SDC Materials and Methods

**Preparation of replication-defective recombinant adenoviral vectors**

The preparation and construction of replication-defective recombinant adenoviral vector (Adv) a human phosphoglycerate kinase (PGK) promoter to drive COX-1 and PGIS (Adv-COPI) and a PGK alone to serve as control (Adv-PGK) were originated from Dr. Shyue SK (16). In brief, replication-defective recombinant Adv were generated by homologous recombination and amplified in 293 cells. A human COPI cDNA containing the entire coding sequence is subcloned into the E1 and E3 deleted adenovirus shuttle plasmid, which contains a promoter of the human PGK. The adenoviral COPI vector cDNA was kindly provided from Dr. Shyue SK at Institution of Biomedical Science, Academia Sinica. Recombinant Adv stocks were prepared by CsCl gradient centrifugation, aliquoted, and stored at -80°C. Viral titers were determined by a plaque-assay method. These 293 cells were infected with serially diluted viral preparations and then overlaid with low melting-point agarose after infection. Numbers of plaques formed were counted within 2 weeks.

**Animals**

Female Wistar rats (220-250 g) were purchased from BioLASCO Taiwan Co. Ltd. (Taipei) and housed at the Experimental Animal Center of National Taiwan Normal University, at a constant temperature and with a consistent light cycle (light from 07:00 to 18:00 o'clock). Food and water were provided ad libitum. All surgical and experimental procedures were approved by National Taiwan Normal University Animal Care and Use Committee and the animal care and experimental protocols were in accordance with the guidelines of the National Science Council of the Republic of China (NSC 1997).

**Intrarenal arterial delivery of Adv-COPI**

For direct gene delivery, an intrarenal arterial catheter was performed via the left femoral artery as described previously (1). In brief, under avertin anesthesia (400 mg/kg, Acros Organics, NJ, USA), one stretched PE10 tubing was introduced into the left renal
artery via the left femoral artery for infusion (20 µL/min) of $10^8$ pfu of Adv-PGK or Adv-COPI in 0.2 mL of phosphate buffer saline. We ascertained the efficient transgene expression via intrarenal arterial infusion of fluorescent Adv-PGK containing a green fluorescent protein (GFP) gene (2). After Adv-PGK or Adv-COPI transfection into the left kidney, the incision was closed in layers with 3.0 sutures (Ethicon) and the animals were allowed to recover for 7 days. After experiments and blood sampling, all the animals were sacrificed by intravenous KCl. Immunohistochemistry for COX-1 and PGIS protein expression in the kidney was performed. Tissue sections were stained with anti-COX-1 and anti-PGIS (Cayman Chemical Co., Ann Arbor, MI, USA) diluted 1:400 and followed by an avidin-biotinylated horseradish-peroxidase procedure using a commercial kit (ABC Elite; Vector Laboratories).

**Induction of ischemic renal failure**

The method for inducing ischemia renal failure has been reported (1). The rats were anesthetized with avertin and the right kidney was removed. The left renal artery was clamped 45 min with a small vascular clamp for induction of renal ischemia. Sham-operated animals underwent similar operative procedures without occlusion of the left renal artery. Reperfusion was initiated by removal of the clamp for 4 or 24 hours. After experiments, arterial blood was collected for renal functional determination. Blood urea nitrogen (BUN) and plasma creatinine were analyzed using a commercial kit from Sigma (St Louis, MO, USA). The kidney was resected and divided into two parts. One part was stored in 10% neutral buffered formalin for immunocytochemical and in situ apoptotic assay, and another was quickly frozen in liquid nitrogen and stored at –70°C for protein isolation.

**Renal microcirculation measurement**

Vasodilator PGI$_2$ may affect renal microcirculation. A full-field laser perfusion imager (MoorFLPI, Moor Instruments Ltd., Devon, UK) was used to continuously record microcirculatory blood flow intensity in the tested kidney. The amount of blood cells moved
within the region of interest (ROI) is processed to produce a 16-color coded image that correlates with the value of renal blood flow. The ROI in blue is recognized as lower flow, whereas that in red is indicated higher flow. The microcirculatory blood flow intensity of each ROI was displayed as Flux with perfusion unit. The images were real-time recorded and analyzed by the MoorFLPI software version 3.0 (Moor Instruments Ltd.). We also measured total renal blood flow by placing a flow probe (Probe# 0.1VBB517, Transonic Systems, Inc., Ithaca, NY) in left renal artery with a transonic system.

