의학박사 학위논문

Preventive effects of dichloroacetate on renal ischemia-reperfusion injury-induced oxidative stress and inflammation in diabetic mice

Dichloroacetate가 신장 허혈-재관류 손상으로 인한 산화스트레스와 염증 발생에 미치는 예방적 효과

2021년 8월

서울대학교 대학원

의학과 중개의학 전공

강 아 름

Dichloroacetate가 신장 허혈-재관류 손상으로 인한 산화스트레스와 염증 발생에 미치는 예방적 효과

지도 교수 최 성 희

이 논문을 의학박사 학위논문으로 제출함

2021년 4월

서울대학교 대학원

의학과 중개의학 전공

강 아 름

강아름의 의학박사 학위논문을 인준함

2021년 7월

위 원 장 _____

부위원장 _____

위 원 _____

위 원 _____

위 원 _____

Abstract

Preventive effects of dichloroacetate on renal ischemia-reperfusion injury-induced oxidative stress and inflammation in diabetic mice

Ah Reum Khang

Department of Medicine

Translational Medicine Major

The Graduate School

Seoul National University

Background

Diabetic kidney disease is one of major causes of chronic kidney disease, in which the vulnerability of ischemic insult increases, leading to an acute kidney injury (AKI). Mitochondria, a well-known source of reactive oxygen species (ROS), plays a fundamental role in the pathogenesis of ischemia-reperfusion (IR) injury, which is a leading cause of AKI. Pyruvate dehydrogenase kinase 4 (PDK4) is a mitochondrial matrix enzyme. In this study, the roles of PDK4 in the production of ROS and inflammation after ischemic AKI was investigated.

Materials and Methods

A streptozotocin (STZ)-induced diabetic male C57BL/6J mouse model undergoing ischemia and reperfusion by clamping both renal pedicles was used: sham controls (n = 6), in which the kidneys were surgically exposed; STZ-induced diabetic mice (n = 7); STZ-induced diabetic mice with IR injury(n = 6), in which the kidney were exposed and both renal pedicles were clamped for 37 min and reperfused for 24 h; and STZ-induced diabetic mice with dichloroacetate (DCA, 250 mg/kg, intraperitoneal) treatment and IR injury (n = 6). The cellular apoptosis, production of ROS and inflammatory markers in NRK-52E and mouse primary tubular cells after hypoxia and reoxygenation using a hypoxia work station *in vitro* were evaluated.

Results

The expression of PDK4, not the other PDK isoforms, was induced after IR injury in the diabetic mice, followed by a marked increase in pyruvate dehydrogenase E1α (PDHE1α) phosphorylation. This was accompanied by induction of heightened activation of ROS and the production of tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), interleukin-1β (IL-1β), and monocyte chemoattractant protein-1 (MCP-1). The renal IR injury-induced apoptosis was attenuated upon treatment with DCA, a PDK inhibitor, due to decrease in the expression of PDK4 and phosphorylation of PDHE1α after IR injury. The treatment with either DCA or shPDK4 reduced the burden of oxidative stress and decreased the production of TNF-α, IL-6, IL-1β and MCP-1 after IR injury. Consequently, the maximal respiration of mitochondria and protein expression levels of complex III and V upon IR injury showed improvement after treatment with DCA or shPDK4

in NRK-52E cells. These results suggest that the renal IR injury involves the PDK4-related production of ROS, inflammation, and consequent mitochondrial dysfunction.

Conclusion

Inhibition of PDK4 diminished renal injury, decreased the production of ROS and inflammation, and improved the mitochondrial dysfunction, thereby revealing the critical roles of PDK4 in ischemic reperfusion damage. This study provides another potential target for reno-protection during IR injury. The specific effects of the inhibition of PDK4 on the mitochondrial dynamics and pathogenesis of renal IR injury need to be further elucidated.

Keywords: ischemia, reperfusion, diabetes, acute kidney injury, pyruvate dehydrogenase kinase 4, dichloroacetic acid, reactive oxygen species, inflammation

Student Number : 2013-31152

Table of Contents

List of Tables

List of Figures

INTRODUCTION

Diabetic kidney disease, also known as diabetic nephropathy is one of the major causes of chronic kidney disease (CKD) and the primary cause of end-stage kidney disease worldwide [1]. Ischemic insult to the diabetic kidneys is an important mechanism for the onset and progression of diabetic nephropathy, resulting in tubulointerstitial fibrosis and reduced renal function [2-4]. Patients with Type 2 diabetes exhibit an increased risk for acute kidney injury (AKI) compared with those without diabetes, even after the adjustment for numerous known risk factors including CKD [5]. Streptozotocin (STZ)-induced diabetic mice show higher ischemia-vulnerability than the non-diabetic controls [6]. In addition, extensive inflammation and tubulointerstitial fibrosis are evident in diabetic rats after ischemia, while non-diabetic rats almost completely recover from the functional impairment of the kidneys [7]. AKI episodes are associated with accumulative risk for developing advanced chronic kidney dysfunction in diabetic patients during a long-term follow-up, independent of other major risk factors for the progression of the disease [8].

AKI is a clinical condition defined by an abrupt decrease in the kidney function accompanied by a fatal and rapid loss of the renal excretory function, which is represented by elevated serum creatinine and accumulation of uremic waste products [9]. AKI is caused by several factors, including intrinsic (*e.g.*, acute tubular necrosis, ischemia, acute interstitial nephritis, glomerulonephritis, presence of nephrotoxins), pre-renal (*e.g.*, hypovolemia, heart failure, and sepsis), and post-renal factors (*e.g.*, postrenal obstructive nephropathy and renal stone). It is

renowned as one of the most devastating complications of hospitalized patients. According to the Kidney Disease Improving Global Outcomes (KDIGO) definitions of AKI, its incidence was reported to be 18.3% in the hospitalized patients, which was more common in the patients with sepsis and critical illness, as well as those that underwent cardiovascular surgeries (~50%) [10]. AKI is independently associated with increased hospital mortality in critically ill patients, and the progression of CKD [11-14]. Therefore, it is important to prevent or minimize the harmful effects of AKI, which will further help to attenuate the risk of progressive CKD in diabetic patients; however, an effective therapeutic strategy has not yet been developed for AKI.

In many cases, AKI ensues from transient renal ischemia and reperfusion [2]. Ischemia-reperfusion (IR) injury comes out when the blood flow to a tissue is cut off for a few minutes to several hours (ischemia) and then recovered (reperfusion) [15]. Typically, IR injury induces organ damage after various events, such as myocardial infarction, cerebrovascular occlusion and organ transplantation [16]. Acute ischemic insult is common in the kidneys of diabetic patients, subsequently leading to CKD or end-stage kidney disease [2-4]. Despite the effort of intensive clinical and preclinical studies in IR renal injury, effective interventions to prevent or reduce the clinical IR injury have not yet been developed [17, 18]. Although previous insights into this phenomenon focused on the heart ischemic disease [19-21], a variety of ischemic disorders, including AKI, intestinal ischemia, stroke, and graft rejection after transplantation, have recently been reported to share a common underlying pathophysiology [15]. Not only ischemia, but also reperfusion itself can paradoxically induce cell death independent of the ischemic episode by a process

known as reperfusion injury [16]. The reoxygenation of an organ that has previously suffered an ischemic period is an additional impetus for the occurrence of the fatal exacerbation of tissue injury [15]. Chouchani *et al*. have recently identified a unifying mechanism for ROS production, during which superoxide was generated through reverse electron transport at complex I of the mitochondrial respiratory chain upon reperfusion [22].

Mitochondria are growingly acknowledged to play a principal role in the pathogenesis of IR injury, which has led to an increased interest in the application of mitochondria-targeted therapies for the management of AKI [23]. The pyruvate dehydrogenase complex (PDC) catalyzes the oxidative decarboxylation of pyruvate, resulting in acetyl Coenzyme A and nicotinamide adenine dinucleotide in mitochondria. It is a central metabolic node of tricarboxylic acid (TCA) cycle flux and is tightly regulated in humans by any one of the four pyruvate dehydrogenase kinases (PDK1, PDK2, PDK3 and PDK4) and pyruvate dehydrogenase phosphatase, deactivating and activating it, respectively [24, 25]. PDK4 is mainly expressed in the skeletal muscle, heart, pancreatic islets and kidneys [24, 25]. PDK4 is upregulated in diabetes or under starvation, while insulin treatment reduces the expression of the PDK4 protein in the diabetic state [26, 27]. Mitochondria are well-known key sources of reactive oxygen species (ROS), which are generated by an early burst of superoxides upon reperfusion, and this has been reported across a range of tissues, including the kidney tissues [28, 29]. Inhibition of PDK, especially PDK4 attenuates the development of cisplatin-induced AKI by reducing the levels of mitochondrial ROS and preserving the mitochondrial function, which further prevents the renal tubular cell apoptosis [30].

A better understanding of the pathophysiology of acute IR injury will facilitate the identification of promising novel therapeutic or preventive agents for widespread application in clinical practice. The present study addressed the hypothesis that IR renal injury in diabetic mice induced the expression of PDK4 in the kidney tissues and the pharmacological inhibition of PDK4 reduced the burst of ROS and production of inflammatory cytokines in IR renal injury, leading to an improved mitochondrial function. I used a diabetic mouse model to prove the hypothesis and to determine the specific role of PDK4 in IR renal injury. Furthermore, this study assessed the effects of pharmacological inhibition of PDK4 on the production of ROS, inflammation, and subsequent mitochondrial function following IR renal injury.

MATERIALS and METHODS

Animal model of ischemia-reperfusion injury in kidney

All experimental procedures were proved by the Institutional Animal Care and Use Committee of Kyungpook National University (KNU 2020-0132). All animal procedures were carried out following the appropriate institutional guidelines for animal research. Nine-week-old C57BL/6J male mice (DooYeol Biotech, Seoul, Republic of Korea) were administered a single intraperitoneal injection of STZ (Sigma-Aldrich, St. Louis, MO), 50 mg/kg daily for five consecutive days to induce diabetes. After 2 weeks, the STZ-injected diabetic mice were selected if they exhibited fasting blood glucose levels exceeding 250 mg/dL. The mice were randomly divided into four study groups after 20 weeks: sham controls (n = 6), in which the kidneys were surgically exposed; STZ-induced diabetic mice (n = 7); STZ-induced diabetic mice with IR injury (n = 6), in which the kidney were exposed and both renal pedicles were clamped for 37 min and reperfused for 24 h; STZ-induced diabetic mice with dichloroacetate (DCA) treatment and IR injury (n = 6). Before IR injury, 250 mg/kg of DCA was administered intraperitoneally once a day for 5 weeks.

Mice were anesthetized by an intraperitoneal injection of 50 mg/kg pentobarbital sodium (Entobar®, Hanlim Pharmaceuticals, Republic of Korea) and were placed on a heater to maintain the body temperature. A bilateral flank incision was made, and the renal vein and artery were clamped for 37 min. For the control group, sham operations were performed without clamping the vessels. The surgical wounds were then closed and sterilized with povidone. Twenty-four hours after the

operation, the mice were sacrificed and the blood samples and both kidneys were collected. The collected kidneys were frozen in liquid nitrogen and stored at –80 ºC for further analysis. Serum was obtained from the blood samples using a microtainer (BD, Franklin Lake, NJ). The serum blood urea nitrogen (BUN) and serum creatinine levels were measured using automatic analyzer 7020 (Hitachi, Osaka, Japan).

