Oxidative Medicine and Cellular Longevity

Oxidative stress in end-stage renal disease: pathophysiology and potential interventions

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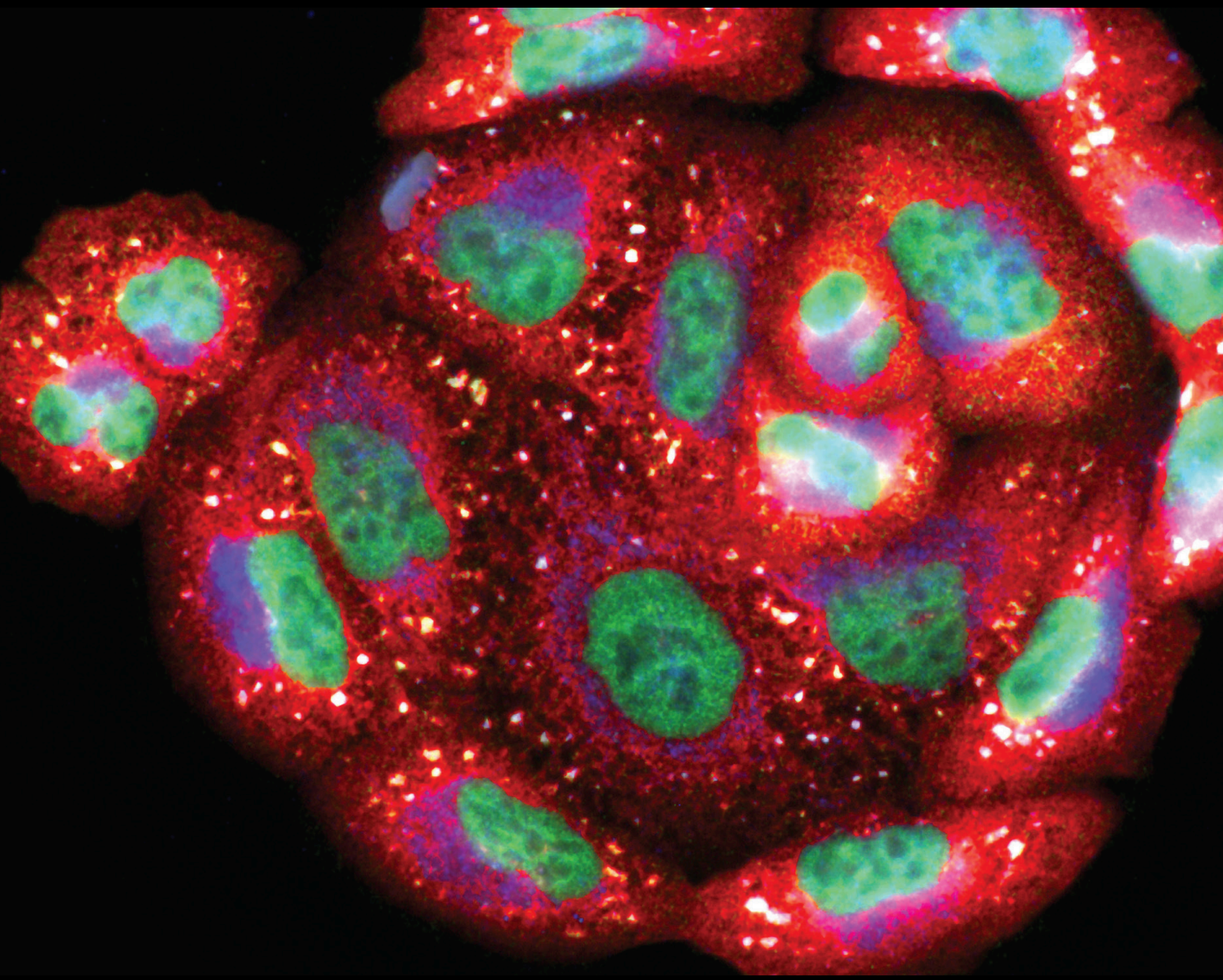
Guest Editors: Vassilios Liakopoulos, Evangelia Dounousi, and Patrick B. Mark



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Editorial

Oxidative Stress in End-Stage Renal Disease: Pathophysiology and Potential Interventions

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Chronic kidney disease (CKD) is a major public health problem worldwide with continuously growing epidemic characteristics and heavy cardiovascular (CV) comorbidity. CKD and CV risk have a parallel course, and CV disease is the leading cause of death in end-stage kidney disease (ESKD) patients, accounting for about 50% of mortality [1].

