Chinese Journal of Physiology 58(6): 420-430, 2015
DOI: 10.4077/CJP.2015.BAD324

Effect of Adenoviral Catalase Gene Transfer on Renal Ischemia/Reperfusion Injury in Rats

Chih-Ching Yang[1, 2], Ship-Ping Hsu[3], Kuo-Hsin Chen[4], and Chiang-Ting Chien[5]

[1]*Department of Internal Medicine, National Yang-Ming University, Taipei 11221*
[2]*Department of Planning, Ministry of Health and Welfare, Executive Yuan, Taipei 11558*
[3]*Department of Internal Medicine, Far-Eastern Memorial Hospital, New Taipei City 22056*
[4]*Division of General Surgery, Far-Eastern Memorial Hospital, New Taipei City 22056*
and
[5]*Departments of Life Science, National Taiwan Normal University, Taipei 11677*
Taiwan, Republic of China

Abstract

Ischemia/reperfusion (I/R) may through overt H_2O_2-induced pathophysiologic mechanisms lead to renal dysfunction. We explore whether catalase (CAT) protein overexpression by adenoviral CAT gene (Adv-CAT) transfection may improve ischemia/reperfusion-induced renal dysfunction. We augmented renal CAT expression by intrarenal arterial Adv-CAT administration with renal venous clamping in avertin-anesthetized female Wistar rats. After Adv-CAT transfection, we examined the CAT expression, location and effects on blood urea nitrogen (BUN) and urinary tubular injury biomarkers by biochemical assays, microcirculation by a laser perfusion imager, renal H_2O_2 amount by a chemiluminescent analyzer and molecular mechanisms including cytosolic cytochrome C leakage, apoptosis, autophagy and phospho-Akt (p-Akt)/phospho-endothelial nitric oxide (p-eNOS)/nitric oxide (NO) signaling by western blotting, immunohistochemistry and immunofluorescence. Adv-CAT enhanced 2.6-fold renal CAT protein expression primarily located in the proximal and distal tubules and renal vessels. Ischemia/reperfusion increased cytosolic cytochrome C leakage, renal H_2O_2-dependent level, autophagic Beclin-1/Atg5-Atg12/LC3 II expression, apoptotic Bax/Bcl-2/caspase 3/poly-(ADP-ribose)-polymerase fragments (PARP) expression and terminal deoxynucleotidyl transferasemediated nick-end labeling (TUNEL) stains and BUN and urinary glutathione S-transferase (GST) levels leading to proximal tubular injury. Ischemia/reperfusion also decreased renal microvascular blood flow associated with the inhibited renal expression of p-Akt and p-eNOS and NO production. Adv-CAT significantly improved the reduction in renal microvascular blood flow, reduced ischemia/reperfusion-enhanced oxidative stress, Beclin-1/Atg5-Atg12/LC3 II-meidated autophagy, Bax/Bcl-2/caspase 3/PARP-mediated apoptotic signaling, TUNEL stains, urinary GST level, and restored the p-Akt/p-eNOS/NO signaling in the kidney. Treatment of phosphatidylinositol 3-kinase inhibitors, wortmannin and LY294002, deleted Adv-CAT-induced p-Akt/p-eNOS/NO protective signaling. In conclusion, our results suggest Adv-CAT gene transfer counteracts H_2O_2-induced ischemia/reperfusion injury through preserving p-Akt/p-eNOS/NO pathway in the rat kidney.

Key Words: adenoviral catalase, apoptosis, autophagy, ischemia/reperfusion (I/R), kidney, nitric oxide

Introduction

Ischemia/reperfusion (I/R) impairs kidney function and structure *via* the burst release of reactive oxygen species (ROS) including superoxide anion ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2) (3, 4). These ROS damage

Corresponding authors: [1] Chiang-Ting Chien, Ph.D., Department of Life Science, National Taiwan Normal University, No. 88, Sec. 4, Tingzhou Rd., Taipei 11677, Taiwan, R.O.C. Tel: +886-2-77346312, Fax: +886-2-29312904, E-mail: ctchien@ntnu.edu.tw; and [2] Kuo-Hsin Chen, M.D., Division General Surgery, Far-Eastern Memorial Hospital, No. 21, Sec. 2, Nanya S. Rd., Banciao Dist., New Taipei City 22056, Taiwan R.O.C. Tel: +886-2-89667000 ext. 2811, E-mail: chen.kuohsin@gmail.com
Received: September 21, 2014; Revised (Final Version): December 20, 2014; Accepted: January 6, 2015.
 ISSN : 0304-4920. http://www.cps.org.tw

lipids, proteins and nucleic acids contributing to abnormal signal transduction or cellular dysfunction and initiate the cascade of autophagy, apoptosis, pyroptosis or necrosis (4-6, 8). $O_2^{\bullet-}$ has a very short half-life and is rapidly converted to less-reactive H_2O_2 by superoxide dismutases and subsequently reduced to H_2O by catalase and glutathione peroxidase (1). Since H_2O_2 serves as a substrate for Fenton reaction to generate the highly reactive and toxic hydroxyl radical, catalase is believed to play a role in cellular antioxidant defense mechanisms by limiting the accumulation of H_2O_2. Mammalian catalase is a 240-kDa homotetrameric heme-containing protein located exclusively in the peroxisome and abundantly expressed in liver, lungs, and kidneys (13). In the kidneys, endogenous catalase is primarily localized to the renal proximal tubular cells (16, 23). Cells overexpressing catalase are more resistant to H_2O_2 toxicity and oxidant-mediated injury (2), whereas the overexpression of glutathione peroxidase is not protective against renal injury in diabetic mice (9).

The increased ROS amount provoked during exacerbated oxidative stress and inflammation enhances Bax/Bcl-2 ratio to trigger caspase 3/poly-(ADP-ribose)-polymerase (PARP) mediated apoptosis formation, Beclin-1/Atg5-12/LC3 II mediated autophagy and caspase 1/IL-1β mediated pyroptosis in damaged renal tubules (8). Mitochondrial dysfunction following oxidative injury is possibly an early and critical event in these types of programmed cell death, because ROS triggers cytoplasmic cytochrome C release to affect caspase 8, caspase 3 and caspase 1 activity (8, 17, 20, 30). In the endothelial cells, the production of H_2O_2, an endothelium-dependent contracting factor, through NAD(P)H oxidase activation causes smooth muscle contraction in the rat renal artery (10). Several preconditioning methods require phosphatidylinositol 3-kinase (PI3K)/Akt signaling for enhancing endothelial nitric oxide synthase (eNOS) expressions, phosphorylation of eNOS (p-eNOS) at Ser-1177, and nitric oxide (NO) production to afford cardiovascular protection (14, 18, 31). However, H_2O_2 downregulates PI3K/Akt signaling, p-eNOS expression and NO level and leads to oxidative injury and thrombosis (7, 14).

Based on the above information, the excess ROS especially H_2O_2 should be detoxified to prevent renal I/R injury. Although catalase is endogenously expressed in the cells, the expression level of the enzyme is insufficient to prevent severe I/R injury. Therefore, overexpression of catalase may be promising for prevention of I/R injury. However, there are few studies reporting therapeutic effects of catalase gene delivery on renal I/R injury. In this study, using a replication-defective adenoviral vector, we aimed to evaluate the effects of catalase gene transfer on I/R kidneys by exploring the responses of programmed cell death and p-Akt/p-eNOS signaling.