Cell culture

NRK-52E rat kidney tubular epithelial cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA, CRL-1571™) and cultured in the Dulbecco's modified Eagle medium (DMEM) with high glucose (Gibco, Grand Island, NY) supplemented with 5 % heat-inactivated fetal bovine serum (FBS, Hyclone, Logan, UT) and 1 % penicillin/streptomycin (P/S, Gibco). Cells were seeded on 100 mm dish (Corning, Kennebunk, ME) at a density of 1×10^6 cells/plate or 60 mm dish (Corning) at 3×10^5cells/plate before the experiments.

Isolation of mouse primary kidney tubular cells was performed as described previously [30]. Briefly, the primary mouse proximal tubules were isolated from 0.1% collagenase (Gibco) dissection solution-treated kidneys of 4-week-old male C57BL/6J (wild type) and pyruvate dehydrogenase 4 knockout (PDK4 KO) C57BL/6J mice [31] originating from The Jackson Laboratory (Bar Harbor, ME). After incubation for 2 h, the tubules were filtered using a 200 μm nylon mesh (pluriSelect, El Cajon, CA), followed by flushing the cells in a 85 μm mesh (pluriSelect) with DMEM/F12 media (Gibco), 1% heat-inactivated FBS, 15 mM HEPES, 2 mM L-glutamine, 50 nM Hydrocortisone (Sigma), 0.55 mM sodium

pyruvate (Sigma), ITS 100X (Gibco), 10 ml/L 100× nonessential amino acid (Gibco), and penicillin/streptomycin (Gibco). The tubules were then centrifuged for 5 min at 1000 rpm and resuspended in DMEM/F12 medium. The tubules were then seeded on a collagen-coated plate (Corning) for 48 h, and culture medium was replaced every 2 days. On the seventh day, the cells were split and seeded as 4×10^5 cells in a 60 mm dish (Corning) for further experiments [30].

In vitro model of hypoxia-reoxygenation (HR) injury

NRK-52E cells were seeded onto 60 mm dishes and incubated for 24 h. The cells were washed twice with HBSS (Gibco) and exposed to hypoxic conditions (1 % O_2) in a serum free-HBSS medium, with and without 2 or 5 mM DCA (Sigma) for 6 h in a pre-conditioning hypoxia work station (Ruskinn, INVIVO$_2$400, Pencoed, UK). Then, reperfusion was performed 2 h after replenishing the cells with normal culture media with or without DCA (2 or 5 mM). The mouse primary tubular cells were exposed to hypoxic conditions for 4 h, and reperfusion was performed for 3 h. For the knockdown experiment, hypoxia-reoxygenation was performed 16 h after infecting the cells with adenoviral shGFP or shPDK4 (at 50 multiplicity of infection), which was amplified.

Quantitative real-time polymerase chain reaction analysis

RNA was extracted from the mouse kidney tissues and NRK-52E cells using QIAzol (QIAGEN, Germantown, MD), and cDNA was synthesized from 4 μg total RNA using oligo dT primer (Thermo Scientific, Graiciuno, Vilnius). Quantitative real-time polymerase chain reaction analysis (qRT-PCR) was performed by Viia7

instrument (Applied Biosystems, Foster City, CA) with SYBR green reagent (Applied Biosystems) [32]. The expression of mouse 36B4 was used as an internal control. Mouse and rat primer sequences for qRT-PCR are described in **Table 1**.

Gene	Sequence (5'→3')
Mouse Pdk1	Forward-CACCACGCGGACAAAGG Reverse-GCCCAGCGTGACGTGAA
Mouse Pdk2	Forward-CCCCGTCCCCGTTGTC Reverse-TCGCAGGCATTGCTGGAT
Mouse Pdk3	Forward-GGAGCAATCCCAGCAGTGAA Reverse-TGATCTTGTCCTGTTTAGCCTTGT
Mouse Pdk4	Forward-CCATGAGAAGAGCCCAGAAGA Reverse-GAACTTTGACCAGCCTGTCTACAA
Mouse Arbp	Forward-ACCTCCTTCTTCCAGGCTTT Reverse-CTCCAGTCTTTATCAGCTGC
Rat TNF- α	Forward-ATGATCCGAGATGTGGAACT Reverse-AGGAATGAGAAGAGGCTGAG
Rat IL-6	Forward-TGCCTTCTTGGGACTGATGTT Reverse-TACTGGTCTGTTGTGGGTGGTA
Rat IL-1β	Forward-CTCTGTGACTCGTGGGATGATG Reverse-GTGCAGCTGTCTAATGGGAACA
Rat Mcp-1	Forward-TGTGAACTTGACCCATAAATCTGAA Reverse-TGCTTGAGGTGGTTGTGGAA
Rat Arbp	Forward-ACCTCCTTCTTCCAGGCTTT Reverse-CTCCAGTCTTTATCAGCTGC

Table 1. Sequences of primers used for quantitative-RT-PCR

Western blot analysis

The frozen tissues and cells were lysed with the protein lysis buffer (20 mmol/L Tris-HCl, pH 7.4, 1% NP-40, 5 mmol/L EDTA, 2 mmol/L Na_3VO_4, 100 mmol/L NaF, 10 mmol/L $Na_4P_2O_7$, 100 μmol/L PMSF, 7 μg/ml aprotinin, 7 μg/mL leupeptin, and phosphatase cocktail inhibitors). The tissues and cell lysates were centrifuged for 10 min at 10000 rpm and then the supernatant was collected. The total protein concentration was determined by using a BCA Protein Assay kit (Thermo Scientific). Proteins were separated on 10–12 % SDS-polyacrylamide gels and transferred to PVDF membranes (Millipore, County Cork, Ireland) [30, 32]. The following antibodies were used for detecting the protein expression levels: anti-PDK1 (ENZO Life Science, Farmingdale, NY); anti-PDK2 (Santa Cruz, Dallas, TX); anti-PDK3 (AbFrontier, Seoul, Republic of Korea); anti-PDK4 (Abcam, Cambridge, MA); antiphsophrylation of pyruvate dehydrogenase E1α (anti-pPDHE1α Ser232, Calbiochem, San Diego, CA); pyruvate dehydrogenase (Thermo Scientific); anti-cleaved caspase-3 (Cell Signaling Technology, Barvely, MA); β-actin (Sigma).

Histological and immunohistochemical analysis

Mouse kidneys were fixed using 4% paraformaldehyde (PFA) for 24 h and embedded in paraffin. Then, 4 μm-thick serial sections were deparaffinized in xylene, rehydrated through descending grades of ethanol, and stained with hematoxylin & eosin (H&E) or periodic acid-Schiff (PAS). Immunohistochemical analysis was performed as described previously [30]. Briefly, the sections were permeabilized with IHC-Tek epitope retrieval solution (IHC world, MD) for 45

min, incubated with 3% hydrogen peroxide (DUKSAN science, Seoul, Republic of Korea) for 15 min and blocked with UltraVision protein block (LabVision Corporation, CA) for 10 min. Sections were then incubated with primary antibodies against p-PDHE1α (Ser232, Calbiochem), 4-hydroxynenenal (4-HNE, Abcam) and nitrotyrosine (NT) for 16 h. H&E and PAS stained section were examined using light microscopy (Olympus BX53 upright microscope, Tokyo).

TdT-mediated dUTP nick end labeling (TUNEL) staining

Paraffin-embedded 4 μm-thick kidneys sections were deparaffinized and stained in accordance with manufacturer's instructions (In situ Cell Detection Kit, Roche, Mannheim). Then, the kidneys sections were permeabilized with Proteinase K (20 μg/ml in 10 mM Tris/HC1, pH 7.4–8; Sigma-Aldrich) for 1 h at 37°C in an incubator. Then, the mouse primary kidney tubular cells were fixed for 15 min with 4% PFA, permeabilized with 0.1% TritonX-100, and used for staining [30]. The kidneys sections and cells were mounted with the mounting medium (Vecta laboratories, Burlingame, CA). Fluorescence images were captured using an OLYMPUS 1X81 inverted microscope system.

Annexin V analysis

NRK-52E cells were collected, resuspended in 1× binding buffer, and incubated with fluorescein isothiocynate (FITC) and propidium iodide (PI) at room temperature for 15 min in the dark. Analysis was performed using FITC-Annexin V apoptosis detection kit I (BD bioscience, Franklin Lakes, NJ) and Accuri™ C6 cytometer (BD bioscience) within 1h as per the manufacturer's instructions.

Measurement of the mitochondrial oxygen consumption rate (OCR)

NRK-52E cells were seeded on XF-24 flux analyzer 24-well culture plates (Agilent Technologies, Santa Clara, CA) at a density of 4×10^5 cells/well and incubated for 24 h. The cells were kept in a hypoxia work station for 6 h, followed by reperfusion in an XF base medium (Agilent Technologies) for 1 h. OCR was measured as described previously [30]. The cells were incubated in an XF-based minimal DMEM (Agilent Technologies) with 24.5 mM D-glucose (Sigma-Aldrich), 1 mM sodium pyruvate (Sigma-Aldrich), and 1× GlutaMAX™ (Gibco) at 37℃ in an incubator. The following chemicals were used in this study; 2 μmol/L Oligomycin A (Sigma-Aldrich), 10 μmol/L Carbonyl cyanide 3-chlorophenylhydrazone (CCCP, Sigma-Aldrich), 1 μmol/L Rotenone (Sigma-Aldrich) and 5 μmol/L Antimycin A (Sigma-Aldrich). OCR was measured using Seahorse XF-24 Flux analyzer (Agilent Technologies). 4', 6-diamidino-2-phenylindole (DAPI) stained cells were counted for normalization using the ImageXpress Micro Confocal Microscope (Molecular Devices, San Jose, CA).

Statistical analyses

All data are expressed as the mean ± standard error of the mean (SEM). Statistical analyses were performed using an unpaired Student's *t*-test and a *p*-value < 0.05 was considered to be statistically significant.

RESULTS

Expression of PDK4 is upregulated in ischemia-reperfusion renal injury in diabetic mice

To the best of my knowledge, there has been no evidence indicating the role of PDK in the fundamental response of the kidney to IR injury. Therefore, in this study, the RNA and protein levels of PDK in IR injury were investigated. A diabetic mouse model, in which diabetes was induced by the injection of STZ (50 mg/kg daily for 5 d), was used to evaluate the expression of PDK during IR injury. C57BL/6J mice (9 weeks old) were randomly divided into four study groups as follows: sham controls (n = 6), in which the kidneys were surgically exposed; STZ-induced diabetic mice (n = 7); STZ-induced diabetic mice with IR injury (n = 6), in which the kidneys were exposed and both renal pedicles were clamped for 37 min and reperfused for 24 h; and STZ-induced diabetic mice with IR injury that underwent treatment with DCA (250 mg/kg, i.p) (n = 6). Histopathological assessment of renal tissues revealed remarkable tissue damage with severe tubular damage, lysis, and necrosis after IR injury (**Figure 1A**). The mRNA expression levels of PDK4 were also significantly increased in the group of STZ-induced diabetic mice with IR injury (**Figure 1B**). Further, the protein levels of PDK, as determined by western blotting, followed the same pattern as the mRNA expression of PDK (**Figure 1C**). Moreover, the phosphorylation of PDHE1α, a target protein of PDK, was significantly increased as revealed by immunohistochemical studies of the STZ-induced diabetic mice with IR injury due to the induction of PDK4

expression (**Figure 1D**). Phosphorylation of PDHE1α was enhanced in the STZ-induced diabetic mice with IR injury, as compared to the sham controls or the STZ-induced diabetic mice without IR injury (**Figure 1E**). These results demonstrate that IR injury induces the mRNA expression and increases the protein levels of PDK4 in diabetic mice.