During the past decades, atherosclerosis and CV disease have been associated, at least partially, with excessive overproduction of reactive oxygen species (ROS) and oxidative stress (OS) has emerged as a novel risk factor for CV mortality in CKD and ESKD patients [2]. OS occurs when the formation of ROS exceeds the buffering ability of the naturally occurring endogenous antioxidant defense mechanisms, thus resulting in injury and oxidation of cells and tissues and ultimately leading to CV disease. Overproduction and accumulation of ROS is present even at early CKD stages, progresses along with eGFR decline to ESKD, and is significantly reversed after kidney transplantation. Due to their high reactivity and ephemeral nature, direct and accurate measurement of ROS is very difficult. An alternative approach for assessing the redox status in CKD and ESKD is to measure the products resulting from protein, lipid, DNA, or carbohydrate damage caused by free radicals. Plasma protein carbonyls (PCO) might serve as reliable biomarkers of OS in CKD; since they are chemically stable with long half-life, their sampling is relatively easy, and there are several validated accurate detection methods; and moreover, PCO reflects accurately the state and degree of OS [3]. The oxidation of biomolecules by ROS starts very early in CKD, progresses in parallel with deterioration of kidney function, and is further exacerbated in ESKD. Circulating PCO levels are higher in patients with early CKD compared to healthy individuals and are gradually increased with reduction of estimated glomerular filtration rate (eGFR) [3]. Compared to predialysis CKD stage 4, ESKD patients undergoing dialysis present significantly increased OS. This is attributed to various factors. Typically, in this stage, physicians give strict dietary restrictions to dialysis patients to avoid consumption of fruits and vegetables that are rich in potassium to prevent hyperkalemia, thus resulting in a reduced intake of dietary antioxidants, vitamins, and flavonoids. Moreover, a certain amount of antioxidants (such as vitamin C and trace elements) is lost during every hemodialysis (HD) session. However, the main trigger for OS in HD is the contact of patients' blood with the bioincompatible, artificial dialysate, and membrane, resulting in activation of white blood cells and overproduction of ROS, after 10 minutes of every HD session [4]. Other HD-related factors causing overproduction of free radicals include the infusion of iron, anemia and inflammation, malfunctioning fistulae, the use of central venous catheters, and the use of heparin and erythropoietin agents [4]. In PD, where nearly all the above factors are absent, one might expect that the OS should be minimal. This is not the case at all; PD patients

experience increased OS compared to predialysis CKD patients, but much less than HD patients. In PD, the main culprit for OS is the bioincompatible dialysate, which progressively damages the peritoneal membrane. Among proteins that are subjected to oxidative modification of their structure and function in dialysis patients, albumin is a well-established marker of nutritional and inflammation status and an independent predictor of all-cause mortality. Although PD patients present lower PCO levels and oxidized albumin levels than HD, it should be noted that a certain amount of serum albumin is also lost during PD procedure [3].

The clinical implications of OS in CKD are serious and cover a vast area of adverse events, including inflammation, atherosclerosis, CV disease, progression of CKD to ESKD, and death from any cause. Among these, the association of OS with inflammation and atherosclerosis is undisputed. Endothelial dysfunction (ED), the hallmark of atherosclerosis presents early in CKD, is triggered by OS and inflammation and is associated with CV mortality [5]. Since the first stage of ED is the oxidation of lipids and the formation of foam cells, it is crucial to investigate the pathophysiologic mechanisms underlying this process.

Proprotein convertase subtilisin/kexin type 9 (PCSK9), by regulating the expression of low-density lipoprotein (LDL) cholesterol receptor, is implicated in inflammation and ED of CKD patients. Dounousi et al. [6] performed a cross-sectional study enrolling 92 predialysis CKD patients (stages II–IV) and found that, although not correlated with eGFR, proteinuria, OS, and inflammation, plasma PCSK9 levels were associated with lipid parameters and ED, assessed by soluble intercellular adhesion molecule-1 levels. Moreover, treatment with statins increases circulating PCSK9 levels in this population and might be of benefit. Another enzyme that is involved in the pathogenesis and development of OS, inflammation, and ED through regulation of the cholesterol efflux and oxidative transformation of LDL cholesterol is soluble epoxide hydrolase 2 (EPHX2), a potential therapeutic target for CV disease [7]. In another prospective study including 118 diabetic kidney disease patients, we found that genetic variations of the EPHX2 gene (rs27411335 and rs11780592) were associated with increased oxidized LDL and carotid intima medium thickness and predicted all-cause mortality [8], indicating thus a possible genetic background in these populations.

Besides CV disease, OS is also implicated in the pathophysiology of CKD progression and various types of kidney diseases, including Balkan endemic nephropathy (BEN) [9]. The exact pathophysiological mechanisms underlying this chronic tubulointerstitial nephropathy disease have not yet been fully elucidated. OS, fibrosis, and inflammation are thought to play a role in the development and progression of BEN, but existing data are limited. Veljkovic et al. performed a cross-sectional study including 50 patients diagnosed with BEN and 38 healthy controls and found that, compared to controls, BEN patients exhibited significantly increased systemic lipid and protein oxidation status, assessed by plasma thiobarbituric acid reactive substances (TBARS) and advanced oxidation protein products (AOPPs), respectively [10]. However, in urine, only AOPP levels were significantly higher compared to controls; the urine local lipid oxidation state was not different among groups, probably due to the reduced urine lipid content.