*Measurements of renal eicosanoids levels in the renal tissue and renal venous plasma*

Adv-COPI administration might selectively augment PGI$_2$ production in the renal tubules and vessels of the kidneys, therefore, the levels of eicosanoids in the kidney and renal venous plasma were evaluated. Renal tissue was gently homogenized in 1 mL ice-cold buffer (0.05 mol/L Tris at pH 7.0, 0.1 mol/L NaCl, 0.02 mol/L EDTA). The homogenized tissues and plasma samples were centrifuged at 55000×g for 1 hour. The supernatants were acidified and passed through a Sep-Pak C18 cartridge. Eicosanoids were analyzed using enzyme immunoassay PGE$_2$ and 6-keto-PGF$_{1\alpha}$ (PGI$_2$) kits (R&D System) following the manufacturer's protocols.

*Isolation of renal proximal (PT) and distal tubules (DT)*

We studied the effect of Adv-COPI on ROS, cell viability, autophagy and apoptosis formation in PT and DT cells. Under avertin anesthesia, the kidneys were flushed with 20 mL of ice-cold Krebs-Henseleit-saline buffer (KHS, Sigma) via an aortal catheter. The isolation of PT and DT was performed as previously described (1). Briefly, the kidneys were perfused with 10 mL of KHS containing 0.1% Type IV collagenase. The 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 x 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. Induction of H/R of the PT and DT was performed as following. The cultures were placed in a baseline condition of 95% O₂/5% CO₂ at 37°C for 30 min. Hypoxia was induced by gassing with 95% N₂/5% CO₂ for 15 min, whereas reoxygenation was induced by reintroduction of 95% O₂/5% CO₂ for 30 min.

Cell viability was determined with a Trypan blue dye exclusion test. Apoptosis was assayed in triplicate using the terminal deoxynucleotidyl transferase-mediated nick-end labeling method (1). Autophagic vacuoles were labeled with 0.05 mmol/L monodansylcadaverine (MDC) (31). After labeling, the cells were washed four times with PBS and immediately fixed with 4% paraformaldehyde and observed under a fluorescence microscope (Leica model DMRD, Wetzlar, Germany). The amounts of ROS in PT and DT (10⁶ cells/mL) were measured by the lucigenin-enhanced CL method. For immunoblotting analysis of PT and DT, 1x10⁶ 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% bromphenol 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 hour at 140 V. After electrophoretic transfer of the separated polypeptides to nitrocellulose, the membrane was probed with the apoptosis-related antibodies as described above.

**Western blotting**

We examined the expression of P47, Rac-1, Beclin-1, caspase 3 and PARP in DT and PT and COX-1, PGIS, P47, Rac-1, cytosolic cytochrome C, and lamin of the kidney tissue by the western immunoblotting and densitometry (1). Briefly, the homogenized proteins concentrations were determined by BioRad Protein Assay (BioRad Laboratories). Ten µg of protein was electrophoresed as described below. We used the primary antibodies, COX-1 (Cayman Chemical Co.), PGIS (Cayman Chemical Co.), P47 (Santa Cruz Biotechnology, Inc.), Rac-1 (Abcam), lamin (Cell Signaling), Beclin-1 (BD Biosciences, San Jose, CA), caspase 3
(CPP32/Yama/Apopain, Upstate Biotechnology, Lake Placid, NY), monoclonal mouse antihuman PARP (Promega, Madison, WI), and monoclonal mouse anti-mouse β-actin (Sigma). All of these antibodies cross-react with the respective rat antigens.

**In situ demonstration of oxidative stress, autophagy and apoptosis formation**

The histology and histological scoring of renal sections for acute tubular necrosis were determined with hematoxylin & eosin as described previously (15). We evaluated the level of renal apoptosis (indicating with the number) (1), autophagy (3) and 4-hydroxynonenal (4-HNE) accumulation (indicating with the area) (3) in the paraffin-embedded sections of kidney tissues with immunocytochemistry. The terminal deoxynucleotidyl transferase-mediated nick-end labeling method (TUNEL) was performed to measure apoptotic production in the damaged kidney. Renal sections were stained with methyl green and TUNEL-avidin-biotin-complex technique. Twenty high-power (×400) fields were randomly selected and analyzed. Renal sections were also stained immunohistochemically for presence of an autophagy marker of Beclin-1 (AnaSpec, Inc., San Jose, CA, USA) diluted at 1:50. The percentage of Beclin-1/autophagy was calculated as Beclin-1-stained area/total area × 100% and analyzed by Adobe Photoshop 7.0.1 image software analysis. All the histologic sections were analyzed using a Sonix Image Setup (Sonix Technology Co., Ltd) containing image analyzing software Carl Zeiss AxioVision Rel.4.8.2 (Future Optics & Tech. Co. Ltd., Hangzhou, China).

**Statistical analysis**

All the data were expressed as mean ± standard error mean. Differences within groups were evaluated by paired t-test. One-way analysis of variance 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. We used Sigma Plot 12.0 for graphs preparation.