in both PT and DT cultures. Equal loading of protein was displayed by β-actin.

Indirect evidence has shown that ROS have an important role in I/R injury; however, obtaining direct proof is hampered because of the high reactivity and the short biologic half-life of ROS. A number of techniques have been developed for study of the production and mechanisms of ROS in I/R tissue. These include electron spin resonance spectroscopy (35), the NBT reduction method (15,28), DAB-Mn^{2+} cytochemistry (2), and measurement of the induction or depletion of endogenous ROS scavengers (7,14,16,36,37). In this study, we used an enhanced CL method to study ROS production. This method has been applied to measurement of ROS production in cultured cells (22), in the whole-blood system (32,38), in isolated perfused organs (3,39), and in lung and brain *in vivo* (2,26). We showed that the level of lucigenin- or MCLA-enhanced CL detected from the renal surface was correlated with that in whole blood from renal venous efflux or from systemic arterial ROS measured by the lucigenin-enhanced CL method. In either method, a significant level of CL was detected only in reperfused ischemic kidneys but not in sham-operated kidneys or in ischemic kidneys without reperfusion. This strongly suggests a role of reperfusion in the induction of ROS generation and, subsequently, apoptosis formation.

By using an *in situ* vascular NBT perfusion technique, we showed that the cellular source of ROS synthesis was mainly the PT epithelial cells in the I/R kidney. The apoptotic cell death as well as the ROS production in PT cells can be ameliorated by SOD treatment before reperfusion. In contrast, SOD treatment did not seem to minimize apoptosis in DT cells in I/R kidneys. We also noted that, in culture, the number of apoptotic cells and the amounts of ROS formed are more evident in PT cells than in DT cells subjected to H/R insult. Kiyama *et al.* (31) reported that upon exposure to transient ischemia, the DT of the kidney often escape the severe damage that afflicts the PT. The resistance to I/R injury by DT epithelial cells may be attributed to a relative abundance of the manganese SOD gene in DT, whereas low levels of expression were detected in PT (31). This could also explain the generally low level of ROS formation in DT cells. As expected, addition of SOD or FC_4S for scavenging ROS in culture provided significant protection against H/R-induced apoptosis in PT but had only a minor effect in DT cells.

Our results indicate that, as in many organs, the increased Bax, CPP32, and PARP levels could contribute to apoptotic cell death in I/R kidneys. The expression patterns of these proteins in kidneys subjected to I/R as well as in PT and DT cultures subjected to H/R are similar. The level of these proteins seems not to explain the different degree of apoptosis in PT and DT cells in culture. Perhaps multiple and complex proteins, in addition to these four proteins (Bax, Bcl-2, CPP32, and PARP), are involved in this process. Thus, the marked difference between the PT and DT in their susceptibility to ischemic and/or hypoxic injury is attributable to their intrinsic cellular properties, which include endogenous antioxidants, *e.g.*, SOD).

In summary, the present study indicates that ROS are produced in significant amounts in PT epithelium under reperfusion conditions and therefore may be responsible for the apoptotic death of these cells. In DT epithelial cells in I/R kidneys, unlike PT, mechanisms other than formation of ROS may prevail for the induction of apoptosis. Treatment with SOD and other free-radical scavengers as well may be effective for the prevention of ROS-mediated apoptosis in PT.

Acknowledgments

The authors thank Yih-Jen Lin and Hsiau-Yuan Su for expert technical assistance. This work was supported by the National Taiwan University Hospital (NTUH89A014, NTUH89A023, and 89S2003), in part by the National Science Council of the Republic of China (NSC89-2320-B-002-119 and NSC89-2314-B-002-148), and by the Mrs. Hsiu-Chin Lee Kidney Research Fund to C.T.C.