During the past decade, there is accumulating evidence suggesting that OS plays a central role in the pathogenesis and development of diabetic kidney disease (DKD) [11]. However, the exact sites and mechanisms underlying this association have not yet been fully understood, mainly because most of the existing studies are experimental. In both *in vitro* (hyperglycemic kidney tubular epithelial cells) and *in vivo* (mouse and human kidney cells with DKD), mitochondrial general control of amino acid synthesis 5-like 1- (GCN5L1-) derived acetylation of the endogenous antioxidant manganese superoxide dismutase induces OS-mediated kidney injury, suggesting a potential novel pathway of DKD and a possible new therapeutic target [12]. To counterbalance the deleterious effects of OS in CKD and ESKD, the supplementation of exogenous antioxidants has been suggested, with the most promising being to-date the fat-soluble vitamin E in HD patients and the powerful scavenger N-acetylcysteine (NAC) in PD. An interesting approach to battle the oxidative burst caused by the exposure of blood to the artificial membrane was the coating of HD dialyzers with vitamin E. These vitamin E-coated membranes have been shown to increase the levels of vitamin E, suppress OS and inflammation markers, and improve anemia status in HD patients [13]. In predialysis CKD, it has been hypothesized that the disturbance of balance between antioxidants and prooxidants in favor of the latter might be a risk factor for CKD progression. Ilori et al. performed a large prospective study including 19,461 participants from the Reasons for Geographic and Racial Differences in Stroke (REGARDS) cohort study [14]. The authors calculated a score assessing oxidative balance by combining 13 popular prooxidants and antioxidants that were determined before enrollment, by using lifestyle and dietary assessment. After a median follow-up period of 3.5 years, the authors found that a higher score (which is indicative of higher levels of exogenous antioxidants) was correlated with significantly lower CKD prevalence. Therefore, it was hypothesized that exogenous administration of antioxidants might abrogate CKD progression. Among the supplements that were examined in CKD populations, bardoxolone methyl and pentoxifylline were shown to significantly protect from deterioration of the kidney function [15]; however, we need more well-designed trials examining novel and more powerful antioxidants.

In experimental prediabetic animal models, Akinnuga et al. showed that bredemolic acid improved glucose homeostasis and markers of kidney function, decreased malondialdehyde (a marker of lipid peroxidation status), and increased the levels of various antioxidants, including glutathione peroxidase, superoxide dismutase, and total antioxidant capacity [16]. The authors hypothesized that these findings might suggest a possible renoprotective effect of this agent, through its antioxidant effects, in an experimental induced prediabetic state. To further investigate new therapeutic, antioxidant strategies in DKD, Huang et al. performed a mixed *in vitro* and *in vivo* study and examined

the possible beneficial effect of short fatty acid supplementation (acetate, propionate, and butyrate) in streptozotocin-induced type 2 diabetes/high-fat diet and DKD mice and in glomerular mesangial cells from high glucose-induced mouse models [17]. Administration of fatty acids, especially butyrate, decreased insulin resistance, prevented proteinuria development and eGFR decline in animals, and suppressed the hyperglycemia-derived OS in mouse glomerular cells, thus suggesting a potential renoprotective effect of short fatty acids, through improvement of OS.

Antioxidant agents may also have a role in the prevention of acute kidney injury (AKI) that may result from nephrotoxic agents or treatments, because the main pathophysiologic pathway in these cases is formation of ROS [18]. NAC has been widely used to prevent contrast-induced nephropathy, a common complication following the exposure to imaging iodinated contrast media [19]. To protect tumor patients treated with cisplatin from AKI, amifostine is usually prescribed as an add-on chemoprotective drug; however, this drug has several side effects. An experimental antioxidant agent (XH-003) has been shown to exert chemoprotective properties similar to that of amifostine, but without causing the adverse side effects of the drug. In the experimental study by Liu et al., HX-003 was shown to decrease the cisplatin-derived AKI through reduction of free radicals and upregulation of the activity of the antioxidant enzymes superoxide dismutase, catalase, and glutathione peroxidase [20].