Materials and Methods

Preparation of Recombinant Adenoviral Vector

The procedures used for the preparation of the recombinant adenoviral vector have been described previously (19). We constructed in the replication-defective recombinant adenoviral vector (Adv) a human phosphoglycerate kinase (PGK) promoter to drive catalase expression (Adv-CAT) and a PGK alone to serve as control (Adv-PGK). A recombinant adenovirus with catalase gene (Adv-CAT) was made by homologous recombination and amplified in human embryonic kidney (HEK) 293 cells and provided from Dr. S.K. Shyue at Institution of Biomedical Science, Academia Sinica (19). Viruses stocks were purified by CsCl density gradient centrifugation, aliquoted, and stored at -80°C. Viral titers, plaque-forming units (pfu), were determined by plaque-forming assay that HEK 293 cells were infected with serially diluted viral preparations and overlaid with low melting-point agarose after infection. Numbers of plaques formed were counted within 2 weeks.

Animals

Female Wistar rats (200-250 g) purchased from BioLASCO Taiwan Co. Ltd. (Taipei) were 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*. The use of female rats rather than male rats was due to the easy introduction of a PE50 tubing through the urethra for urine collection and no gender difference on ischemia/reperfusion induced renal injury (4). All surgical and experimental procedures were approved by National Taiwan Normal University Animal Care and Use Committee and the animal care and experimental protocols are in accordance with the guidelines of the National Science Council of the Republic of China (NSC 1997).

Experimental Design

Rats were randomly assigned into 6 groups (n = 6 each): sham operated Adv-PGK control, sham operated Adv-CAT control, Adv-PGK with renal I/R, Adv-CAT with renal ischemia/reperfusion, Adv-CAT with renal I/R treated PI3K inhibitors wortmannin and Adv-CAT with renal I/R treated LY294002. Wortmannin (500 μg/kg) and LY294002 (1000 μg/kg) (Sigma, St. Louis, MO, USA) were administered intra-

peritoneally daily for 7 days to the rat since Adv-CAT transfection. To ascertain the transgene expression at the region of Adv infusion, we infused adenoviruses containing a green fluorescent protein, Adv-GFP, into the left kidney and determined the location of GFP in rat kidneys 4 h later (n = 6). The kidneys with Adv-PGK infusion were used as negative control.

Intrarenal Arterial Gene Delivery

For direct gene delivery to kidney, an intrarenal arterial PE10 tube was introduced from the left femoral artery as described previously (5). Under avertin anesthesia (400 mg/kg, ACROS ORGANICS, NJ, USA), the Adv solution was administered (5). Adv-PGK or Adv-CAT of 10^8 pfu in 0.2 ml of saline was administered into the left kidney *via* the intrarenal arterial catheter at the rate of 20 μl/min. After Adv-PGK or Adv-CAT treatment, the incision was closed in layers with 3.0 suture (Ethicon, Inc., Somerville, NJ, USA), and the animals were allowed to recover. Seven days after Adv-PGK or Adv-CAT treatment, these rats were subjected to renal I/R injury or sham operation.

Intrarenal Arterial H_2O_2 Infusion

To infuse H_2O_2 into the renal circulation, an intrarenal tubing as described above was inserted into the left renal artery. Renal microcirculation was determined in the basal state and for 60 min period during an intrarenal infusion of H_2O_2 (30 μmol) in the left renal artery described by Salahudeen *et al*. (25).

Induction of Renal Ischemia

Under anesthesia, the rats were tracheotomized. For induction of ischemia in the left kidney, the right kidney was removed and the left renal artery was clamped 45 min by a small vascular clamp (4). Sham-operated animals underwent similar operative procedures without occlusion of the left renal artery. Reperfusion was initiated by removal of the clamp for 4 h. After I/R insults, arterial blood was collected for renal functional determination. Blood urea nitrogen (BUN) was analyzed using a commercial kit from Sigma (St. Louis, MO, USA). Urine samples were collected before and after I/R periods from the PE50 urethral tubing. Concentrations of urinary α glutathione S-transferase (GST) by using a commercially available kit (Biotrin International, Dublin, Ireland) was measured according to the manufacturer's instructions. This urinary biomarker is a specific indicator of proximal tubular damage (3). The kit is a quantitative solid-phase immunoassay specific for rats.

The kidney was resected and divided into two parts. One part was stored in 10% neutral buffered formalin for immunocytochemic and *in situ* apoptotic assay, and another was frozen in liquid nitrogen and stored at –70°C for protein isolation.

In Vivo *Chemiluminescence Recording for H_2O_2 Amount*

The H_2O_2 levels during baseline or I/R were detected from the left kidney surface by intrarenal arterial infusion of luminol solution and by using a Chemiluminescence Analyzing System (CLA-ID3, Tohoku Electronic In. Co., Sendai, Japan) (4). The real-time displayed chemiluminescence signal was recognized as H_2O_2 level from the kidney surface.

Renal Microvascular Blood Flow Measurement

A full-field laser perfusion imager (MoorFLPI, Moor Instruments Ltd., Devon, UK) was used to continuously record microcirculatory blood flow intensity in the kidney as described previously (29). 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 identified as higher flow. The microcirculatory blood flow intensity of each ROI was displayed as perfusion unit. The images were real-time analyzed by the MoorFLPI software version 3.0 (MoorFLPI, Moor Instruments Ltd., Devon, UK).

Renal NO Measurement

In vivo measurement of renal NO was performed using a NO detector (INC-020, NO electrode; Inter Medical Co., Ltd.), amplified by an electrochemical amplifier (IMEC-601/601A; Inter Medical Co., Ltd.) and recorded in a physiologic recording system (Power Lab 8/30; AD instruments) as described previously (22).

In Situ *Demonstration of Catalase Location, Autophagy and Apoptosis Formation*

The catalase location in the treated kidney was stained with anti-catalase antibody (GeneTex Inc., Hsinchu, Taiwan, ROC) by immunohistochemistry. The method for the terminal deoxynucleotidyl transferase-mediated nick-end labeling (TUNEL) was performed to evaluate apoptosis formation and LC3 II stains for autophagy for mation (6). Twenty high-power (×400)

fields were randomly selected for each section, and the value of each programmed cell death was analyzed using a Sonix Image Setup (Sonix Technology Co., Ltd., Hsinchu, Taiwan, ROC) containing image analyzing software Carl Zeiss AxioVision Rel.4.8.2 (Future Optics & Tech. Co. Ltd., Hangzhou, Zhejiang, PRC).