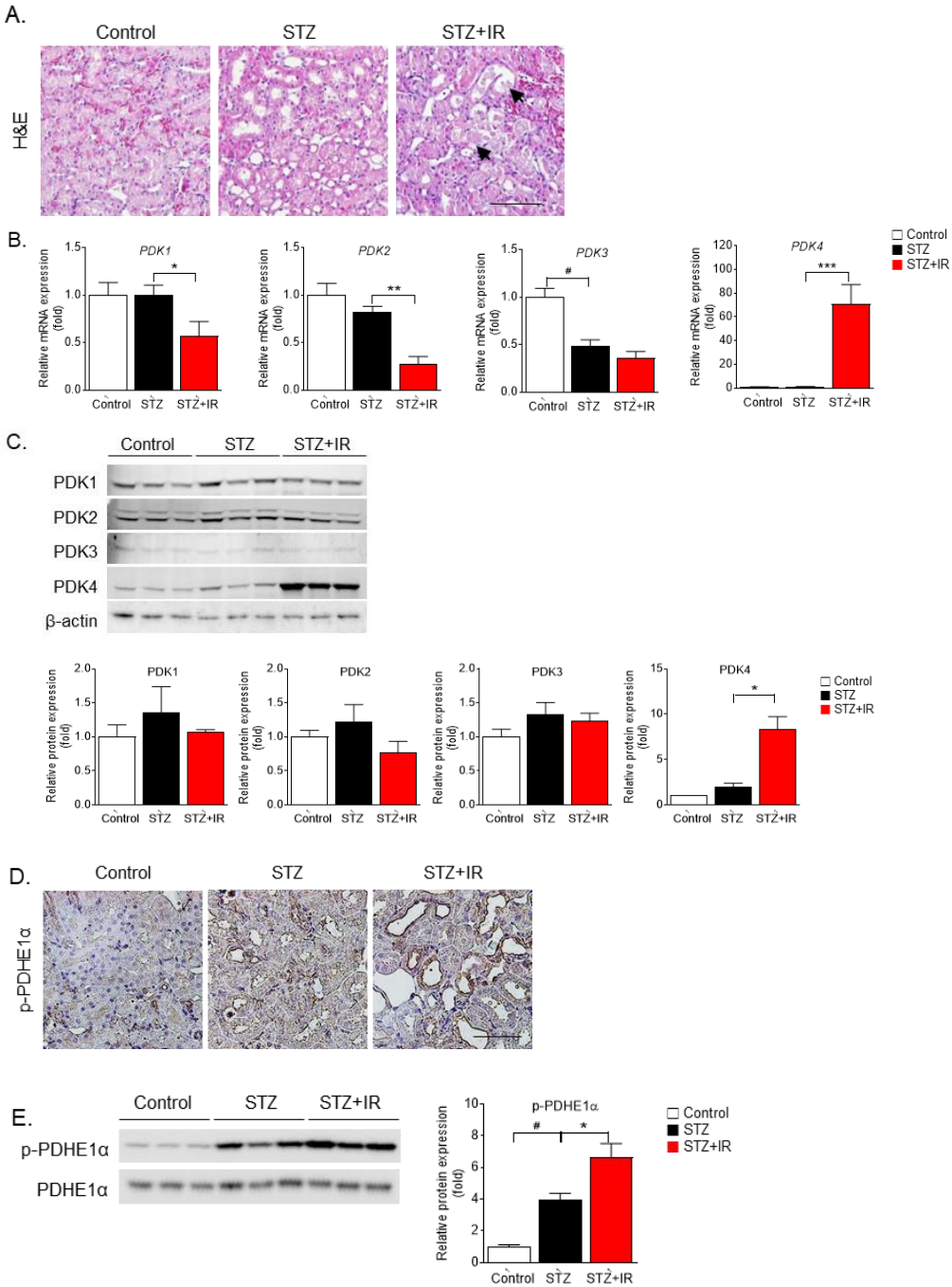


Figure 1. PDK4 is induced in ischemia-reperfusion renal injury in diabetic mice. A. Hematoxylin and eosin staining in mouse kidneys (original magnification = 200×, scale bar: 100 μm, arrow: damaged tubules). B. Relative mRNA

expression of PDK isoforms in mice kidney. C. Protein expression and quantitative graph of PDK isoforms in mice kidney. D. Immunohistochemical image of p-PDHE1α expression in mice kidney (original magnification: 200×, scale bar: 100 μm, arrow: positive regions). E. Protein expression and quantitative graph of p-PDHE1α in mice kidney tissues. $^{\#}p<0.01$ versus Control; $^{*}p<0.05$, $^{**}p<0.001$, $^{***}p<0.01$ versus STZ. Data are the mean ± standard error of the mean (SEM). PDK, pyruvate dehydrogenase kinase; PDHE1α, pyruvate dehydrogenase E1α; STZ, streptozotocin

DCA mitigates IR injury

To determine whether inhibition of PDK attenuates IR injury, I investigated the severity of IR renal injury and the expression levels of PDK isoforms in diabetic mice with IR injury after the administration of DCA *in vivo*. The findings of this study revealed that DCA treatment reduced tubular damage in diabetic mice, leading to less extensive tubular dilatation with cellular lysis and sloughed debris (**Figure 2A**). Compared with the sham-operated control, 37 min of bilateral renal ischemia followed by 24 h of reperfusion resulted in a marked increase in blood urea nitrogen and serum creatinine levels in the STZ-induced diabetic mice (**Figure 2B**). Treatment with DCA, significantly attenuated the IR injury-induced renal dysfunction in the diabetic mice. After five weeks of DCA treatment, no differences in blood glucose levels and body weight were observed between the treated and un-treated diabetic mice groups one day before IR injury (**Figure 3**). IR injury-induced mRNA and protein expressions levels of PDK4 were prominently diminished after DCA treatment (**Figure 2C and 2D**). Along with the decreased expression of PDK4, the phosphorylation of PDHE1α was also alleviated upon DCA treatment (**Figure 2E and 2F**). These results suggest that pretreatment with DCA before IR injury mitigates IR renal injury in diabetic mice.

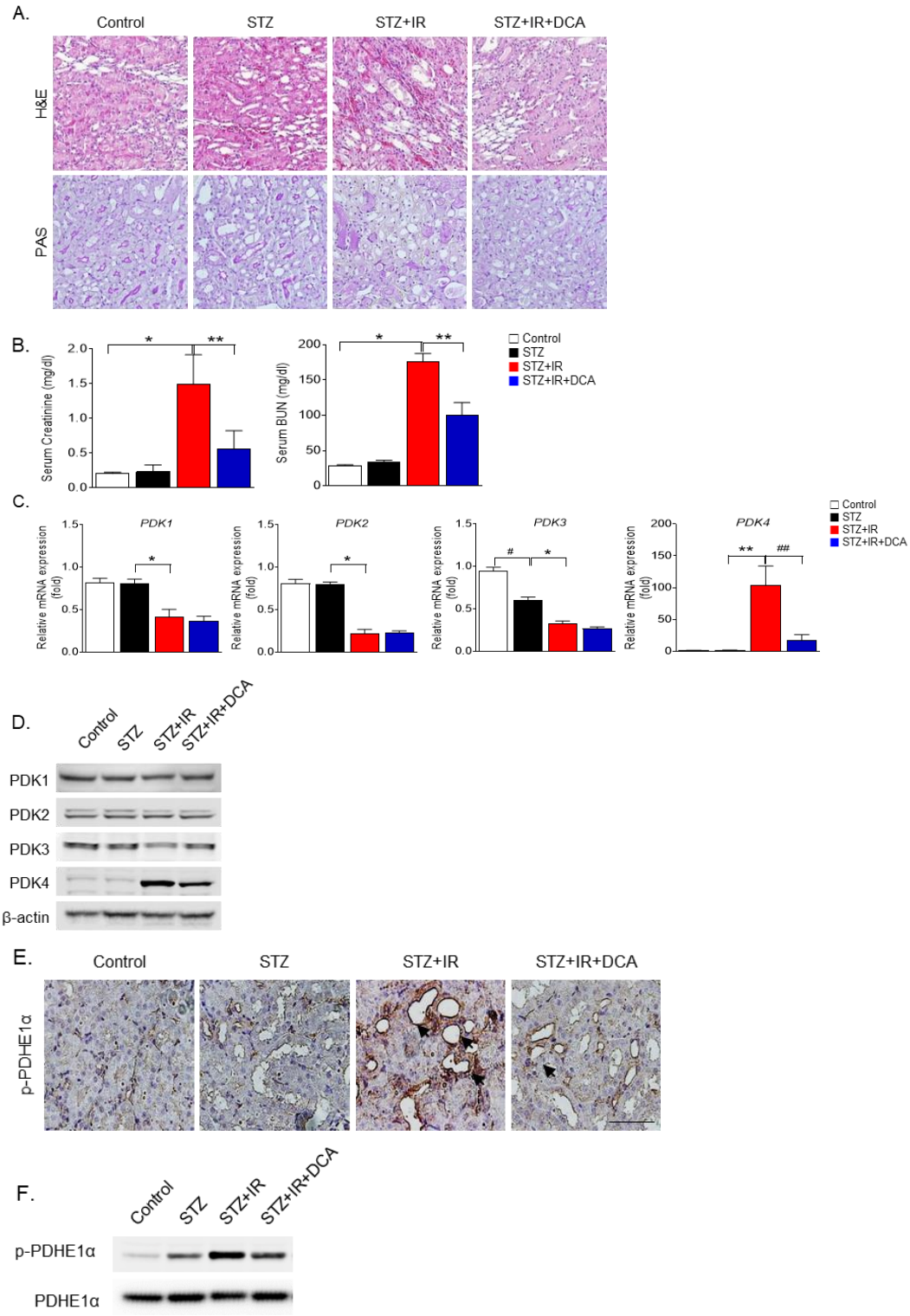

A.
Control
STZ
STZ+IR
STZ+IR+DCA
H&E
PAS
B.
Serum Creatinine (mg/dl)
Serum BUN (mg/dl)
Control
STZ
STZ+IR
STZ+IR+DCA
C.
PDK1
PDK2
PDK3
PDK4
Relative mRNA expression (fold)
D.
Control
STZ
STZ+IR
STZ+IR+DCA
PDK1
PDK2
PDK3
PDK4
β-actin
E.
Control
STZ
STZ+IR
STZ+IR+DCA
p-PDHE1α
F.
Control
STZ
STZ+IR
STZ+IR+DCA
p-PDHE1α
PDHE1α

Figure 2. **DCA mitigates IR injury.** A. Hematoxylin and eosin staining and periodic acid-Schiff staining in mouse kidneys (original magnification = 200×, scale bar: 100 μm, Arrow: damaged tubules). B. Serum creatinine and BUN in mice, $^{*}p<0.01$ versus STZ; $^{**}p<0.01$ versus STZ+IR. C. Relative mRNA expression of PDK isoforms in mice kidney tissues. $^{\#}p<0.01$ versus Control; $^{*}p<0.01$, $^{**}p<0.05$ versus STZ; $^{\#\#}p<0.05$ versus STZ+IR. D. Protein expression of PDK isoforms in mice kidney. E. Immunohistochemical image of p-PDHE1α expression in mice kidney (original magnification: 200×, scale bar: 100 μm, arrow: positive regions). F. Protein expression of p-PDHE1α in mice kidney tissues. Data are the mean ± standard error of the mean (SEM). IR, ischemia-reperfusion; PDK, pyruvate dehydrogenase kinase; PDHE1α, pyruvate dehydrogenase E1α; BUN, blood urea nitrogen; STZ, streptozotocin.