This special issue is compatible and consistent with our attempt to elucidate the pathophysiologic mechanisms through which OS affects cells, tissues, organs, and biomolecules and its impact on health outcomes in CKD and ESKD. The increasing knowledge of the pathophysiology might provide further insights in the management of OS and in the evaluation of novel, therapeutic, antioxidant treatments that might benefit CKD and ESKD patients at the clinical level. This is an ongoing process, and we still need more evidence and data.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Research Article

Association between PCSK9 Levels and Markers of Inflammation, Oxidative Stress, and Endothelial Dysfunction in a Population of Nondialysis Chronic Kidney Disease Patients

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Proprotein convertase subtilisin/kexin 9 (PCSK9) plays an important role in lipid metabolism while available literature regarding its involvement in the pathogenesis of atherosclerosis and in the expression of genes associated with apoptosis and inflammation is constantly increasing. Patients with chronic kidney disease (CKD) experience disproportionately increased cardiovascular morbidity and mortality due to dyslipidemia, accelerated atherosclerosis, inflammation, oxidative stress, and other risk factors. In the present cross-sectional study, we investigated the possible association of serum PCSK9 levels with markers of inflammation, oxidative stress, and endothelial damage in patients with CKD. *Patients and Methods*. Ninety-two patients with CKD stages II-*IV* (eGFR CKD-EPI 47.3 ± 25.7 ml/min/1.73 m^2, mean age 66 years, 51 men) were included in the study. Plasma PCSK9 levels were correlated with comorbidities (arterial hypertension, diabetes mellitus, and history of cardiovascular disease), renal function indices (eGFR, proteinuria–UPR/24 h), lipid parameters (LDL-cholesterol, HDL-cholesterol, triglycerides, Lp(a), APO-A1, and APO-B), and soluble biomarkers of inflammation, oxidative stress, and endothelial damage (hs-CRP, fibrinogen, 8-epiPGF2a, ox-LDL, IL-6, TNF-α, sICAM-1, and sVCAM-1). *Results*. The mean plasma value of PCSK9 was 278.1 ng/ml. PCSK9 levels showed direct correlation with serum triglycerides ($p = 0.03$), Lp(a) ($p = 0.01$), and sICAM-1 levels ($p = 0.03$). There was no significant correlation between PCSK9 levels and indices of the renal function, other lipid profile parameters, inflammatory markers, or comorbidities. Multiple regression analysis showed a significant effect of Lp(a) on PCSK9 levels, and for each unit of higher Lp(a), an increase by 3.082 is expected (95% CI: 0.935-5.228, $p = 0.006$). At the same time, patients receiving statins are expected to have on average 63.8 ng/ml higher PCSK9 values compared to patients not receiving statins (95% CI: 14.6-113.5, $p = 0.012$). *Conclusion*. Plasma levels of PCSK9 in nondialysis CKD patients are correlated with endothelial dysfunction and lipid metabolism parameters. Statin intake increases PCSK9 levels significantly in this patient population. PCSK9 levels are not correlated with the severity of kidney disease. Major prospective studies are necessary to investigate the role of PCSK9 in the atherosclerotic cardiovascular outcome in CKD.

1. Introduction

Chronic kidney disease (CKD) has been characterized as an atherosclerosis multiplier increasing disproportionally the incidence of fatal and nonfatal cardiovascular events from the early stages [1]. Almost a quarter of patients with mild to moderate CKD have been reported to die mainly due to atherosclerotic cardiovascular disease (ASCVD) before the initiation of renal replacement therapy while non-ASCVD becomes dominant at more advanced stages of CKD [2, 3]. Atherosclerosis, beyond being a lipid disorder, is characterized by major inflammatory properties and has been implicated in the pathogenesis of arterial plaque formation and rupture and in clinical outcomes [4, 5]. Chronic inflammation, oxidative stress, and endothelial dysfunction separately and through their crosstalk have direct implications in the pathogenesis of atherosclerosis in CKD patients and have been established as nontraditional cardiovascular risk factors in this frail population [6–9].

Dyslipidemia, on the other hand, is one of the traditional cardiovascular risk factors. Lipid metabolism dysregulations are well recognized in patients with CKD [10, 11]. The common pattern of lipid disorders in a CKD patient consists of increased levels of triglycerides (TG) and lipoprotein (a) (Lp(a)), reduced levels of high-density lipoprotein cholesterol (HDL-C), and normal or slightly reduced total cholesterol (T-Chol) and low-density lipoprotein cholesterol (LDL-C) [10, 11]. Proprotein convertase subtilisin/kexin type 9 (PCSK9) has been identified as a central regulator of the LDL-receptor (LDL-R) expression by binding on the hepatocyte LDL-R, causing a reduction in the number of LDL-R and a subsequent enhancement in circulating LDL-C levels [12]. In CKD patients, existing literature is conflicting about possible association of PCSK9 plasma levels with the renal function. In the largest published observational study in two independent cohorts of nondialysis patients, Rogacev et al. showed no association of PCSK9 with renal function or with cardiovascular mortality [13]. Monoclonal antibodies against PCSK9 (PCSK9i) have emerged as safe and efficient hypolipidemic agents. In large clinical trials, PCSK9i manage to reduce LDL-C to target levels and Lp(a) levels by 20-30% with additional cardiovascular benefit when added to statin treatment in high-risk populations [14]. Nevertheless, efficacy of the PCSK9i alirocumab in CKD stage 3 patients was estimated in an analysis that pooled data from eight phase III ODYSSEY trials showing an efficacy and safety profile similar to that of patients with preserved eGFR [15].