Isolation and Hypoxia/Reoxygenation Injury of Renal Proximal Tubules

Under urethane anesthesia, the kidneys from female Wistar rats (n = 6) were flushed with 20 ml of ice-cold Krebs-Henseleit-saline buffer (KHS) *via* an aortal catheter. The isolation of proximal tubules was performed as described previously (4). In brief, the kidneys were flushed with KHS and then perfused with 10 ml of 0.1% Type IV collagenase in KHS (Sigma). Minced renal cortices were incubated at 37°C in 30 ml of 0.1% collagenase-KHS for 25 min, continuously gassed with 95% O_2/5% CO_2, and gently agitated. Proximal tubular cells were obtained 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, USA) for 30 min at 4°C. The band enriched in proximal tubules was withdrawn and washed two times with ice-cold KHS. Proximal tubular cells were cultured and identified by the alkaline phosphatase activity (a marker enzyme for proximal tubular cells) using a spectrophotometric *p*-nitrophenylphosphate assay (4). The proximal tubular cells were further processed for cytologic examination after formal fixation and coloration with periodic acid-Schiff. The characterization of proximal tubular cells was confirmed by their granular yellow cytoplasm and pink brush border membrane. Induction of hypoxia/reoxygenation of the renal tubule cells was performed as described previously (4). 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. After experiments, the cellular proteins were obtained for Western blotting.

Western Blotting and Immunofluorescence

For cytochrome C staining, Adv-PGK and Adv-CAT treated proximal tubular cells with hypoxia/reoxygenation and Western blotting were performed as described before (5). These samples were incubated overnight at 4°C with the primary antibodies, cytochrome C rabbit polyclonal antibody and heat shock protein 60 goat polyclonal antibody (Santa Cruz Biotechology, Inc., Santa Cruz, CA, USA) and then incubated in anti-goat rhodamine- and anti-rabbit fluorescein isothiocyanate (FITC)-conjugated secondary antibody (Santa Cruz Biotechology, Inc.), respectively. These immunofluorescence images were taken by a laser scanning confocal system (MRC 1000, Bio-Rad Laboratories, Hercules, CA, USA). Mitochondrial leakage of cytochrome C to cytosol (cytosolic cytochrome C) is required for triggering apoptotic pathway (5). The proximal tubular cells were subjected to differential centrifugation to obtain the cytosolic fractions. Protein concentration was determined by a BioRad Protein Assay (BioRad Laboratories, Hercules, CA, USA). Ten μg of protein was electrophoresed as described below. The primary antibody of polyclonal rabbit antihuman cytochrome C (Santa Cruz Biotechology, Inc.) was used at 1:1000.

The expression levels of catalase, apoptosis-related proteins including Bax, Bcl-2, caspase 3, PARP, and autophagy-related proteins Beclin-1, Atg5-Atg12 and LC3 I and II expression were determined by Western blotting in proximal tubular cells and kidney tissues. Western blotting method has been described elsewhere (8). Antibodies raised against Atg5-Atg12 (Gene Tex, Alton Parkway, Irvine, CA, USA), Bax (Chemicon, Temecula, CA, USA), Bcl-2 (Transduction, Bluegrass-Lexington, KY, USA), Beclin-1 (Cell Signaling Technology, Inc., Danvers, MA, USA), the activation fragments (17 kDa of cleaved product) of caspase 3 (CPP32/Yama/Apopain, Upstate Biotechnology, Lake Placid, NY, USA), catalase (GeneTex Inc., Hsinchu, Taiwan), cytochrome C (Sigma, St. Louis, MO, USA), LC3 II (Cell Signaling Technology, Inc.), PARP (N-terminal peptide from the p85 fragment, Promega, Madison, WI, USA), Akt (Chemicon), p-Akt (Santa Cruz Biotechology, Inc.), e-NOS (Cell Signaling Technology, Inc.), p-eNOS (Cell Signaling Technology, Inc.) and β-actin (Sigma) were used. The density of the band with the appropriate molecular mass was determined semi-quantitatively by densitometry using an image analyzing system (Alpha Innotech, San Leandro, CA, USA).

Statistical Analysis

All data were expressed as the mean ± standard error of the mean (SEM). Differences within groups were evaluated by a paired *t*-test. One-way analysis of variance (ANOVA) was used to compare differences among groups. Intergroup comparisons were made by Duncan's multiple-range test. Differences were obtained as significant for $P < 0.05$. Sigma Plot 12.0 was used for graphs preparation. All statistical analyses were performed by using the SPSS software system (SPSS Inc., Chicago, IL, USA).