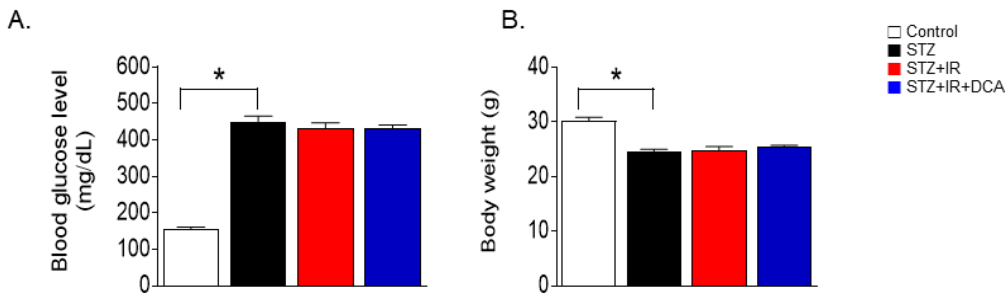


Figure 3. Blood glucose level and body weight are comparable between DCA-treated and untreated diabetic mice groups before IR injury. Blood glucose level (A) and body weight were measured on one day before IR injury. $^{*}p<0.01$ versus Control. Data are the mean ± standard error of the mean (SEM). IR, ischemia-reperfusion.

DCA and PDK knockdown attenuates IR-induced apoptosis of renal cells in diabetic mice

IR renal injury is associated with cellular apoptosis, which might contribute to renal dysfunction. To evaluate the effect of PDK inhibition on the apoptosis of renal cells in IR renal injury model using diabetic mice, I performed TUNEL assay and flow cytometry analysis with Annexin V/PI staining in the mouse primary tubular and NRK-52E cells. To study the mechanism by which PDK contributes to IR injury of diabetic kidney tissue, I established an *in vitro* model using high glucose-conditioned renal proximal tubular cells (NRK-52E) and mouse primary tubular cells, which were incubated in the hypoxia work station for 6 h, followed by reoxygenation for 2 h. A significant induction of apoptosis was observed in the NRK-52E cells with hypoxia-reoxygenation (HR) (**Figure 4A**). The mRNA expression levels of PDK4 in both NRK-52E and mouse primary tubular cells was found to be increased upon HR (**Figure 4B and 4C**). TUNEL assay revealed that the proportion of apoptotic cells were increased in the STZ-induced diabetic mice with IR injury, as compared to those in the mice without IR injury (**Figure 5A**). The cleaved caspase-3 protein levels were markedly increased in the diabetic mice with IR injury as compared to the diabetic mice without IR injury, which decreased further after DCA treatment (**Figure 5B**). The reduction of the number of apoptotic cells and the protein levels of cleaved caspase-3 upon DCA treatment suggests the potential reno-protective effect of PDK inhibition in IR injury. Annexin V/PI staining also showed that HR injury could strongly induce apoptosis and necrosis of NRK-52E cells and that treatment with DCA markedly reduced the degree of cell death (**Figure 5C**). TUNEL assay using mouse primary tubular cells revealed

that the number of apoptotic cells was less in the DCA treated cells than the untreated cells (**Figure 5D**). Furthermore, DCA treatment of both NRK-52E and mouse primary tubular cells showed markedly reduced protein levels of cleaved caspase-3 in a dose-dependent manner, when compared with the untreated cells (**Figure 5E and 5F**). To further verify the role of PDK4 in hypoxic kidney damage, its levels were analyzed in primary tubular cells isolated from the PDK4 knockout mice. It was found that the primary tubular cells from PDK4 knockout mice showed reduced protein levels of cleaved caspase 3, as compared to those in the wild type mice (**Figure 5G**).

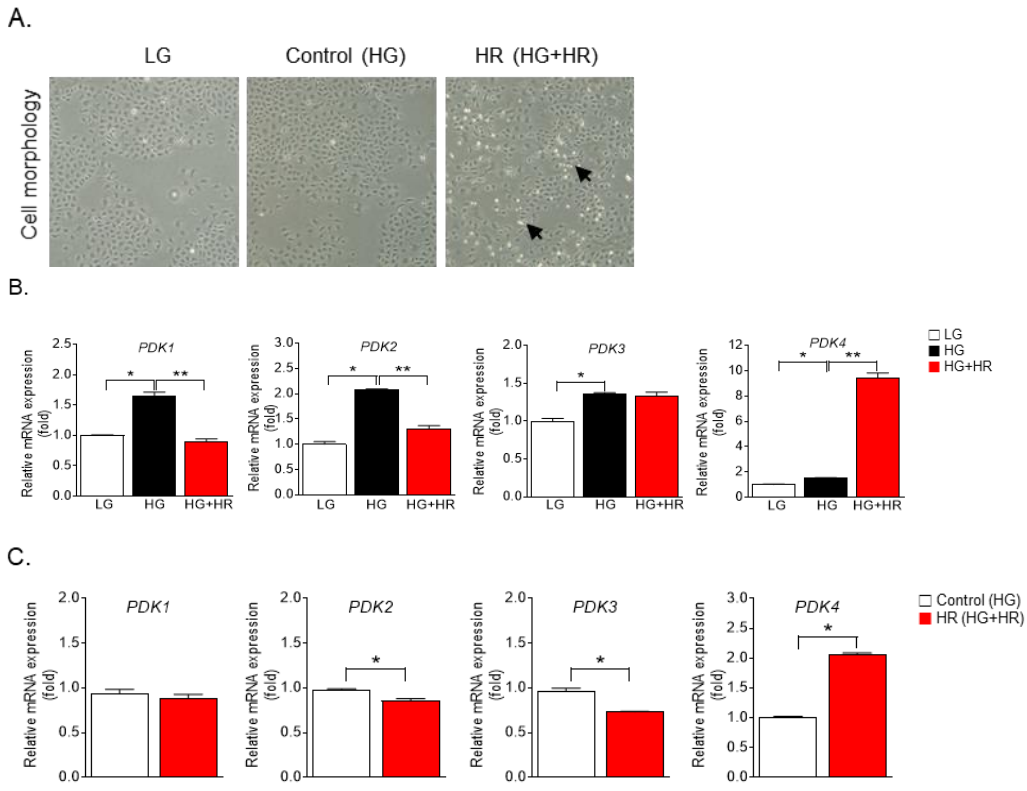
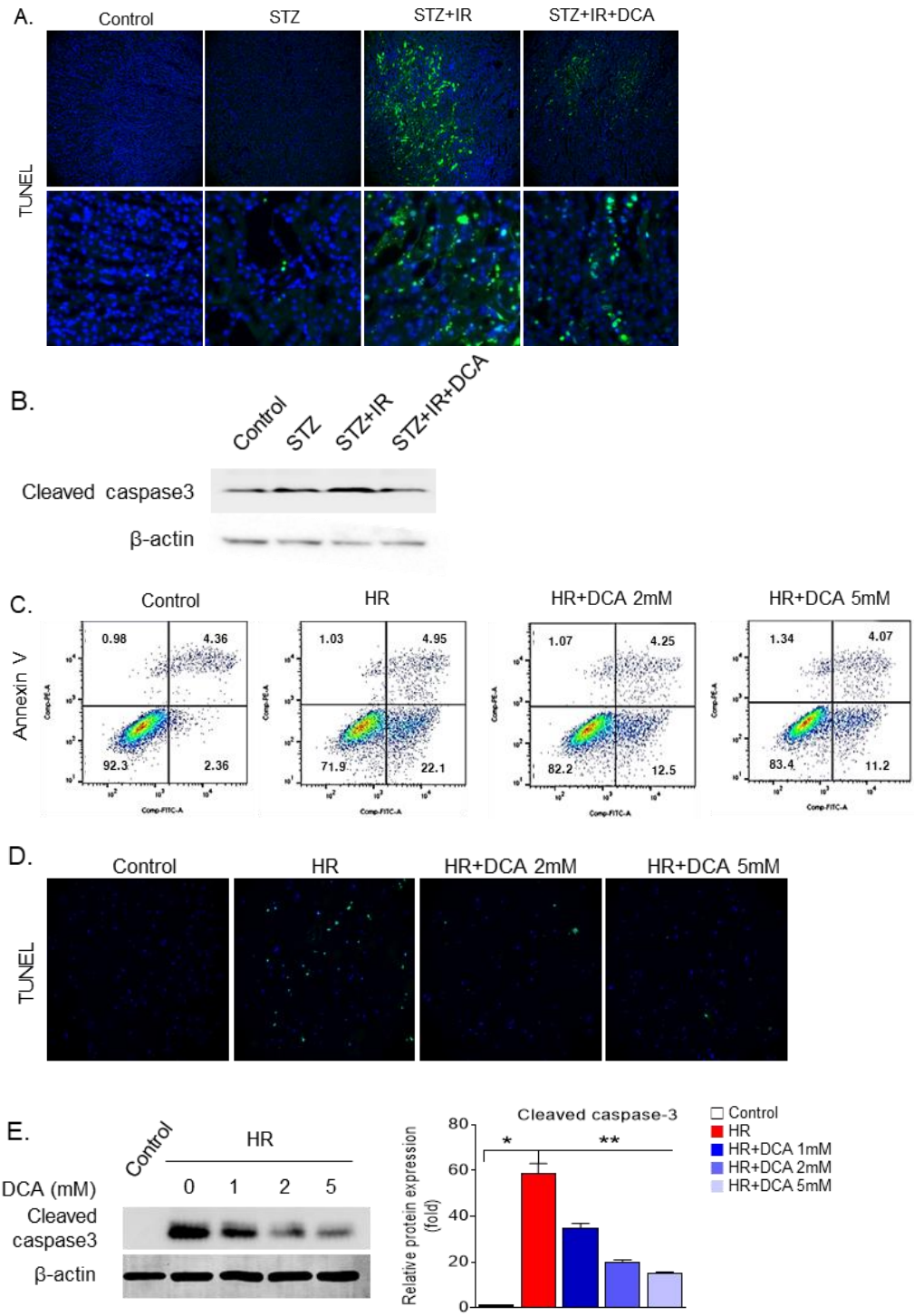


Figure 4. PDK4 is increased in NRK-52E and mouse primary tubular cells after hypoxia-reoxygenation. A. Cell morphology of NRK-52E (original magnification: 40×, arrows: apoptotic cells). B. Relative mRNA expression of PDK isoforms in NRK-52E (hypoxia, 6 h; reoxygenation, 2 h). $^{*}p<0.01$ versus low glucose (LG); $^{**}p<0.01$ versus high glucose (HG). C. Relative mRNA expression of PDK isoforms in mouse primary tubular cells (hypoxia, 4 hours; reoxygenation, 3 hours), $^{*}p<0.01$ versus HR. Data are the mean ± standard error of the mean (SEM). PDK4, pyruvate dehydrogenase kinase 4.