Beyond the key role of PCSK9 on lipid disorders and management, emerging evidence points out its determining implication in the inflammatory arm of atherosclerosis. Experimental data have shown the upregulatory effect of proinflammatory and oxidative stress factors on the PCSK9 expression and vice versa, accordingly the possible modulatory role of PCSK9 on inflammation mediators [16]. Tumor necrosis factor alpha (TNF-α) resulted in the induction of PCSK9 mRNA and protein expression in HepG2 cells and vascular smooth muscle cells (VSMCs) [17], while oxidized low-density lipoprotein (ox-LDL) upregulated the PCSK9 expression in different cells, such as vascular endothelial cells (ECs), VSMCs, macrophages, and dendritic cells [18–23]. The administration of lipopolysaccharide in a PCSK9 knockout mice (PCSK9$^{-/-}$) reduced plasma levels of TNF-α and interleukin 6 and 10 (IL-6, IL-10) [24], whereas knockdown of PCSK9 inhibited the inflammatory response in macrophages promoted by ox-LDL [19]. Moreover, PCSK9 has been implicated in endothelial dysfunction by upregulating the expression of intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) in endothelial cells [16].

Experimental results are supported only by a limited number of clinical studies investigating the putative role of PCSK9 in the inflammatory process. Plasma levels of inflammatory cytokines, TNF-α, IL-6, and IL-8 were decreased in septic patients with a PCSK9 loss of function allele [24] while patients with bacteremia had increased PCSK9 plasma levels in a direct association with CRP [25]. Moreover, CRP as a marker of inflammation and ASCVD has been positively associated with plasma levels of PCSK9 both in patients with acute coronary syndromes and stable coronary disease [26]. In the same line, fibrinogen levels have been associated with PCSK9 levels in patients with stable coronary disease independently of traditional cardiovascular risk confounding [27].

Despite the emerging role of PCSK9 in ASCVD [28], there is lack of robust clinical data investigating the interplay of PCSK9 with markers of inflammation, oxidative stress, and endothelial dysfunction in high-risk patients such as CKD patients. Having this in mind, we designed a cross-sectional, observational study to investigate possible association of PCSK9 with inflammation markers, including high-sensitivity C-reactive protein (hs-CRP), IL-6, TNF-α, oxidative stress markers, 8-isoprostanes (8-epiPGF2a), and ox-LDL, and endothelial dysfunction markers such as (sICAM-1) and (sVCAM-1) in a nondialysis population of stable CKD patients. As a secondary outcome, we investigated the possible association of PCSK9 levels with renal function markers, lipid metabolism markers, and echocardiographic indices.

2. Patients and Methods

2.1. Participants. Ninety-two stable, CKD stage II-*I*V adults from the outpatient clinics of the Nephrology Department of our Tertiary Hospital were included in this single-center, cross-sectional, observational study. Twenty healthy volunteers attending the Outpatient Obesity and Lipid Clinic of the University Hospital of Ioannina, Greece, were recruited for the study in order to serve as a control group. The exclusion criteria were a recent major cardiovascular event (within the past 3 months before recruitment), active infection or history of infection in the last month, severe heart failure (NYHA IV) and/or severe valvulopathy, hepatic cirrhosis, active malignancy, and current immunosuppression treatment. All participants after being informed in detail provided signed informed consent to participate in the study. The study was approved by the Scientific Committee of the University Hospital of Ioannina, Greece, while all requirements of Helsinki Declaration were met.

2.2. Anthropometric Variables—Biochemical Parameters. On recruitment, all patients underwent a detailed review of their medical history and a careful clinical examination. The protocol of the study included recording demographic characteristics, smoking habits and alcohol consumption, primary renal disease, history of comorbidity and medication, anthropometric measurements, and measurement of blood pressure and heart rate. A routine full hematologic and biochemical screening was performed. All laboratory measurements were carried out after an overnight fast while water consumption was allowed. Plasma (using EDTA as an anticoagulant) and serum were prepared from blood samples and stored in aliquots at -80°C. Lipid profile assessment included serum levels of T-Chol, TC, and HDL-C that were determined enzymatically on an Olympus AU600 clinical chemistry analyzer (Olympus Diagnostica, Hamburg, Germany), LDL-C was calculated using the Friedewald formula, and Lp(a), apolipoprotein A1 (APO-A1), and apolipoprotein B (APO-B) were measured with a Behring Holding GmbH analyzer (Liederbach, Germany). All the above measurements were conducted in the laboratory facilities of the University Hospital of Ioannina. For the calculation of the estimated glomerular filtration rate (eGFR, ml/min/1.73 m^2), the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation was used [29]. Proteinuria was estimated in a 24-hour urine collection (UPR, mg/24 h).