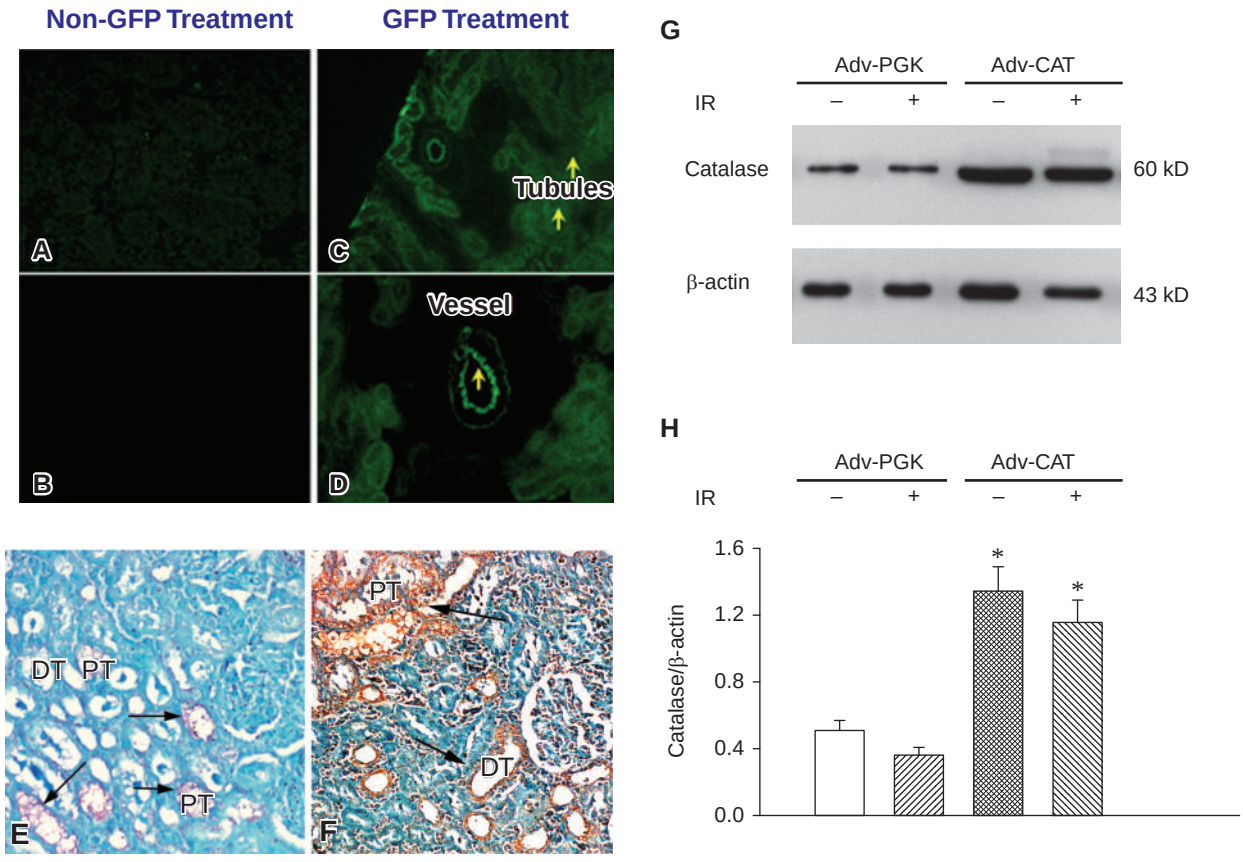

Fig. 1. Effect of Adv-CAT gene transfer to the rat kidney. There is no fluorescent intensity in the control kidney with Adv-PGK treatment (A, B). After 4 h of intrarenal arterial administration of Adv-GFP, renal tubules (C) and renal vessel (D) but not glomeruli display green fluorescence. With immunohistochemical analysis for confirmation of successful transfection and subsequent protein transcription of CAT gene, the renal proximal tubules (PT) and distal tubules (DT) (F) display a positive brownish color in the kidney section post 7 days of transfection. The kidney with Adv-PGK transfer displays a very mild brownish-colored stain in the PT but not in the DT after 7 days of transfection (E). In response to I/R injury, the effect of Adv-PGK and Adv-CAT treatment on renal CAT protein levels post 7 days transfection is displayed in G. Adv-CAT but not Adv-PGK increases renal CAT protein expression. Statistical data from six of rats in each group is shown in H. $*P < 0.05$ when compared with Adv-PGK control.

Results

Intrarenal Arterial Adv-CAT Increases CAT Expression in the Kidney In Vivo

We infused Adv-GFP into the left kidney of normal rats to determine the efficiency of intrarenal adenovirus vector infusion. After 4 h of transfection, the green fluorescence was identified in the renal vessels and tubules (Fig. 1, C and D) of Adv-GFP kidney, but not observed in Adv-PGK kidney (Fig. 1, A and B). We evaluated CAT protein expression 7 days after transfection. By CAT immunohistochemistry, the proximal and distal tubular cells (Fig. 1F) from Adv-CAT-treated kidneys are highly stained with brownish CAT stains when compared to Adv-PGK-treated kidneys with less CAT stains (Fig. 1E).

With Western blotting, compared with Adv-PGK control, Adv-CAT enhanced maximal CAT protein expression around 2.6 folds (Fig. 1, G and H). In response to I/R, the level of CAT expression was depressed by 20% in Adv-PGK and by 16% in Adv-CAT group. However, the higher level of CAT expression was recognized in Adv-CAT than Adv-PGK group.

Intrarenal Adv-CAT Improves Renal Microcirculation in Response to I/R and Intrarenal H_2O_2 Infusion

I/R (Fig. 2A) and intrarenal H_2O_2 infusion (Fig. 2B) markedly decreased renal microvascular blood flow in both Adv-PGK and Adv-CAT rats. However, the statistic data of percentage change of perfusion units indicated that Adv-CAT treatment significantly and efficiently restored IR- (Fig. 2C) or intrarenal H_2O_2 administration-induced (Fig. 2D)

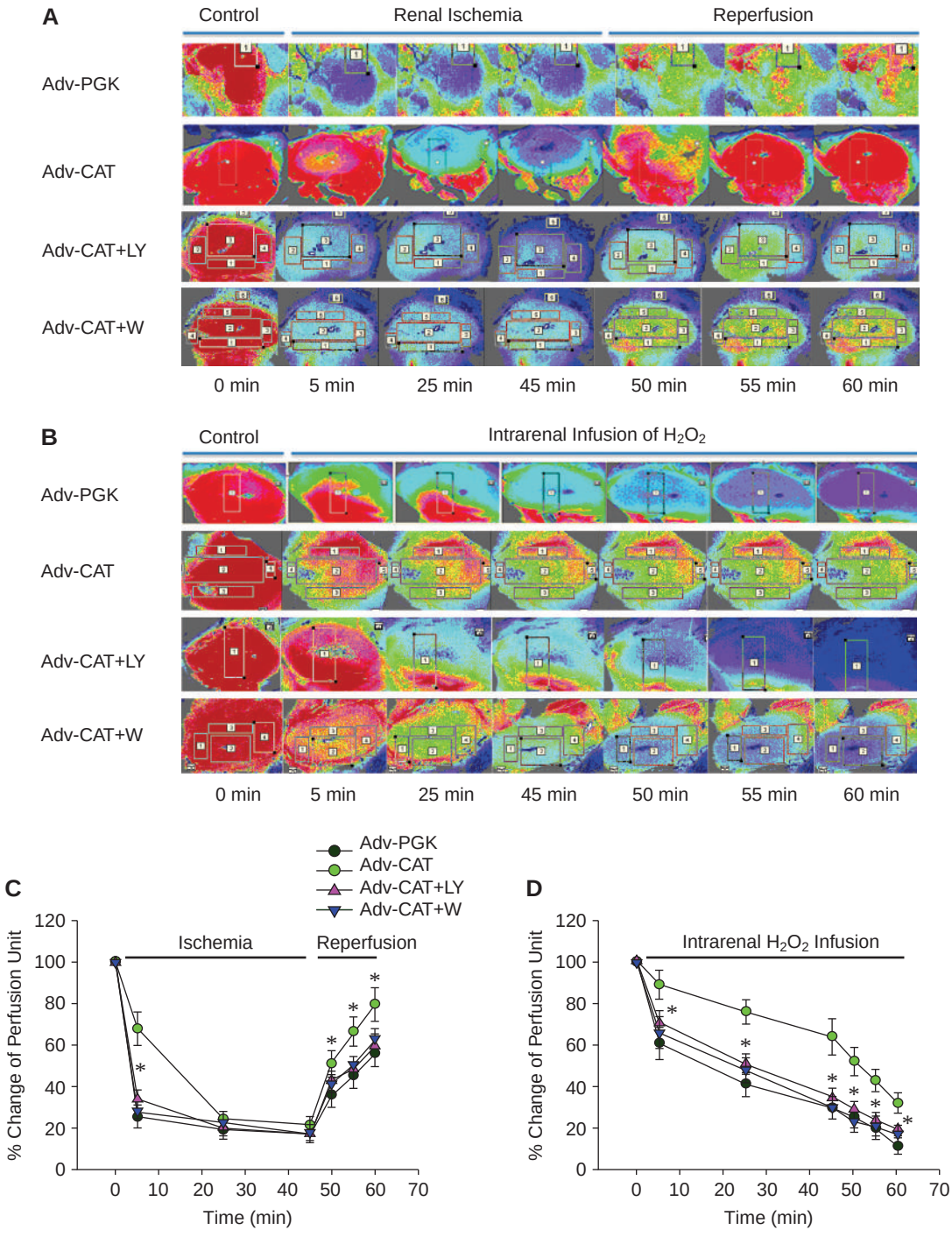

Fig. 2. Effect of I/R and intrarenal H_2O_2 infusion on renal microvascular blood flow in the Adv-PGK, Adv-CAT, Adv-CAT with LY, and Adv-CAT with W treated rats. (A) Typical laser speckle imaging perfusions are displayed on a 16-level color palette in Adv-PGK, Adv-CAT, Adv-CAT with LY, and Adv-CAT with W rats subjected to 45-min ischemia followed by reperfusion. (B) Typical laser speckle imaging perfusions are displayed in Adv-PGK, Adv-CAT, Adv-CAT with LY, and Adv-CAT with W rats subjected to 60-min intrarenal H_2O_2 administration. Statistic data (n = 6 in each group) of percentage change of perfusion units responding to I/R and intrarenal H_2O_2 infusion are shown in C and D. LY, LY294002; W, wortmannin. *P < 0.05 Adv-CAT *vs.* Adv-PGK.

reduction in renal microvascular blood flow when compared to Adv-PGK rats. When compared to Adv-PGK kidney, a slower reduction of perfusion units during early ischemia and a faster recovery of perfusion units during reperfusion periods were noted in the Adv-CAT kidney.