A.
Control
STZ
STZ+IR
STZ+IR+DCA
TUNEL
B.
Control
STZ
STZ+IR
STZ+IR+DCA
Cleaved caspase3
β-actin
C.
Control
HR
HR+DCA 2mM
HR+DCA 5mM
Annexin V
0.98
4.36
92.3
2.36
1.03
4.95
71.9
22.1
1.07
4.25
82.2
12.5
1.34
4.07
83.4
11.2
D.
Control
HR
HR+DCA 2mM
HR+DCA 5mM
TUNEL
E.
Control
HR
DCA (mM)
0
1
2
5
Cleaved caspase3
β-actin
Cleaved caspase-3
Relative protein expression (fold)
80
60
40
20
0
*
**
Control
HR
HR+DCA 1mM
HR+DCA 2mM
HR+DCA 5mM

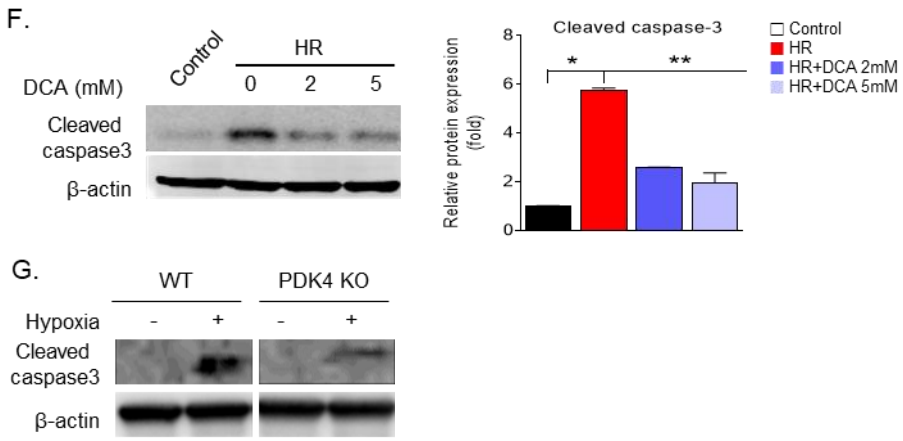


Figure 5. DCA and PDK knockdown attenuates IR-induced apoptosis in mice and NRK-52E cells. A. TUNEL staining in mice kidney (original magnification: 200×, scale bar: 200 μm, arrow: TUNEL positive tissues). B. Protein expression of cleaved caspase-3 in mice. C. Representative Annexin V/PI stain in NRK-52E cells. D. TUNEL staining in mouse primary tubular cells (original magnification: 200×, scale bar: 100 μm, arrow: TUNEL positive cells). Protein expression of cleaved caspase-3 in NRK-52E cells (E) and mouse primary tubule cells (F). G. Protein expression of cleaved caspase-3 in mouse primary tubular cells isolated from WT and PDK4 KO mice. $^{*}p<0.01$ versus Control; $^{**}p<0.01$ versus HR. Data are the mean ± standard error of the mean (SEM). IR, ischemia-reperfusion; PDK, pyruvate dehydrogenase kinase; TUNEL, TdT-mediated dUTP nick end labeling; WT. wild-type; KO, knockout.

DCA attenuates IR-induced oxidative stress and inflammation in diabetic mice and NRK-52E cells

As ROS and inflammation are recognized as essential effectors of IR injury, the production of ROS and inflammatory mediators were investigated in a murine model of IR injury with diabetes [33, 34]. **Figure 6A and 6B** display the expression levels of an oxidative stress marker, 4-hydroxynonenal (4-HNE), and nitrotyrosine in the kidneys in sham control, STZ-induced diabetic mice, STZ-induced diabetic mice with IR injury, and the STZ-induced diabetic mice with DCA and IR injury. The findings indicated that the STZ-induced diabetic mice with IR injury exhibited a marked increase in staining for 4-HNE and nitrotyrosine. Furthermore, DCA treatment for 5 weeks before IR injury resulted in a significant reduction of the 4-HNE and nitrotyrosine burden (**Figure 6A and 6B**). Assessment of inflammatory cytokines revealed that STZ-induced diabetic mice with IR injury exhibited higher expression levels of the tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), interleukin-1β (IL-1β), and the monocyte chemoattractant protein-1 (MCP-1) as compared to those without IR injury; and this effect was reversed upon DCA treatment (**Figure 6C**). Moreover, DCA or shPDK4 treatment of NRK-52E cells showed markedly reduced expression levels of TNF-α, IL-6, IL-1β and MCP-1, when compared with those of untreated cells (**Figure 6D and 6E**). These findings suggest that the blockade of PDK4 exhibits potent reno-protective effects by alleviating the renal oxidative stress and extensive inflammation post-IR injury.

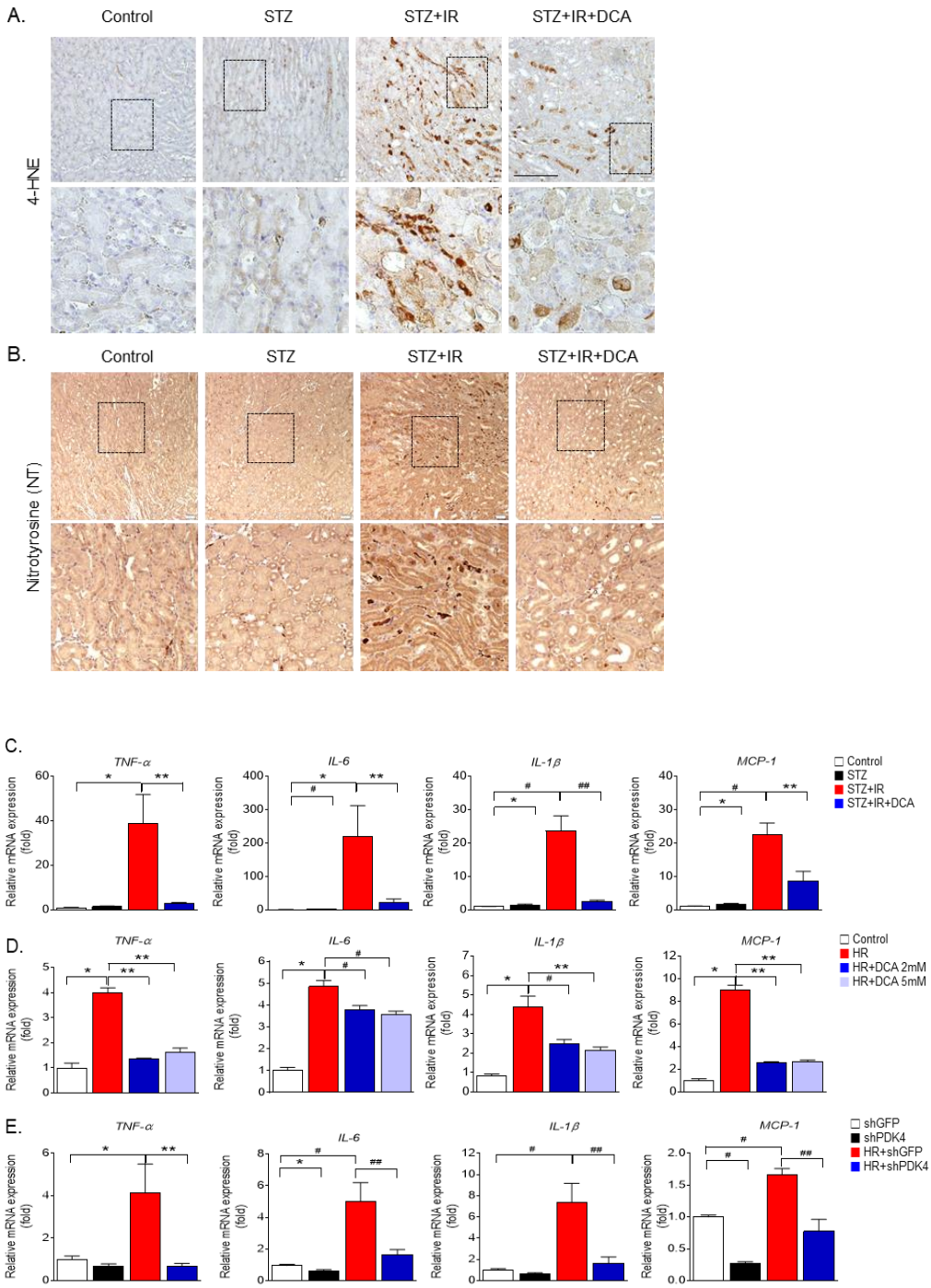


Figure 6. DCA attenuates IR-induced oxidative stress and inflammation in mice and NRK-52E cells. A. 4-HNE staining in mice kidney tissues (original

magnification: 200×, scale bar: 100 μm). B. Nitrotyrosine (NT) staining in mice kidney (original magnification: 200×, scale bar: 100 μm). C. Inflammatory markers in mice. $^{*}p<0.01$ versus Control; $^{\#}p<0.05$, $^{**}p<0.01$, $^{\#\#}p<0.05$ versus STZ+IR. D. Inflammatory markers in NRK-52E with or without DCA, $^{*}p<0.01$ versus Control; $^{**}p<0.01$, $^{\#}p<0.05$ versus HR. E. Inflammatory markers in NRK-52E with shGFP and shPDK4, $^{*}p<0.05$, $^{\#}p<0.01$ versus shGFP; $^{\#\#}p<0.01$, $^{**}p<0.05$, versus HG+shGFP. Data are the mean ± standard error of the mean (SEM). IR, ischemia-reperfusion; PDK, pyruvate dehydrogenase kinase; 4-HNE, 4-hydroxynenenal; STZ, streptozotocin; HR, hypoxia-reoxygenation; HG, high glucose.

DCA attenuates IR-induced mitochondrial dysfunction in kidney injury

As PDK4 is a mitochondrial matrix enzyme, the mitochondrial OCR was measured at 37℃ in an XF24 analyzer in NRK-52E cells for assessment of mitochondrial function. Maximal OCR was substantially increased by DCA or shPDK4 treatment compared to the untreated cells after HR injury (**Figure 7A and 7B**). Additionally, either DCA or shPDK4 treatment revealed a significant improvement in ATP production after HR injury in the NRK-52E cells (**Figure 7A and 7B**). Among the respiratory chain enzymes, the protein expression levels of complex III and V decreased after IR injury and recovered with DCA treatment (**Figure 7C**). These data indicated that inhibition of PDK4 might improve the mitochondrial function in the kidney tissues after IR injury.

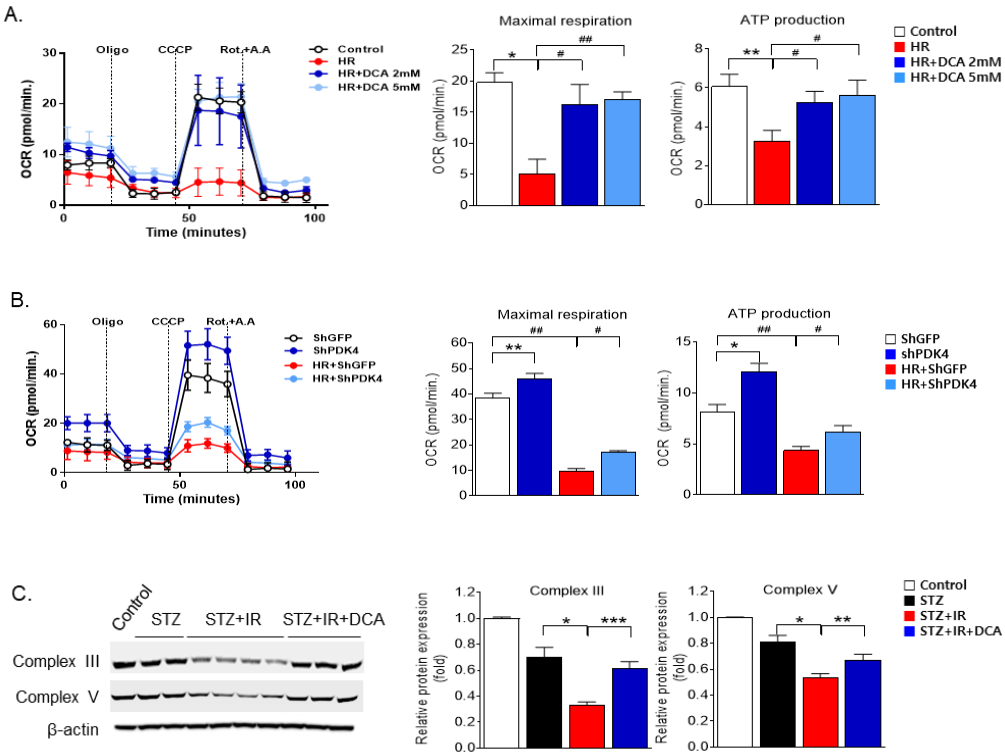


Figure 7. DCA and PDK knockdown attenuates IR-induced mitochondrial dysfunction in mice and NRK-52E cells. A. Mitochondrial oxygen consumption rate in NRK-52E cells with or without DCA. $^{*}p<0.01$, $^{**}p<0.05$ versus Control; $^{\#}p<0.05$, $^{\#\#}p<0.01$ versus HR. B. Mitochondrial oxygen consumption rate in NRK-52E cells with or without shPDK4 (50 MOI). $^{*}p<0.01$, $^{**}p<0.05$ versus shGFP; $^{\#}p<0.05$, $^{\#\#}p<0.01$ versus HR+shGFP. C. Protein expression and quantitative graphs of mitochondrial complex. $^{*}p<0.01$ versus Control; $^{**}p<0.05$, $^{***}p<0.01$ versus STZ+IR. Data are the mean ± standard error of the mean (SEM). DCA, dichloroacetate; PDK, pyruvate dehydrogenase kinase; IR, ischemia-reperfusion; HR, hypoxia-reoxygenation ; STZ, streptozotocin.