2.3. Determination of PCSK9. Plasma PCSK9 concentration was determined by a quantitative sandwich enzyme immunosorbent assay using a commercially available kit according to the instructions provided by the manufacturer (R&D Systems, Inc.) as we previously described [30]. The intra-assay coefficient of variation ranges from 4.1 to 6.5, and the inter-assay coefficient of variation ranges from 4.1 to 6.0.

2.4. Determination of 8-epiPGF2a. Serum levels of 8-epiPGF2a were determined by means of a competitive ELISA using a commercially available kit (Cayman Chemicals, Ann Arbor, MI USA), as we previously described [31, 32]. This method has a specificity of 100% for 8-epiPGF2a, while having minimal crossreactivity with other compounds, mostly 8-isoPGF3a.

2.5. Determination of ox-LDL. Plasma levels of ox-LDL were measured by a competitive enzyme-linked immunosorbent assay using a specific murine monoclonal antibody (4E6) according to the instructions provided by the manufacturer (Mercodia, Uppsala, Sweden) as we previously described [32]. Intra- and interassay coefficients of variation were 6.0% and 7.0%, respectively.

2.6. Determination of IL-6 and TNF-α. Serum IL-6 and TNF-α were measured by a high-sensitivity ELISA (Quantikine HS human IL-6 and TNF-α, Research & Diagnostic Systems Europe Ltd., Abington UK). The sensitivity of the ELISA system was less than 0.5 pg/ml for both IL-6 and TNF-α.

2.7. Determination of hs-CRP, sICAM-1, and sVCAM-1. Serum hs-CRP levels were measured by high-sensitivity immunoturbidimetry (Cobas Integra 800, Roche). Serum levels of the molecules sICAM-1 and sVCAM-1 were measured by a sandwich enzyme immunoassay technique (ELISA) using commercially available standard kits (Quantikine human sICAM-1 and VCAM-1, Research & Diagnostic Systems Europe Ltd., Abington UK). The sensitivity of the ELISA system was less than 2 ng/ml for both sICAM-1 and sVCAM-1.

2.8. Echocardiography. Left ventricular mass (LVM) was assessed by 2D-mode echocardiogram usually within one week and no longer than one month from study entry, by a single cardiologist who followed a predefined protocol for the recordings and measurements and was blinded to the clinical and biochemical data. LV mass was estimated with Devereux formula, and LV mass index (LVMI) was calculated by dividing LV mass with patient's BMI [LVMI = LV mass (g)/BSA (m^2)] [33, 34]. Left ventricle ejection fraction (LVEF) and left ventricle shortening fraction (LVFS) were estimated as well.

3. Statistical Analysis

Frequencies and percentages were used to describe all categorical data collected, while means with standard deviation (normally distributed data) and medians with interquartile range (IQ, nonnormally distributed data) for the scale measurements. Pearson's correlation coefficient or Spearman's rho, depending on the data distribution, was used to assess linear relationships, while differences in the PCSK9 levels between dichotomous data were examined with the independent samples t-test, after checking the normality assumption under the Kolmogorov–Smirnov test. Multiple regression analysis was carried out to test the effect of all independent parameters for their effect on plasma PCSK9 levels. Included parameters were all with a $p < 0.2$ in the univariate analysis (sex, diabetes mellitus, statin treatment, logUPR, Lp(a), TG, CRP, fibrinogen, ox-LDL, and sICAM-1). The level of significance was set at 0.05, and the analysis was conducted using the SPSS v23.0 software.

4. Results

Patients' mean age was 66 years, and 51 (55.4%) were males. Demographic, clinical, and laboratory data and comorbidities of the 92 patients are shown in Table 1. The mean eGFR was 47.3 ± 25.7 mg/ml/1.73 m^2, and the median proteinuria was 323 mg/24 h (IQR, 140-1148 mg/24 h).

The mean PCSK9 plasma level in CKD patients was 278.10 ng/ml in comparison with significantly lower PCSK9 level in 20 control subjects (mean value 156 ± 43 ng/ml) (Table 2). Median hs-CRP was 1.0 (0.3-4.3) mg/l, mean fibrinogen was 433 mg/dl, median IL-6 was 3.1 (1.9-4.5) pg/ml, median TNF-α was 1.9 (1.4-3.0) pg/ml, mean 8-epiPGF2a was 110 pg/ml, ox-LDL was 79.1 U/l, median sICAM-1 was 240 (200-317) ng/ml, and mean VCAM-1 was 917.3 ng/ml.