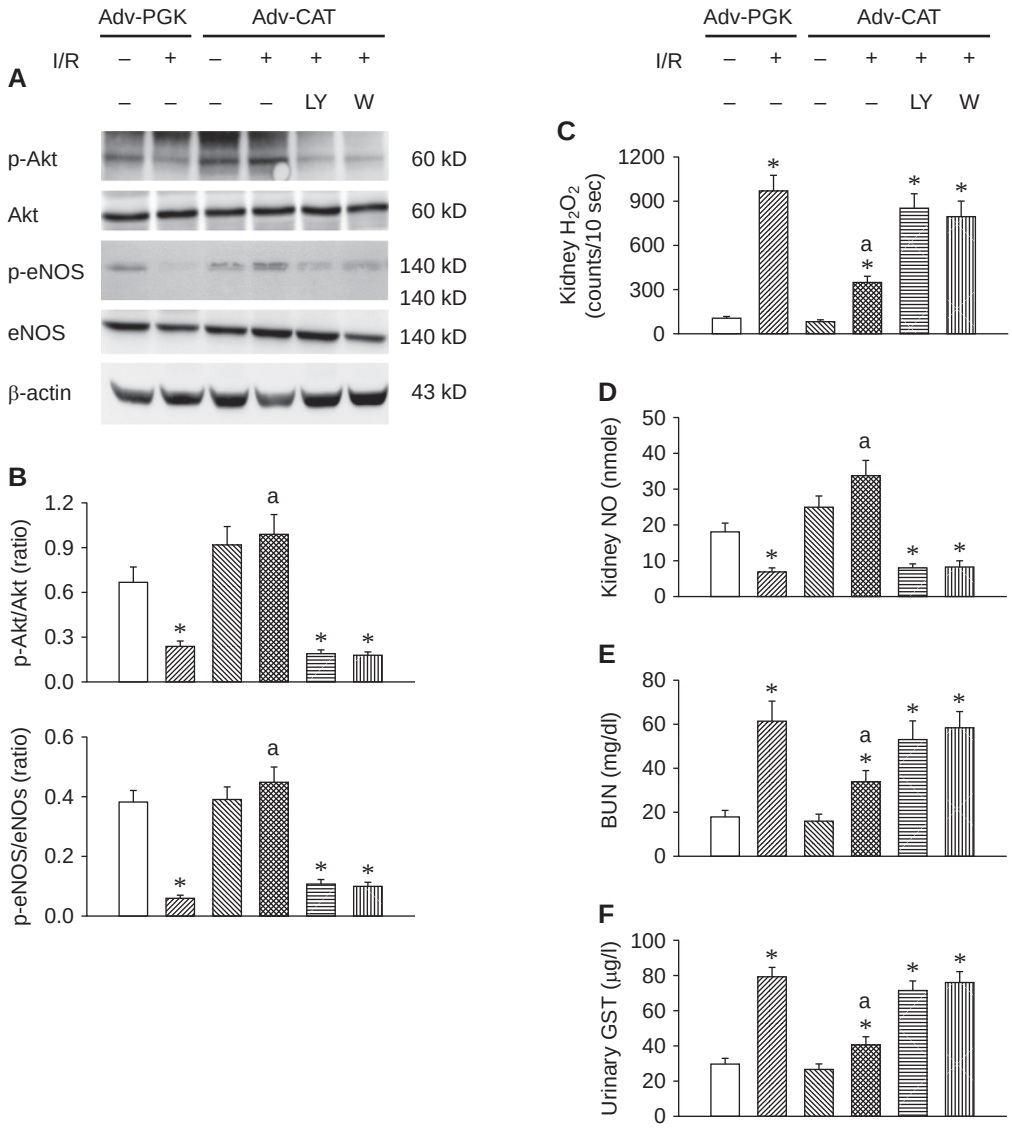

Fig. 3. Effect of Adv-CAT on p-Akt/Akt and p-eNOS/eNOS expression (A) and ratio (B), renal H_2O_2 level (C), kidney NO amount (D), BUN (E) and urinary GST levels (F) in response to I/R injury (n = 6 in each group). Renal I/R produced a significant decrease in p-Akt/Akt and p-eNOS/eNOS ratio and kidney NO amount and an increase in renal H_2O_2, BUN and urinary GST level. The administration of Adv-CAT significantly recovered the p-Akt/Akt and p-eNOS/eNOS ratio and kidney NO amount and decreased renal H_2O_2, BUN and urinary GST levels when compared to Adv-PGK rats with I/R injury. Using phosphatidylinositol 3-kinase inhibitors, LY or W, significantly depressed Adv-CAT-enhanced p-Akt/Akt ratio, p-eNOS/eNOS, and kidney NO amount and increased kidney H_2O_2, BUN and urinary GST levels. LY, LY294002; W, wortmannin. *$P < 0.05$ *vs*. Adv-PGK treated control. [a]$P < 0.05$ *vs.* I/R Adv-PGK.

Adv-CAT Restored I/R-Depressed P-Akt and P-eNOS Expressions

The expression of renal p-Akt, Akt, p-eNOS and eNOS in response to several stimuli was displayed in Fig. 3A. In response to I/R, the ratio in p-Akt/Akt and p-eNOS/eNOS was significantly ($P < 0.05$) decreased in the kidneys (Fig. 3B). Adv-CAT treatment seemed to have a tendency but not significantly to increase p-Akt/Akt and p-eNOS/eNOS ratios in the baseline control. In response to I/R, however, there was a significantly higher p-Akt/Akt and p-eNOS/eNOS ratio in Adv-CAT than that in Adv-PGK kidneys. Administration of intraperitoneal LY294002 and wortmannin significantly ($P < 0.05$) suppressed the Adv-CAT-enhanced p-Akt/Akt and p-eNOS/eNOS ratio. Under baseline conditions, Adv-CAT treatment did not affect the kidney H_2O_2 level (Fig. 3C), NO amount (Fig. 3D), BUN (Fig. 3E) and urinary GST levels (Fig. 3F) when compared to Adv-PGK treatment.