DISCUSSION

Mitochondria are complex intracellular organelles exhibiting a variety of important functions. The renal tubule is densely packed with them to power the daily solute transport in the body. The dysfunction of mitochondria is thought to be crucial for the pathogenesis of AKI. IR injury depletes cell energy during ischemia and generates a burst of ROS and inflammatory mediators during reperfusion, which induces downstream tubular cell damage and apoptosis [35]. Intracellular ROS are produced mainly as a result of the incomplete reduction of oxygen by the damaged mitochondria. Despite previous studies reporting positive effects of antioxidants in the animal models of AKI and renal injury, the use of antioxidant therapy in clinical studies has been limited. N-acetylcysteine (NAC) is a glutathione precursor and a representative antioxidant, which acts as an ROS scavenger due to its sulfhydryl group. It has been protective against IR, cisplatin, and rhabdomyolysis-induced AKI in animal models. It ameliorates the decline in kidney function and systemic oxidative stress, increases renal glutathione levels, and reduces renal interstitial inflammation [36-38]. However, it has no effect on urinary isoprostanes, the specific and stable indices of oxidative injury, suggesting that it performs other activities that are either additive or unrelated to its activity as an ROS scavenger [38]. Moreover, NAC had no overall benefit in preventing or treating contrast-induced AKI in humans [39]. Similar to contrast-induced AKI, the administration of NAC before, during, or after cardiac or abdominal aortic surgery to preserve renal function has largely failed to show any benefit [40]. Likewise, previous studies using non-specific antioxidants have failed to show its specific

effects in human AKI. These findings suggest that the agents could not be competently delivered to the appropriate regions of the cell to prevent oxidative stress. As mitochondrial ROS generation upon reperfusion is increasingly recognized as a key contributor to AKI, the appropriate methods of delivering therapeutic agents inside the mitochondria should be developed to attenuate the production of mitochondrial ROS.

There is growing interest in the application of mitochondria-targeted therapies for the prevention or management of AKI [23]. The coenzyme Q10 (CoQ10), which is a component of the electron transport chain and plays specific roles in mitochondrial electron carrier and free radical scavenger, has been shown to prevent mitochondrial dysfunction in humans [41]. MitoQ, a derivative of CoQ10, targets the mitochondria and improves the renal function during various kidney diseases including IR renal injury in animal models [42, 43]. Succinate accumulation in mitochondria during ischemia, which has been reported by several researchers previously, receives attention due to its leading role of ROS production upon reperfusion [44-46]. In recent work, succinate is reported to store electron in the absence of oxygen, which is accumulated during ischemia due to conversion of fumarate by reversal of succinate dehydrogenase (mitochondrial complex II). There is a burst of superoxide produced via the reverse electron transport from succinate dehydrogenase to complex I in the mitochondrial respiratory chain upon reperfusion [22]. Inhibition of succinate dehydrogenase with malonate reduces the production of ROS following reperfusion, which leads to a reduction of infarct size in the isolated hearts of mice [22]. Succinate accumulation can therefore be considered as a universal signature of ischemia and a potential electron source for

the production of ROS upon reperfusion. Valls-Lacalle *et al.* reported that treatment with malonate at initiation of reperfusion reduced the infarct size in the isolated mice hearts mediated via a reduction in the succinate re-oxidation, followed by a reduction in ROS production and attenuation of the mitochondrial permeability during reperfusion [47]. Succinate-mediated ROS production is emerging as a potential mitochondria-targeting strategy for intervention during IR injury.

PDKs phosphorylate and inactivate PDC, which is an inner-mitochondrial-membrane enzyme complex that regulates the entry point of pyruvate into the TCA cycle. They demonstrate tissue-specific abundance and distinctive regulation patterns in mammals. PDK1 is responsible for the Warburg effect in cancer cells, which is associated with cancer metabolism. PDK1 has been reported to be one of the target genes of hypoxia-inducible factor-1 (HIF-1), which is an oxygen-sensing transcription factor, under hypoxic conditions [48, 49]. Activation of glycolytic genes by HIF-1 is considered to be responsible for cellular adaptation to the state of low oxygen concentration via increased conversion of glucose to lactate. HIF-1-dependent induction of PDK1 actively inhibits mitochondrial pyruvate metabolism and respiration in the hypoxic human B lymphocyte cell line [48, 49]. This induces a decrease in the mitochondrial oxygen consumption, thereby resulting in relatively increased concentration of intracellular oxygen. Upregulation of PDK3 by HIF-1 has been observed in cancer cell lines under hypoxic conditions and in proliferative stem cells for metabolic adaptation to build biomass and preserve the redox balance [50-52]. Previous studies have demonstrated the upregulation of PDK4 in several peripheral tissues, including the heart, skeletal muscle, adipose tissues and kidneys

in starved states [26, 53, 54]. PDK4 protein levels are relatively low in the tissues of a well-fed mouse. However, these levels increase under conditions such as fasting, when it is necessary to down-regulate the aerobic glucose oxidation to spare pyruvate for gluconeogenesis [55, 56]. Furthermore, expression of PDK4 is significantly increased in the liver, heart, and skeletal muscles under diabetic conditions and in high inorganic phosphate-treated vascular smooth muscle cells, and cisplatin-injured renal tissues under extremely stressed conditions [30, 32, 53, 57-59]. Oxidative stress, which is mainly caused by the excessive accumulation of ROS, and impaired mitochondrial function play important roles in the pathogenesis of cisplatin-induced AKI [30]. This study demonstrates that PDK4 is also upregulated after IR-induced renal injury and tubular damage that causes cellular apoptosis. Other PDK isoforms show no significant change with IR injury. It was further revealed that the kidney injury and tubular apoptosis were significantly attenuated by reduced production of ROS and inflammation upon inhibition of PDK, associated with a marked decrease in PDK4 expression. PDK4 inhibitor can preserve mitochondrial respiratory capacity with reduced mitochondrial oxidative stress and inflammation during IR injury. These findings indicate that PDK4 is mechanistically linked with mitochondrial dysfunction in the renal tissues under stressful stimuli, such as IR injury or presence of cisplatin. Though the causes of AKI vary widely, the modulation of PDK4 seems to play a critical role in the attenuation of AKI.

AKI is common in diabetes and potentially causes CKD or end-stage kidney disease. Recent large cohort studies have reported that AKI may cause more severe consequences in diabetic patients; resulting in significantly lower rates of recovery,

higher mortality rates, and progression toward more advanced kidney disease, independent of other risk factors [8, 60]. According to KDIGO definition, the odds ratio of mortality was 2.0-, 3.4-, and 10.1-fold in the patients with AKI stages 1, 2, and 3, respectively, compared with those without AKI after complete adjustment of other demographic characteristics and risk factors [10]. The adjusted hazard ratio among the individuals with AKI is 2.67 (95% confidence interval, 1.99–3.58) for new or progressive CKD [14]. Using animal models, Goor *et al.* and Peng *et al.* have already reported the high vulnerability of STZ-induced diabetic rats or mice to IR renal injury [61, 62]. Ongoing studies are investigating mechanisms underlying accelerated kidney injury in diabetes mellitus with AKI. A preclinical study evaluating the long-term effects of acute renal ischemic injury in obese diabetic rats has revealed that post-ischemic inflammation is a crucial factor in the acceleration of CKD in obesity/diabetes [63]. Moreover, Gao *et al.* have documented that a TNF-α neutralizing antibody can decrease the susceptibility to IR renal injury in diabetic mice, thereby providing evidence that supports the role of the inflammatory response involving TNF-α [64]. The present study showed that IR enhances the expression levels of PDK4 in the kidneys of a diabetic mouse model and increases oxidative stress and inflammation-induced kidney injury. The mRNA expression of TNF-α was markedly increased after renal IR, which indicates that TNF-α plays a possible role as a mediator in enhancing the susceptibility of diabetic mice to IR injury. DCA, a PDK inhibitor, noticeably alleviates the IR injury-induced expression of TNF-α. IR injury also increases the mRNA expression of MCP-1, which has been associated with the development of tissue fibrosis. MCP-1 is a chemokine that activates monocytes and macrophages

and mediates the tubulointerstitial inflammation that precedes fibrosis [65, 66]. MCP-1 stimulates the expression of IL-6, cell adhesion molecules, and other inflammatory factors that contribute to renal tubular fibrosis [67]. Inhibition of PDK4 attenuates the increased expression of MCP-1 following IR injury. These data indicate that the expression levels of TNF-α, IL-6, IL-1β, and MCP-1 increase with enhanced expression of PDK4 upon IR injury. Furthermore, a decrease in the expression levels of inflammatory or fibrosis markers after PDK inhibition, supports the fact that PDK4 and mitochondria play critical roles in causing the hypoxia-reoxygenation damage in diabetic mice.

Although this study presented crucial information on the role of PDK4 in IR injury, it has certain limitations. First, we did not compare the severity of IR injury between control mice and the STZ-induced diabetic mice with IR injury. As the vulnerability of animals or patients with diabetes to AKI caused by various factors is already well reported, I specifically examined the role of PDK4 in renal IR injury of diabetic mice in this study. Second, the more specific and detailed signaling pathways, as well as related mediators responsible for the increased expression of PDK4 in the kidney after IR injury were not investigated in this study. To delineate the upstream mechanism of increased PDK4 expression in the kidney with IR injury, detailed molecular studies should be performed. Third, as PDK4 inhibitor was only administered prior to IR injury, it is unclear whether post-administration of the PDK4 inhibitor has any beneficial effects in IR injury. Most cases of AKI are not predictable in the clinical environment. Therefore, the administration of PDK4 inhibitor in advance may be useful in AKI-prone conditions, such as sepsis and critical illness. Finally, due to species differences

between mice and humans, these results need to be interpreted with due caution. Although many issues remained unsolved, these findings still provide a potential strategy for preventing AKI.

In summary, the findings of this study indicate that IR injury enhances the expression of PDK4 in the kidney of the diabetic mouse model. PDK4 inhibition mitigates kidney injury by decreasing the ROS production and inflammation, suggesting a critical role for PDK4 in causing IR damage. Therefore, inhibition of PDK4 may prove to be an effective target for reno-protection during IR injury. The roles of PDK4 need to be further elucidated and established in terms of mitochondrial functions and dynamics in renal IR injury.