References

1. Kilgore KS, Lucchesi BR: Reperfusion injury after myocardial infarction: The role of free radicals and the inflammatory response. *Clin Biochem* 26: 359–370, 1993
2. Kontos CD, Wei EP, Williams J, Kontos HA, Polvisshock JT: Cytochemical detection of superoxide in cerebral inflammation and ischemia in vivo. *Am J Physiol* 263: H1234–H1242, 1992
3. Okuda M, Lee HC, Kumar C, Chance B: Oxygen radical generation during ischemia-reperfusion in the isolated perfused rat liver monitored by enhanced chemiluminescence. *Circ Shock* 38: 228–237, 1992
4. Strassberg SM, Howard TK, Molmenti EP, Hertl M: Selecting the donor liver: Risk factors for poor function after orthotopic liver transplantation. *Hepatology* 20: 829–838, 1994

5. Bonventre JV, Weinberg JM: Kidney preservation ex vivo for transplantation. *Annu Rev Med* 43: 523–553, 1992
6. Schumer M, Colombel MC, Sawczuk IS, Gobe G, Connor J, O'Toole KM, Olsson CA, Wise GJ, Buttyan R: Morphologic, biochemical, and molecular evidence of apoptosis during the reperfusion phase after brief periods of renal ischemia. *Am J Pathol* 140: 831–838, 1992
7. Zager RA, Fuerstenberg SM, Baehr PH, Myerson D, Torok-Storb B: An evaluation of antioxidant effects on recovery from postischemic acute renal failure. *J Am Soc Nephrol* 4: 1588–1597, 1994
8. Hunter T: Protein kinase and phosphatase: The yin and yang of protein phosphorylation and signaling. *Cell* 80: 225–236, 1995
9. Stamler JS: Redox signaling: Nitrosylation and related target interactions of nitric oxide. *Cell* 78: 931–936, 1994
10. Ueda N, Walker PD, Hsu SM, Shah SV: Activation of a 15-kDa endonuclease in hypoxia/reperfusion injury without morphologic features of apoptosis. *Proc Natl Acad Sci USA* 92: 7202–7206, 1995
11. Majno G, Joris I: Apoptosis, oncosis, and necrosis: An overview of cell death. *Am J Clin Pathol* 146: 3–15, 1995
12. Farber E: Programmed cell death: Necrosis versus apoptosis. *Mod Pathol* 7: 605–609, 1994
13. Hockenbery D: Defining apoptosis. *Am J Pathol* 146: 16–19, 1995
14. Chien CT, Chen CF, Chiang LY, Lai MK: Novel water-soluble hexa(sulfony)fullerenes attenuates apoptosis formation after ischemic renal failure. *Fullerene Sci Techn* 7: 529–540, 1999
15. Wei EP, Povlishock JT, Kontos HA, Moskowitz MA: Oxygen radicals in cerebral ischemia. *Am J Physiol* 263: H1356–H1362, 1992
16. Paller MS, Hoidal JR, Ferris TF: Oxygen free radicals in ischemic acute renal failure in the rat. *J Clin Invest* 74: 1156–1164, 1984
17. Buttke TM, Sandstrom PM: Oxidative stress as a mediator of apoptosis. *Immunol Today* 15: 7–10, 1994
18. Basile DP, Liapis H, Hammerman MR: Expression of bcl-2 and bax in regenerating rat renal tubules following ischemic injury. *Am J Physiol* 272: F640–F647, 1997
19. Kaushal GP, Singh AB, Shah SV: Identification of gene family of caspases in rat kidney and altered expression in ischemia-reperfusion injury. *Am J Physiol* 274: F587–F595, 1998
20. Cohen GM: Caspases: The executioners of apoptosis. *Biochem J* 326: 1–16, 1997
21. McGown AJ, Ruiz-Ruiz MC, Gorman AM, Lopez-Rivas A, Cotter TG: Reactive oxygen intermediate(s) (ROI): Common mediator(s) of poly(ADP-ribose)polymerase (PARP) cleavage and apoptosis. *FEBS Lett* 39: 299–303, 1996