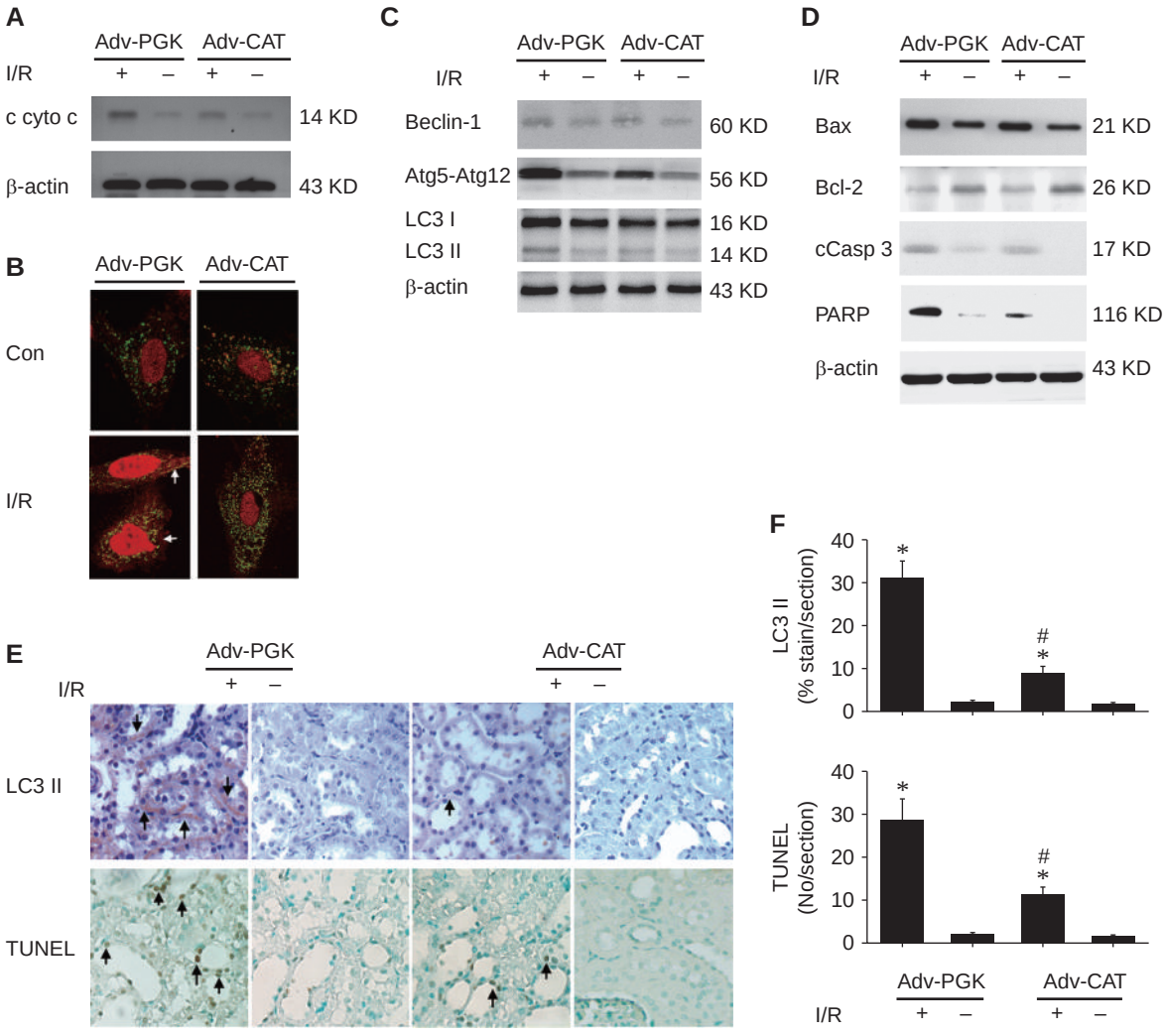

Fig. 4. Effect of Adv-CAT on I/R induced cytosolic cytochrome c (c cyto c) release, LC3 II-mediated autophagy and PARP/TUNEL-apoptosis in the rat primary proximal tubular cells and kidneys. Ten μg of cytosolic cytochrome c (c cyto c) (A) was analyzed in triplicate from the rat primary proximal tubular cells by Western blotting. (B) With confocal fluorescence microscope, I/R induced a morphological change in mitochondria (green fluorescence of HSP60 marker) and increased c cyto c (red fluorescence) leakage from the impaired mitochondria in the Adv-PGK treated primary proximal tubular cells. Adv-CAT treatment inhibited red fluorescence of c cyto c leakage from mitochondria. The total protein of autophagy-related Beclin-1, Atg5-Atg12, and LC3 I/II (C), and apoptosis-mediated Bax, Bcl-2, cleaved casapse 3 (cCasp 3), PARP and β-actin (D) was assayed from the rat kidneys by Western blotting. (E) In immunohistochemistry, I/R increased LC3 II-mediated autophagy and TUNEL-apoptosis in the Adv-PGK treated rat kidneys. Adv-CAT reduced the stains of autophagy and apoptosis in these I/R kidneys. The statistic data (n = 6 in each group) are displayed in F. $^{*}P < 0.05$ *vs.* Adv-PGK treated control. $^{\#}P < 0.05$ *vs.* I/R Adv-PGK.

In response to I/R, there was a significant increase in kidney H_2O_2, BUN and urinary GST levels and a decrease in kidney NO amount in Adv-PGK kidneys. Adv-CAT significantly reduced renal H_2O_2, BUN and urinary GST levels and increased NO amount when compared to Adv-PGK group.

Adv-CAT Restored I/R-Depressed P-Akt and P-eNOS Expressions

Fig. 4A by Western blotting showed I/R enhanced cytosolic cytochrome c leakage in PT cells. In contrast, Adv-CAT treatment inhibited cytosolic cytochrome c release in PT cells. By confocal immunofluoresence, I/R increased the red fluorescent cytochrome cleakage to cytosol in PT cells (Fig. 4B), whereas Adv-CAT inhibited I/R induced cytosolic cytochrome c release. We further found that I/R markedly increased autophagy-related Beclin-1, Atg5-Atg12, and LC3 II expression (Fig. 4C), and apoptosis-mediated Bax/Bcl-2 ratio, cleaved casapse 3, and PARP expression (Fig. 4D) in the Adv-PGK kidneys when compared to their

sham controls. IR enhanced Bax expression and depressed Bcl-2 expression in the Adv-PGK and Adv-CAT kidneys. Adv-CAT greatly depressed I/R-enhanced autophagy-related proteins expression and apoptosis-mediated proteins expression when compared to Adv-PGK group. By immunohistochemistry, we found that the LC3 II and TUNEL stains were not evident in the Adv-PGK and Adv-CAT kidneys without I/R injury. Our data confirmed I/R-enhanced the LC3 II and TUNEL stains in Adv-PGK treated kidneys (Fig. 4E). These parameters were also significantly depressed by Adv-CAT treatment (Fig. 4F).

Discussion

Hepatic delivery of the catalase gene by adenovirus vectors has been reported to efficiently improve I/R-induced or CCl_4-induced hepatic injury (28). In this study, we found that Adv-CAT mildly upregulated catalase protein expression by 2.6 folds in the kidneys through intrarenal arterial route. Adv-CAT treatment successfully and efficiently reduced I/R-induced a depression in renal microcirculation, an impairment of mitochondrial function, an increase of autophagic and apoptotic cell death and BUN level through the enhancement of pAkt/p-eNOS/NO signaling.