4.1. Associations of Kidney Function Parameters. Age, systolic BP, UPR, uric acid, PTH, and LVMI increased with decreasing eGFR ($p < 0.05$ for age and $p < 0.001$ for all other

Table 1: Demographic characteristics, clinical and laboratory data, and comorbidities of the 92 CKD patients and the 20 controls.

Parameters	CKD patients ($n = 92$)	Controls [20]
Age (years)	65.8 ± 12.45	35 ± 6
Gender (male), *n* %	51 (55.4%)	6 (30%)
BMI (kg/m^2)	47.29 ± 25.68	22.7 ± 2.0
SBP (mmHg)	142 ± 19	111 ± 12
DPB (mmHg)	81 ± 11	67 ± 8
Hypertension, *n* %	80 (74%)	0
Diabetes mellitus, *n* %	26 (28.3%)	0
History of CAD, *n* %	25 (27%)	0
Statin treatment, *n* %	33 (36%)	0
eGFR/CKD-EPI (ml/min/1.73 m^2)	47.3 ± 25.7	89.5 ± 18.3
UPR (mg/24 h)	323 (140, 1148)	—
T-Chol (mg/dl)	209 ± 47	172 ± 27
TG (mg/dl)	163 ± 88	67 ± 32
HDL (mg/dl)	52 ± 15	57 ± 11
LDL (mg/dl)	124 ± 39	102 ± 21
Lp(a) (mg/dl)	11.1 (8.0, 29.3)	8 (1-24)
APO-A1 (mg/dl)	139 ± 30	159 ± 23
APO-B (mg/dl)	93 ± 28	65 ± 15
Albumin (mg/dl)	4.23 ± 0.40	4.4 ± 0.30
Uric acid (mg/dl)	6.82 ± 1.64	5.1 ± 1.35
PTH pg/ml	74 (47, 121)	—
Hb (g/dl)	13.0 ± 1.6	13.6 ± 1.4
HbA1c (%)	6.1 (5.7, 7.2)	—
LVMI (g/m^2)	135.7 ± 47.1	—
EF (%)	69 ± 10	—
FS (%)	37 ± 8	—

Table 2: Levels of PCSK9, inflammation, oxidative stress, and endothelial dysfunction markers in the 92 CKD patients and the 20 controls.

PCSK9 (ng/ml)	278.10 ± 80.2	156.2 ± 43.1
CRP (mg/l)	1.0 (0.3, 4.3)	1.0 (1.0, 2.2)
Fibrinogen (mg/dl)	433 ± 174	312 ± 136
IL-6 (pg/ml)	3.1 (1.9, 4.5)	0.8 (0.5, 2.6)
TNF-α (pg/ml)	1.9 (1.4, 3.0)	0.7 (0.1, 3.3)
8-epiPGF2a (pg/ml)	110 (92, 138)	45 ± 19
ox-LDL (U/l)	79.1 ± 23.9	42 ± 15
sICAM-1 (ng/ml)	240 (200, 317)	224 ± 20
sVCAM-1 (ng/ml)	917.3 ± 377.1	602.9 ± 145

parameters), whereas BMI and hemoglobin decreased with advanced CKD ($p < 0.001$ for both). Among lipid profile markers, only APO-A1 was significantly associated with eGFR in a direct fashion ($p = 0.001$). Kidney function expressed as eGFR showed significant inverse association with fibrinogen ($p < 0.001$), IL-6 ($p < 0.05$), TNF-α ($p < 0.001$), 8-epiPGF2a ($p < 0.001$), and sVCAM-1 ($p < 0.001$).

Urine protein daily excretion was strongly associated (in a direct fashion) with TG ($p < 0.001$), LVMI ($p < 0.001$), 8-epiPGF2a ($p = 0.007$), and sVCAM-1 ($p < 0.001$).

We did not find a significant association between PCSK9 levels and markers of the kidney function in the nondialysis CKD population of our study.

4.2. Associations between Inflammation, Oxidative Stress, and Endothelial Dysfunction Markers. Associations between inflammation, oxidative stress, and endothelial dysfunction markers are shown in Table 3. High-sensitivity CRP was found to have a direct association with fibrinogen ($p < 0.001$), IL-6 ($p < 0.001$), 8-epiPGF2a ($p < 0.001$), and sICAM-1 ($p < 0.001$). Interleukin-6 significantly associated in a direct manner with fibrinogen ($p < 0.001$), TNF-α ($p = 0.001$), and sVCAM-1 ($p < 0.001$), while TNF-α and sVCAM-1 had a direct strong association ($p < 0.001$). sVCAM-1 was found to correlate directly with sICAM-1 as well ($p < 0.001$). Significant associations of oxidative stress markers were direct association of 8-epiPGF2a with hs-CRP, sICAM-1, and sVCAM-1 ($p < 0.001$, $p = 0.001$, and $p = 0.003$, respectively), while ox-LDL associated directly with lipid metabolism parameters, TG, T-Chol, LDL-C, and APO-B ($p < 0.001$ for all) and with Lp(a) ($p = 0.02$).