22. Rembish SJ, Trush M: Further evidence that lucigenin-derived chemiluminescence monitors mitochondrial superoxide generation in rat alveolar macrophage. *Free Radic Biol Med* 17: 117–126, 1994
23. Mochida S, Masaki N, Ohta Y, Matsui A, Ogata I, Fujiwara K: In situ detection of oxidative stress in rat hepatocytes. *J Pathol* 167: 83–89, 1992
24. Calabrese F, Valente M, Pettenazzo E, Ferraresso M, Burra P, Cadrobbi R, Cardin R, Bacelle L, Parnigotto A, Rigotti P: The protective effect of L-arginine after liver ischemia/reperfusion injury in a pig model. *J Pathol* 183: 477–485, 1997
25. Kricka LJ: Chemiluminescence and bioluminescence. *Anal Chem* 65: 460R–462R, 1993
26. Takahashi A, Nakano M, Mashiko S, Inaba H: The first observation of O_2^- generation in in situ lungs of rats treated with drugs to induce experimental acute respiratory distress syndrome. *FEBS Lett* 261: 369–372, 1990
27. Dirnagl U, Lindauer U, Them A, Schreiber S, Pfister HW, Koedel U, Reszka R, Freyer D, Villringer A: Global cerebral ischemia in the rat: Online monitoring of oxygen free radical production using chemiluminescence in vivo. *J Cereb Blood Flow Metab* 15: 929–940, 1995
28. Hagen TM, Huang S, Curnutte J, Fowler P, Martinez V, Wehr CM, Ames BN, Chisari FV: Extensive oxidative DNA damage in hepatocytes of transgenic mice with chronic active hepatitis destined to develop hepatocellular carcinoma. *Proc Natl Acad Sci USA* 91: 12808–12812, 1994
29. Gavrieli Y, Sherman Y, Ben-Sasson SA: Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation. *J Cell Biol* 119: 493–501, 1992
30. Hsu SM, Raine L, Fanger H: Use of avidin-biotin-peroxidase complex (ABC) in immunoperoxidase techniques: A comparison of ABC and unlabeled antibody (PAP) procedure. *J Histochem Cytochem* 29: 577–580, 1981
31. Kiyama S, Yoshioka T, Burr IM, Kon V, Fogo A, Ichikawa I: Strategic locus for the activation of the superoxide dismutase gene in the nephron. *Kidney Int* 47: 536–546, 1995
32. Lu FJ, Lin JT, Wang HP, Huang WC: A simple, sensitive, non-stimulated photon counting system for detection of superoxide anion in whole blood. *Experientia* 52: 141–144, 1996
33. Vinay P, Gougoux A, Lemieux G: Isolation of a pure suspension of rat proximal tubules. *Am J Physiol* 241: F403–F411, 1981
34. Guder WG, Ross BD: Enzyme distribution along the nephron. *Kidney Int* 26: 101–111, 1984
35. Vasquez-Vivar J, Hogg N, Pritchard KA, Martasek P, Kalyanaraman B: Superoxide anion formation from lucigenin: An electron spin resonance spin-trapping study. *FEBS Lett* 403: 127–130, 1997
36. Halliwell B, Gutteridge JMC, Cross CE: Free radicals, antioxidants, and human disease: Where are we now? *J Lab Clin Med* 119: 599–620, 1992
37. Gwinner W, Landmesser U, Brandes RP, Kubat B, Plasger J, Eberhard O, Koch K, Olbricht C: Reactive oxygen species and antioxidant defense in puromycin aminonucleoside glomerulopathy. *J Am Soc Nephrol* 8: 1722–1731, 1997
38. Sun JS, Tsuang UH, Chen IJ, Huang WC, Hang YS, Lu FJ: An ultra-weak chemiluminescence study on oxidative stress in rabbits following acute thermal injury. *Burns* 24: 225–231, 1998
39. Paky A, Michael JR, Burke-Wolin TM, Wolin MS, Gurtner GH: Endogenous production of superoxide by rabbit lungs: Effects of hypoxia or metabolic inhibitors. *J Appl Physiol* 74: 2868–2874, 1993