The use of antioxidants like superoxide dismutase or vitamin C may provide some renal protection against I/R injury, however, a lower efficiency and some adverse effects like kidney stone induction should be considered. The application of targeting protection by the modified adenoviral vector to the renal tubules and vessels could efficiently relieve I/R-induced damage. This technique does not induce nephrotoxicity and $CD4^+/CD8^+$-mediated immune response in the treated kidneys (5). In addition, the procedure of intrarenal arterial catheterizing technique plus temporarily renal venous clamping did not evoke significant nephron morphologic changes (5, 6, 29). Recently, an intrarenal arterial catheter used for renal sympathetic denervation has been well done in the clinical application. *Via* our developed target therapy techniques, we have successfully corrected the I/R-induced nephron dysfunction by transferring Adv-bcl-2, Adv-bcl-xL, Adv-cyclo-oxygenase-1/prostacyclin synthase genes (5, 6, 29) and Adv-CAT to the targeted nephron and vessel segments.

In response to oxidative stress, apoptosis-inducing factor (AIF) releases from mitochondria, translocates to the nucleus and triggers DNA fragmentation. During post-ischemic reperfusion, excess ROS production from mitochondria initiates apoptosis *via* the release of cytochrome c from mitochondria. Overexpression of MnSOD, CuZnSOD, catalase or glutathione peroxidase prevented AIF translocation from mitochondria to the nucleus implicating non-specific protection due to reducing ROS load (32). Overexpressin of Bcl-2 or Bcl-xL inhibits I/R-triggered cytosolic Bax translocation to mitochondria, suppresses cytosolic cytochrome c release and consequently reduces apoptosis and autophagy in the kidney (5, 6). In the kidney with unilateral ureteral obstruction, the hypoxic/ischemic condition also enhances mitochondrial cytochrome c release into cytosol leading to autophagy, apoptosis and pyroptosis (8). Activating NF-E2-related nuclear factor erythroid-2 signaling by sulforaphane depresses mitochondrial stress-related autophagy, apoptosis and pyroptosis in kidney (8). These reports implicate that the protection of mitochondria against oxidative stress by antioxidant genes would reduce three types of programmed cell death.

The expression level of p-Akt/p-eNOS contributes to detrimental or protective effects of the tissue/organ. Elevated H_2O_2 in the impaired arteriovenous fistulas from chronic kidney diseases animals or cell death caused a significant decrease in p-Akt levels and an activated Bax/caspase-3 signaling (7, 14). Activation of PI3K/Akt signaling and subsequent enhancement of eNOS, phospho-Ser473-Akt, and phospho-Ser1177-eNOS by several treatments increases NO production, and reduces vascular ROS, oxidative stress, and ICAM-1 expression in the damaged tissue (7, 18). Our study also found that the decreased p-Akt/p-eNOS/NO signaling by I/R injury is associated with the reduction of renal microcirculation, the increase of renal H_2O_2 amount BUN, autophagy and apoptosis consequently leading to proximal tubular injury by the elevation of urinary GST level. Adv-CAT reverses all these parameters through PI3K/Akt dependent pathway. In addition, we found that Adv-CAT can significantly attenuate intrarenal arterial H_2O_2 infusion-induced renal vasoconstriction.

Makino *et al*. (21) indicated the superoxide dismutase mimetic infusion failed to prevent superoxide dismutase inhibitor-induced hypertension, unless catalase was coinfused. This information implicates H_2O_2 not $O_2^{-\cdot}$ possibly plays a role in renin/angiotensin-induced systemic hypertension and renal vasoconstriction. Direct infusion of H_2O_2 into the kidney results in reduction of renal microcirculation in our study and increases of mean arterial pressure (20). Insufficient catalase activity and accumulated H_2O_2 might impair the renal structure and function. Reducing catalase activity promotes oxidative stress, telomerase deficiency (24), and diabetic renal injury (14). Overepxression of catalase can attenuate tubular apoptosis, hypertension and tubulointerstitial fibrosis in several types of kidney injury models (11, 12, 26, 27). In this study, we use internal arterial route to administer Adv-CAT and to upregulate catalase expression primarily in the proximal, distal tubules

and renal vessels. This technique for induction of catalase overexpression efficiently and safely rescues I/R-induced renal injury.

In conclusion, we suggest that Adv-CAT gene transfer counteracts I/R induced mitochondrial stress-mediated apoptosis and autophagy through scavenging H_2O_2 and preserving p-Akt/p-eNOS/NO pathway in the rat kidney.

Acknowledgments

This work was supported by the National Science Council of the Republic of China (NSC 102-2320-B-003-001-MY3) to Dr. CT Chien and research fund from Far-Eastern Memorial Hospital (FEMH-2012) to Dr. KS Chen.