REFERENCE

1. Hong, Y.A., T.H. Ban, C.-Y. Kang, S.D. Hwang, S.R. Choi, H. Lee, et al., Trends in epidemiologic characteristics of end-stage renal disease from 2019 Korean Renal Data System (KORDS). Kidney research and clinical practice 2021; **40**: 52.
2. Bienholz, A., B. Wilde, and A. Kribben, From the nephrologist's point of view: diversity of causes and clinical features of acute kidney injury. Clinical kidney journal 2015; **8**: 405-14.
3. Singh, D.K., P. Winocour, and K. Farrington, Mechanisms of disease: the hypoxic tubular hypothesis of diabetic nephropathy. Nature Clinical Practice Nephrology 2008; **4**: 216-26.
4. Schiffer, T.A. and M. Friederich-Persson, Mitochondrial reactive oxygen species and kidney hypoxia in the development of diabetic nephropathy. Frontiers in physiology 2017; **8**: 211.
5. Girman, C., T. Kou, K. Brodovicz, C. Alexander, E. O'Neill, S. Engel, et al., Risk of acute renal failure in patients with type 2 diabetes mellitus. Diabetic Medicine 2012; **29**: 614-21.
6. Peng, J., X. Li, D. Zhang, J.-K. Chen, Y. Su, S.B. Smith, et al., Hyperglycemia, p53, and mitochondrial pathway of apoptosis are

involved in the susceptibility of diabetic models to ischemic acute kidney injury. Kidney international 2015; **87**: 137-50.

7. Melin, J., O. Hellberg, L.M. Akyürek, Ö. Källskog, E. Larsson, and B.C. Fellström, Ischemia causes rapidly progressive nephropathy in the diabetic rat. Kidney international 1997; **52**: 985-91.

8. Thakar, C.V., A. Christianson, J. Himmelfarb, and A.C. Leonard, Acute kidney injury episodes and chronic kidney disease risk in diabetes mellitus. Clin J Am Soc Nephrol 2011; **6**: 2567-72.

9. Kellum, J.A., N. Lameire, P. Aspelin, R.S. Barsoum, E.A. Burdmann, S.L. Goldstein, et al., Kidney disease: improving global outcomes (KDIGO) acute kidney injury work group. KDIGO clinical practice guideline for acute kidney injury. Kidney international supplements 2012; **2**: 1-138.

10. Zeng, X., G.M. McMahon, S.M. Brunelli, D.W. Bates, and S.S. Waikar, Incidence, outcomes, and comparisons across definitions of AKI in hospitalized individuals. Clinical Journal of the American Society of Nephrology 2014; **9**: 12-20.

11. Hoste, E.A., G. Clermont, A. Kersten, R. Venkataraman, D.C. Angus, D. De Bacquer, et al., RIFLE criteria for acute kidney injury are associated with hospital mortality in critically ill patients: a cohort analysis. Critical care 2006; **10**: R73.

12. Uchino, S., R. Bellomo, D. Goldsmith, S. Bates, and C. Ronco, An assessment of the RIFLE criteria for acute renal failure in hospitalized patients. Critical care medicine 2006; **34**: 1913-17.

13. Bellomo, R., J.A. Kellum, and C. Ronco, Acute kidney injury. The Lancet 2012; **380**: 756-66.

14. See, E.J., K. Jayasinghe, N. Glassford, M. Bailey, D.W. Johnson, K.R. Polkinghorne, et al., Long-term risk of adverse outcomes after acute kidney injury: a systematic review and meta-analysis of cohort studies using consensus definitions of exposure. Kidney international 2019; **95**: 160-72.

15. Eltzschig, H.K. and T. Eckle, Ischemia and reperfusion—from mechanism to translation. Nature medicine 2011; **17**: 1391-401.

16. Hausenloy, D.J. and D.M. Yellon, Myocardial ischemia-reperfusion injury: a neglected therapeutic target. The Journal of clinical investigation 2013; **123**: 92-100.

17. Cavaille-Coll, M., S. Bala, E. Velidedeoglu, A. Hernandez, P. Archdeacon, G. Gonzalez, et al., Summary of FDA workshop on ischemia reperfusion injury in kidney transplantation. American journal of transplantation 2013; **13**: 1134-48.

18. Lefer, D.J. and R. Bolli, Development of an NIH consortium for

preclinicAl AssESsment of CARdioprotective therapies (CAESAR): a paradigm shift in studies of infarct size limitation. Journal of cardiovascular pharmacology and therapeutics 2011; **16**: 332-39.

19. Braunwald, E. and R.A. Kloner, Myocardial reperfusion: a double-edged sword? The Journal of clinical investigation 1985; **76**: 1713-19.

20. Hearse, D.J., S.M. Humphrey, and G.R. Bullock, The oxygen paradox and the calcium paradox: two facets of the same problem? Journal of molecular and cellular cardiology 1978; **10**: 641-68.

21. Raedschelders, K., D.M. Ansley, and D.D. Chen, The cellular and molecular origin of reactive oxygen species generation during myocardial ischemia and reperfusion. Pharmacology & therapeutics 2012; **133**: 230-55.

22. Chouchani, E.T., V.R. Pell, E. Gaude, D. Aksentijević, S.Y. Sundier, E.L. Robb, et al., Ischaemic accumulation of succinate controls reperfusion injury through mitochondrial ROS. Nature 2014; **515**: 431-35.

23. Hall, A.M. and C.D. Schuh, Mitochondria as therapeutic targets in acute kidney injury. Current Opinion in Nephrology and Hypertension 2016; **25**: 355-62.

24. Jeoung, N.H., Pyruvate Dehydrogenase Kinases: Therapeutic Targets

for Diabetes and Cancers. Diabetes Metab J 2015; **39**: 188-97.

25. Sugden, M.C. and M.J. Holness, Recent advances in mechanisms regulating glucose oxidation at the level of the pyruvate dehydrogenase complex by PDKs. Am J Physiol Endocrinol Metab 2003; **284**: E855-62.

26. Wu, P., J. Sato, Y. Zhao, J. Jaskiewicz, K.M. Popov, and R.A. Harris, Starvation and diabetes increase the amount of pyruvate dehydrogenase kinase isoenzyme 4 in rat heart. Biochem J 1998; **329 (Pt 1)**: 197-201.

27. Popov, K.M., J.W. Hawes, and R.A. Harris, Mitochondrial alpha-ketoacid dehydrogenase kinases: a new family of protein kinases. Advances in second messenger and phosphoprotein research 1997; **31**: 105-11.

28. Chouchani, E.T., V.R. Pell, A.M. James, L.M. Work, K. Saeb-Parsy, C. Frezza, et al., A unifying mechanism for mitochondrial superoxide production during ischemia-reperfusion injury. Cell metabolism 2016; **23**: 254-63.

29. Padanilam, B.J., Cell death induced by acute renal injury: a perspective on the contributions of apoptosis and necrosis. American Journal of Physiology-Renal Physiology 2003; **284**: F608-F27.

30. Oh, C.J., C.-M. Ha, Y.-K. Choi, S. Park, M.S. Choe, N.H. Jeoung, et

al., Pyruvate dehydrogenase kinase 4 deficiency attenuates cisplatin-induced acute kidney injury. Kidney international 2017; **91**: 880-95.

31. Jeoung, N.H. and R.A. Harris, Pyruvate dehydrogenase kinase-4 deficiency lowers blood glucose and improves glucose tolerance in diet-induced obese mice. American Journal of Physiology-Endocrinology and Metabolism 2008; **295**: E46-E54.

32. Park, B.-Y., J.-H. Jeon, Y. Go, H.J. Ham, J.-E. Kim, E.K. Yoo, et al., PDK4 Deficiency suppresses hepatic glucagon signaling by decreasing cAMP levels. Diabetes 2018; **67**: 2054-68.

33. Bonventre, J.V. and A. Zuk, Ischemic acute renal failure: an inflammatory disease? Kidney international 2004; **66**: 480-85.

34. Okusa, M.D., The inflammatory cascade in acute ischemic renal failure. Nephron 2002; **90**: 133-38.

35. Soares, R.O.S., D.M. Losada, M.C. Jordani, P. Évora, and E.S.O. Castro, Ischemia/Reperfusion Injury Revisited: An Overview of the Latest Pharmacological Strategies. Int J Mol Sci 2019; **20**.

36. Panizo, N., A. Rubio-Navarro, J.M. Amaro-Villalobos, J. Egido, and J.A. Moreno, Molecular Mechanisms and Novel Therapeutic Approaches to Rhabdomyolysis-Induced Acute Kidney Injury. Kidney Blood Press Res 2015; **40**: 520-32.

37. Luo, J., T. Tsuji, H. Yasuda, Y. Sun, Y. Fujigaki, and A. Hishida, The

molecular mechanisms of the attenuation of cisplatin-induced acute renal failure by N-acetylcysteine in rats. Nephrology Dialysis Transplantation 2008; **23**: 2198-205.

38. Nitescu, N., S.-E. Ricksten, N. Marcussen, B. Haraldsson, U. Nilsson, S. Basu, et al., N-acetylcysteine attenuates kidney injury in rats subjected to renal ischaemia-reperfusion. Nephrology dialysis transplantation 2006; **21**: 1240-47.

39. Chalikias, G., I. Drosos, and D.N. Tziakas, Prevention of contrast-induced acute kidney injury: an update. Cardiovascular drugs and therapy 2016; **30**: 515-24.

40. Koyner, J.L., R.S. Ali, and P.T. Murray, Antioxidants. Nephron Experimental Nephrology 2008; **109**: e109-e17.

41. Garrido-Maraver, J., M.D. Cordero, M. Oropesa-Avila, A.F. Vega, M. de la Mata, A.D. Pavon, et al., Clinical applications of coenzyme Q10. Front Biosci (Landmark Ed) 2014; **19**: 619-33.

42. Ratliff, B.B., W. Abdulmahdi, R. Pawar, and M.S. Wolin, Oxidant mechanisms in renal injury and disease. Antioxidants & redox signaling 2016; **25**: 119-46.

43. Kizilgun, M., Y. Poyrazoglu, Y. Oztas, H. Yaman, E. Cakir, T. Cayci, et al., Beneficial effects of N-acetylcysteine and ebselen on renal ischemia/reperfusion injury. Renal failure 2011; **33**: 512-17.

44. Taegtmeyer, H., Metabolic responses to cardiac hypoxia. Increased production of succinate by rabbit papillary muscles. Circulation research 1978; **43**: 808-15.

45. Hohl, C., R. Oestreich, P. Rösen, R. Wiesner, and M. Grieshaber, Evidence for succinate production by reduction of fumarate during hypoxia in isolated adult rat heart cells. Archives of biochemistry and biophysics 1987; **259**: 527-35.

46. Ashrafian, H., G. Czibik, M. Bellahcene, D. Aksentijević, A.C. Smith, S.J. Mitchell, et al., Fumarate is cardioprotective via activation of the Nrf2 antioxidant pathway. Cell metabolism 2012; **15**: 361-71.

47. Valls-Lacalle, L., I. Barba, E. Miró-Casas, J.J. Alburquerque-Béjar, M. Ruiz-Meana, M. Fuertes-Agudo, et al., Succinate dehydrogenase inhibition with malonate during reperfusion reduces infarct size by preventing mitochondrial permeability transition. Cardiovascular research 2016; **109**: 374-84.

48. Kim, J.W., I. Tchernyshyov, G.L. Semenza, and C.V. Dang, HIF-1-mediated expression of pyruvate dehydrogenase kinase: a metabolic switch required for cellular adaptation to hypoxia. Cell Metab 2006; **3**: 177-85.

49. Papandreou, I., R.A. Cairns, L. Fontana, A.L. Lim, and N.C. Denko, HIF-1 mediates adaptation to hypoxia by actively downregulating

mitochondrial oxygen consumption. Cell Metab 2006; **3**: 187-97.

50. Lu, C.-W., S.-C. Lin, K.-F. Chen, Y.-Y. Lai, and S.-J. Tsai, Induction of pyruvate dehydrogenase kinase-3 by hypoxia-inducible factor-1 promotes metabolic switch and drug resistance. Journal of Biological Chemistry 2008; **283**: 28106-14.

51. Prigione, A., N. Rohwer, S. Hoffmann, B. Mlody, K. Drews, R. Bukowiecki, et al., HIF1α modulates cell fate reprogramming through early glycolytic shift and upregulation of PDK1–3 and PKM2. Stem cells 2014; **32**: 364-76.

52. Vander Heiden, M.G., L.C. Cantley, and C.B. Thompson, Understanding the Warburg effect: the metabolic requirements of cell proliferation. Science 2009; **324**: 1029-33.

53. Wu, P., P.V. Blair, J. Sato, J. Jaskiewicz, K.M. Popov, and R.A. Harris, Starvation increases the amount of pyruvate dehydrogenase kinase in several mammalian tissues. Arch Biochem Biophys 2000; **381**: 1-7.

54. Jeoung, N.H., P. Wu, M.A. Joshi, J. Jaskiewicz, C.B. Bock, A.A. Depaoli-Roach, et al., Role of pyruvate dehydrogenase kinase isoenzyme 4 (PDHK4) in glucose homoeostasis during starvation. Biochem J 2006; **397**: 417-25.

55. Klyuyeva, A., A. Tuganova, N. Kedishvili, and K.M. Popov, Tissue-

specific kinase expression and activity regulate flux through the pyruvate dehydrogenase complex. J Biol Chem 2019; **294**: 838-51.

56. Sugden, M.C. and M.J. Holness, Mechanisms underlying regulation of the expression and activities of the mammalian pyruvate dehydrogenase kinases. Arch Physiol Biochem 2006; **112**: 139-49.

57. Harris, R.A., B. Huang, and P. Wu, Control of pyruvate dehydrogenase kinase gene expression. Adv Enzyme Regul 2001; **41**: 269-88.

58. Go, Y., J.Y. Jeong, N.H. Jeoung, J.H. Jeon, B.Y. Park, H.J. Kang, et al., Inhibition of Pyruvate Dehydrogenase Kinase 2 Protects Against Hepatic Steatosis Through Modulation of Tricarboxylic Acid Cycle Anaplerosis and Ketogenesis. Diabetes 2016; **65**: 2876-87.

59. Lee, S.J., J.Y. Jeong, C.J. Oh, S. Park, J.Y. Kim, H.J. Kim, et al., Pyruvate Dehydrogenase Kinase 4 Promotes Vascular Calcification via SMAD1/5/8 Phosphorylation. Sci Rep 2015; **5**: 16577.

60. Hsu, C.Y., G.M. Chertow, C.E. McCulloch, D. Fan, J.D. Ordoñez, and A.S. Go, Nonrecovery of kidney function and death after acute on chronic renal failure. Clin J Am Soc Nephrol 2009; **4**: 891-8.

61. Goor, Y., G. Peer, A. Iaina, M. Blum, Y. Wollman, T. Chernihovsky, et al., Nitric oxide in ischaemic acute renal failure of streptozotocin diabetic rats. Diabetologia 1996; **39**: 1036-40.

62. Peng, J., X. Li, D. Zhang, J.K. Chen, Y. Su, S.B. Smith, et al., Hyperglycemia, p53, and mitochondrial pathway of apoptosis are involved in the susceptibility of diabetic models to ischemic acute kidney injury. Kidney Int 2015; **87**: 137-50.

63. Kelly, K.J., J.L. Burford, and J.H. Dominguez, Postischemic inflammatory syndrome: a critical mechanism of progression in diabetic nephropathy. American Journal of Physiology-Renal Physiology 2009; **297**: F923-F31.

64. Gao, G., B. Zhang, G. Ramesh, D. Betterly, R.K. Tadagavadi, W. Wang, et al., TNF-α mediates increased susceptibility to ischemic AKI in diabetes. American Journal of Physiology-Renal Physiology 2013; **304**: F515-F21.

65. Wynn, T.A., Cellular and molecular mechanisms of fibrosis. J Pathol 2008; **214**: 199-210.

66. Lloyd, C.M., A.W. Minto, M.E. Dorf, A. Proudfoot, T.N. Wells, D.J. Salant, et al., RANTES and monocyte chemoattractant protein-1 (MCP-1) play an important role in the inflammatory phase of crescentic nephritis, but only MCP-1 is involved in crescent formation and interstitial fibrosis. J Exp Med 1997; **185**: 1371-80.

67. Schwarz, M., M. Wahl, K. Resch, and H.H. Radeke, IFNgamma induces functional chemokine receptor expression in human

mesangial cells. Clin Exp Immunol 2002; **128**: 285-94.

요약 (국문 초록)

Dichloroacetate가 신장 허혈-재관류 손상으로 인한 산화스트레스와 염증 발생에 미치는 예방적 효과

서울대학교 대학원
의학과 중개의학 전공
강 아 름

배경 당뇨병성 신장 질환은 만성 질환의 주요한 원인이며, 급성 신장 손상의 발생을 유도하는 허혈성 손상에 대한 민감도가 증가하는 것으로 알려져 있다. 반응성 산소종을 생성하는 중요한 세포내 소기관인 미토콘드리아는 급성 신장 손상의 주된 이유인 허혈-재관류 손상의 발병 기전에 핵심적인 역할을 하는 것으로 보고되었다. 이 연구는 미토콘드리아 기질 효소 중 하나인 pyruvate dehydrogenase kinase 4 (PDK4) 가 허혈-재관류 급성 신장 손상 후 발생하는 반응성 산소종과 염증에 끼치는 영향을 streptozotocin-유발 당뇨병 쥐에서 조사하고자 한다.

방법 9주령의 수컷 C57BL/6J 쥐에 streptozotocin 50 mg/kg 를 5일 연속으로 복강내 주사하여 당뇨병 동물 모델을 확립하였다. 고혈당이 확인된

25 마리의 C57BL/6J 쥐를 아래와 같이 분류하였다. 대조군 (6마리), STZ 유발 당뇨병 쥐군 (7마리), STZ 유발 당뇨병 쥐 중 허혈-재관류 손상을 받은 군 (6마리), 그리고 허혈-재관류 손상을 받기 전 dichloroacetate (DCA)를 5주간 250 mg/kg 로 복강 내 주사한 군 (6마리)으로 나누어 PDK4 의 mRNA 및 단백질 발현 정도를 비교하였다. 허혈-재관류 손상은 양쪽 신장의 혈류를 죔쇠로 37분간 고정한 후 다시 죔쇠를 풀고 24시간 동안 재관류하여 허혈-재관류 신손상 모델을 구축하였다. 저산소실 (hypoxia work station)을 이용하여 NRK-52E 세포와 배양한 일차 쥐신세뇨관세포 (mouse primary tubular cells)에 허혈-재관류 손상을 주어, 신장 세포의 세포사멸사, 반응성 산소종의 생성, 그리고 염증 지표를 조사 및 비교하였다.

결과 당뇨병 쥐의 신장 허혈-재관류 손상 후 다른 PDK 동형단백질은 증가하지 않았으나, PDK4는 뚜렷하게 증가하였고, PDK4의 표적 단백질인 pyruvate dehydrogenase E1α (PDHE1α)의 인산화도 유의미하게 증가하였다. 이와 함께 당뇨병 쥐의 신장 조직 및 NRK-52E 세포에서 신장 허혈-재관류 손상 후 반응성 산소종의 증가, 종양괴사인자-α (tumor necrosis factor, TNF-α), 인터루킨-6 (interleukin-6, IL-6), 인터루킨-1β (interleukin-1β, IL-1β) 그리고 단핵구화학주성단백-1 (monocyte chemoattractant protein-1, MCP-1)의 유도가 동반되었다. PDK 억제제인 DCA 치료시 PDK4 단백질 발현 및 PDHE1α의 인산화가 뚜렷이 감소하였으며, 신장 허혈-재관류 손상 유도 cleaved caspase-3 가 DCA 의 용량 의존적으로 감소하며 세포 사멸 또한

감소됨이 관찰되었다. DCA 혹은 small hairpin PDK4 (shPDK4)를 사용한 PDK4 의 억제는 허혈-재관류 손상 후 반응성 산소종의 생성 증가를 약화시켰으며 염증 지표인 TNF-α, IL-6, IL-1β 그리고 MCP-1을 감소시켰음을 확인할 수 있었다. 이에 더하여 DCA 혹은 shPDK4 의 사용은 신장 허혈-재관류 손상으로 인한 미토콘드리아의 최대 산소소비량 (oxygen consumption rate)을 의미 있게 증가시키고, 전자전달계의 complex III 및 complex V 의 단백질 발현을 증가시켜, ATP의 생성을 개선시키는 것을 확인하였다. 이러한 실험 결과는 PDK4 관련 반응성 산소종의 생성, 염증 유발, 그리고 미토콘드리아의 기능 이상이 허혈-재관류로 인해 발생하는 신장 손상에 중요한 역할을 함을 의미한다.

결론 PDK4 억제시 신장 허혈-재관류 손상으로 발생하는 반응성 산소종의 생산 및 염증 유도를 감소시켜 결과적으로 미토콘드리아 기능 이상을 개선시킴으로써 신장 손상의 정도를 경감시킬 수 있었다. 이를 통해서 신장 허혈-재관류 손상에서 PDK4가 결정적인 역할을 하고 있음을 확인할 수 있었다. PDK4는 허혈-재관류로 인한 급성 신장 손상에서 신장 기능을 보존하는 데 활용할 수 있는 중요한 표적이 되므로 추후 연구를 통해 PDK4의 억제가 급성 신장 손상에서 발생하는 미토콘드리아 기능 및 역학적 변화에 어떠한 영향을 미치는지에 대해 살펴볼 필요가 있다.

주요어 : 허혈, 재관류, 급성신장손상, 피루베이트 탈수소효소 키나아제4, 디클로로아세트산, 반응성 산소종, 염증

학 번 : 2013-31152

Acknowledgement

Parts of this thesis accepted in 2021 Diabetes and Metabolism Journal as an original article and will be published near future.

I wishes to express my sincerest gratitude and warm appreciation to the following persons who had contributed much in helping me complete this valuable piece of work.

Professor In Kyu Lee for supporting my graduation thesis and completing my work with the greatest care.

Professor Sung Hee Choi for warm encouragement and valuable advice.

Ph.D Chang Joo Oh, Dong Hoon Kim and **Professor Jae-Han Jeon** to help scientific approach and experiments for my work.

My parents, **Mr. Ki yong Kang and Mrs. Jeong ok Jo**, for supporting me with love and understanding.

My parents-in-law, **Mr. Dong Min Lee and Mrs. Young Ja Kim**, for an important inspiration and endless support.

Mr. Hong yeul Lee, for his constant encouragement throughout my research period.

Lovely, Jun seong and Ye seong, for making me feel alive and being the meaning of my life.

In addition, I would like to thank all of the researchers on whose work I was able to build this work.