References

1. Brownlee, M. Biochemistry and molecular cell biology of diabetic complications. *Nature* 414: 813-820, 2001.
2. Brown, M.R., Miller, F.J. Jr. and Li, W.G. Overexpression of human catalase inhibits proliferation and promotes apoptosis in vascular smooth muscle cells. *Circ. Res.* 85: 524-533, 1999.
3. Chatterjee, P.K., Brown, P.A., Cuzzocrea, S., Zacharowski, K., Stewart, K.N., Mota-Filipe, H., McDonald, M.C. and Thiemermann, C. Calpain inhibitor-1 reduces renal ischemia/reperfusion injury in the rat. *Kidney Int.* 59: 2073-2083, 2001.
4. Chien, C.T., Lee, P.H., Chen, C.F., Ma, M.C., Lai, M.K. and Hsu, S.M. *De novo* demonstration and co-localization of free-radical production and apoptosis formation in rat kidney subjected to ischemia/reperfusion. *J. Am. Soc. Nephrol.* 12: 973-982, 2001.
5. Chien, C.T., Chang, T.C., Tsai, C.Y., Shyue, S.K. and Lai, M.K. Adenovirus-mediated bcl-2 gene transfer inhibits renal ischemia/reperfusion induced tubular oxidative stress and apoptosis. *Am. J. Transplant.* 5: 1194-1203, 2005.
6. Chien, C.T., Shyue, S.K. and Lai, M.K. Bcl-xL augmentation potentially reduces ischemia/reperfusion induced proximal and distal tubular apoptosis and autophagy. *Transplantation* 84: 1183-1190, 2007.
7. Chien, C.T., Fan, S.C., Lin, S.C., Kuo, C.C., Yang, C.H., Yu, T.Y., Lee, S.P., Cheng, D.Y. and Li, P.C. Glucagon-like peptide-1 receptor agonist activation ameliorates venous thrombosis-induced arteriovenous fistula failure in chronic kidney disease. *Thromb. Haemost.* 112: 1051-1064, 2014.
8. Chung, S.D., Lai, T.Y., Chien, C.T. and Yu, H.J. Activating nrf-2 signaling depresses unilateral ureteral obstruction-evoked mitochondrial stress-related autophagy, apoptosis and pyroptosis in kidney. *PLoS One* 7: e47299, 2012.
9. de Haan, J.B., Stefanovic, N., Nikolic-Paterson, D., Scurr, L.L., Croft, K.D., Mori, T.A., Hertzog, P., Kola, I., Atkins, R.C. and Tesch, G.H. Kidney expression of glutathione peroxidase-1 is not protective against streptozotocin-induced diabetic nephropathy. *Am. J. Physiol. Renal Physiol.* 289: F544-F551, 2005.
10. Gao, Y.J. and Lee, R.M. Hydrogen peroxide is an endothelium-dependent contracting factor in rat renal artery. *Brit. J. Pharmacol.* 146: 1061-1068, 2005.
11. Godin, N., Liu, F., Lau, G.J., Brezniceanu, M.L., Chénier, I., Filep, J.G., Ingelfinger, J.R., Zhang, S.L. and Chan, J.S. Catalase overexpression prevents hypertension and tubular apoptosis in angiotensinogen transgenic mice. *Kidney Int.* 77: 1086-1097, 2010.
12. Hasegawa, K., Wakino, S., Yoshioka, K., Tatematsu, S., Hara, Y., Minakuchi, H., Washida, N., Tokuyama, H., Hayashi, K. and Itoh, H. Sirt1 protects against oxidative stress-induced renal tubular cell apoptosis by the bidirectional regulation of catalase expression. *Biochem. Biophys. Res. Commun.* 372: 51-56, 2008.
13. Ho, Y.S., Xiong, Y., Ma, W., Spector, A. and Ho, D.S. Mice lacking catalase develop normally but show differential sensitivity to oxidant tissue injury. *J. Biol. Chem.* 279: 32804-32812, 2004.
14. Huang, S.C., Tsai, Y.F., Cheng, Y.S., Liu, K.H., Li, P.C. and Chien, C.T. Vascular protection with less activation evoked by progressive thermal preconditioning in adrenergic receptor-mediated hypertension and tachycardia. *Chinese J. Physiol.* 52: 419-425, 2009.
15. Hwang, I., Lee, J., Huh, J.Y., Park, J., Lee, H.B., Ho, Y.S. and Ha, H. Catalase deficiency accelerates diabetic renal injury through peroxisomal dysfunction. *Diabetes* 61: 728-738, 2012.
16. Johkura, K., Usuda, N., Liang, Y. and Nakazawa, A. Immunohistochemical localization of peroxisomal enzymes in developing rat kidney tissues. *J. Histochem. Cytochem.* 46: 1161-1173, 1998.
17. Li, H., Wang, P., Sun, Q., Ding, W.X., Yin, X.M., Sobol, R.W., Stolz, D.B., Yu, J. and Zhang, L. Following cytochrome c release, autophagy is inhibited during chemotherapy-induced apoptosis by caspase 8-mediated cleavage of Beclin 1. *Cancer Res.* 71: 3625-3634, 2011.
18. Li, P.C., Yang, C.C., Hsu, S.P. and Chien, C.T. Repetitive progressive thermal preconditioning hinders thrombosis by reinforcing phosphatidylinositol 3-kinase/Akt-dependent heat-shock protein/endothelial nitric oxide synthase signaling. *J. Vasc. Surg.* 56: 159-170, 2012.
19. Lin, S.J., Shyue, S.K., Liu, P.L., Chen, Y.H., Ku, H.H., Chen, J.W., Tam, K.B. and Chen, Y.L. Adenovirus-mediated overexpression of catalase attenuates oxLDL-induced apoptosis in human aortic endothelial cells *via* AP-1 and C-Jun N-terminal kinase/extracellular signal-regulated kinase mitogen-activated protein kinase pathways. *J. Mol. Cell. Cardiol.* 36: 129-139, 2004.
20. Lu, Y., and Chen, L. Mechanisms in adaptation to brain ischemia by ischemic preconditioning. *Adapt. Med.* 4: 20-26, 2012.
21. Makino, A., Skelton, M.M., Zou, A.P. and Cowley, A.W. Jr. Increased renal medullary H_2O_2 leads to hypertension. *Hypertension* 42: 25-30, 2003.
22. Noiri, E., Peresleni, T., Miller, F. and Goligorsky, M.S. *In vivo* targeting of inducible NO synthase with oligodeoxynucleotides protects rat kidney against ischemia. *J. Clin. Invest.* 97: 2277-2383, 1996.
23. Oberley, T.D., Oberley, L.W., Slattery, A.F., Lauchner, L.J. and Elwell, J.H. Immunohistochemical localization of antioxidant enzymes in adult Syrian hamster tissues and during kidney development. *Am. J. Pathol.* 137: 199-214, 1990.
24. Pérez-Rivero, G., Ruiz-Torres, M.P., Díez-Marqués, M.L., Canela, A., López-Novoa, J.M., Rodríguez-Puyol, M., Blasco, M.A. and Rodríguez-Puyol, D. Telomerase deficiency promotes oxidative stress by reducing catalase activity. *Free Radic. Biol. Med.* 45: 1243-1251, 2008.
25. Salahudeen, A.K., Clark, E.C. and Nath, K.A. Hydrogen peroxide-induced renal injury, A protective role for pyruvate *in vitro* and *in vivo*. *J. Clin. Invest.* 88: 1886-1893, 1991.
26. Satoh, M. Benefits of catalase overexpression in renal proximal tubular cells. *Kidney Int.* 77: 1060-1062, 2010.
27. Shi, Y., Lo, C.S., Chenier, I., Maachi, H., Filep, J.G., Ingelfinger, J.R., Zhang, S.L. and Chan, J.S. Overexpression of catalase prevents hypertension and tubulointerstitial fibrosis and normalization of renal angiotensin-converting enzyme-2 expression in Akita mice. *Am. J. Physiol. Renal Physiol.* 304: F1335-F1346, 2013.
28. Ushitora, M., Sakurai, F., Yamaguchi, T., Nakamura, S., Kondoh, M., Yagi, K., Kawabata, K. and Mizuguchi, H. Prevention of hepatic ischemia-reperfusion injury by pre-administration of catalase-expressing adenovirus vectors. *J. Control. Release* 142: 431-437, 2010.
29. Yang, C.C., Chen, K.H., Hsu, S.P. and Chien, C.T. Augmented

renal prostacyclin by intrarenal bicistronic cyclo-oxygenase-1/prostacyclin synthase gene transfer attenuates renal ischemia-reperfusion injury. *Transplantation* 96: 1043-1051, 2013.

30. Yang, Y., Xing, D., Zhou, F. and Chen, Q. Mitochondrial autophagy protects against heat shock-induced apoptosis through reducing cytosolic cytochrome c release and downstream caspase-3 activation. *Biochem. Biophys. Res. Commun.* 395: 190-195, 2010.
31. Yeh, D.Y., Kao, S.J., Feng, N.H., Chen, H.I. and Wang, D. Increased nitric oxide production accompanies blunted hypoxic pulmonary vasoconstriction in hyperoxic rat lung. *Chinese J. Physiol.* 49: 305-312, 2006.
32. Zemlyak, I., Brooke, S.M., Singh, M.H. and Sapolsky, R.M. Effects of overexpression of antioxidants on the release of cytochrome c and apoptosis-inducing factor in the model of ischemia. *Neurosci. Lett.* 453: 182-185, 2009.