4.3. Associations of PCSK9 with Lipid Metabolism Parameters, Inflammation, Oxidative Stress, and Endothelial Dysfunction Markers. PCSK9 levels were directly associated with TG ($p = 0.03$), Lp(a) ($p = 0.01$), and ICAM-1 ($p = 0.03$) (Figure 1). There was a significant positive correlation between PCSK9 levels and statin treatment. Patients on statin treatment had higher PCSK9 levels in comparison with those who did not (318.19 ng/ml vs. 253.57 ng/ml, $p < 0.001$). In our studied CKD population of stages II-IV, PCSK9 plasma levels were not associated with kidney function parameters, diabetes mellitus, and cardiovascular comorbidity or with echocardiography indices.

Multiple regression analysis, as described above, was conducted to test all independent parameters for their effect on plasma PCSK9 levels. The results showed a statistically significant effect of the Lp(a) values, as well as of statin intake. Specifically, for each unit of higher Lp(a), an increase by 3.082 is expected for the PCSK9 values (95% CI: 0.935-5.228, $p = 0.006$). At the same time, patients receiving statins are expected to have on average 63.8 ng/ml higher PCSK9 values compared to patients not receiving statins (95% CI: 14.6-113.5, $p = 0.012$).

5. Discussion

This study shows that in nondialysis CKD patients, plasma levels of PCSK9 are directly associated with the endothelial dysfunction biomarker sICAM-1 and with Lp(a), an established risk factor for myocardial infraction and cardiovascular death in CKD patients [35]. A plethora of experimental

Table 3: Associations between inflammation, oxidative stress, endothelial dysfunction, and lipid metabolism markers in the CKD patients.

		Spearman rho	p value
hs-CRP	Fibrinogen	0.44	<0.001
	8-epiPGF2a	0.32	<0.001
	IL-6	0.27	<0.001
	sICAM-1	0.35	<0.001
IL-6	Fibrinogen	0.32	<0.001
	TNF-α	0.25	0.001
	sVCAM-1	0.31	<0.001
TNF-α	sVCAM-1	0.35	<0.001
sVCAM-1	sICAM-1	0.27	<0.001
8-epiPGF2a	sICAM-1	0.26	0.001
	sVCAM-1	0.23	0.003
ox-LDL	TG	0.30	<0.001
	T-Chol	0.68	<0.001
	LDL-C	0.71	<0.001
	APO-B	0.64	<0.001
	Lp(a)	0.20	0.02

and clinical studies have established the central role of PCSK9 in lipid metabolism while robust evidence supports the implication of PCSK9 in the inflammatory nature of atherosclerosis [16].

Inflammation, oxidative stress, and endothelial dysfunction hold a key role in ASCVD in CKD patients. Studies investigating the role of PCSK9 in atherosclerosis beyond LDL-C regulation completely lack in the CKD high-risk population. In this clinical study, for the first time, we tested the possible association of PCSK9 levels with inflammation, oxidative stress, and endothelial dysfunction biomarkers in a cohort of CKD nondialysis patients. We found that sICAM-1 levels were directly associated with PCSK9 levels. The increased levels of sICAM-1 are shown to be an independent predictor of mortality in predialysis patients with cardiovascular disease [36, 37]. In the basic step of atherosclerotic vascular damage, LDL-C stimulates endothelial cells in the inner layer which express on their surface ICAM-1 and VCAM-1 and further promote the adhesion of circulating inflammatory leucocytes. Serum PCSK9 levels have been evaluated in healthy participants and showed an independent association with arterial stiffness, a well-established risk factor for atherosclerotic cardiovascular disease [38]. Moreover, in patients undergoing coronary angiography for acute coronary syndrome or stable angina, PCSK9 was found to linearly associate with the fraction and amount of necrotic core tissue in coronary atherosclerosis, independently of serum LDL cholesterol levels and statin use [39]. In an animal model, administration of lipopolysaccharides in PCSK9 knockout mice diminished the expression of VCAM-1 from endothelial vascular cells compared with wild type [16]. Release of ICAM-1 from vascular endothelial cells was reduced with PCSK9i alirocumab and anti-PCSK9 vaccine AT04 in the APOE∗3Leiden.CETP transgenic mouse model for hyperlipidemia and atherosclerosis [40, 41].

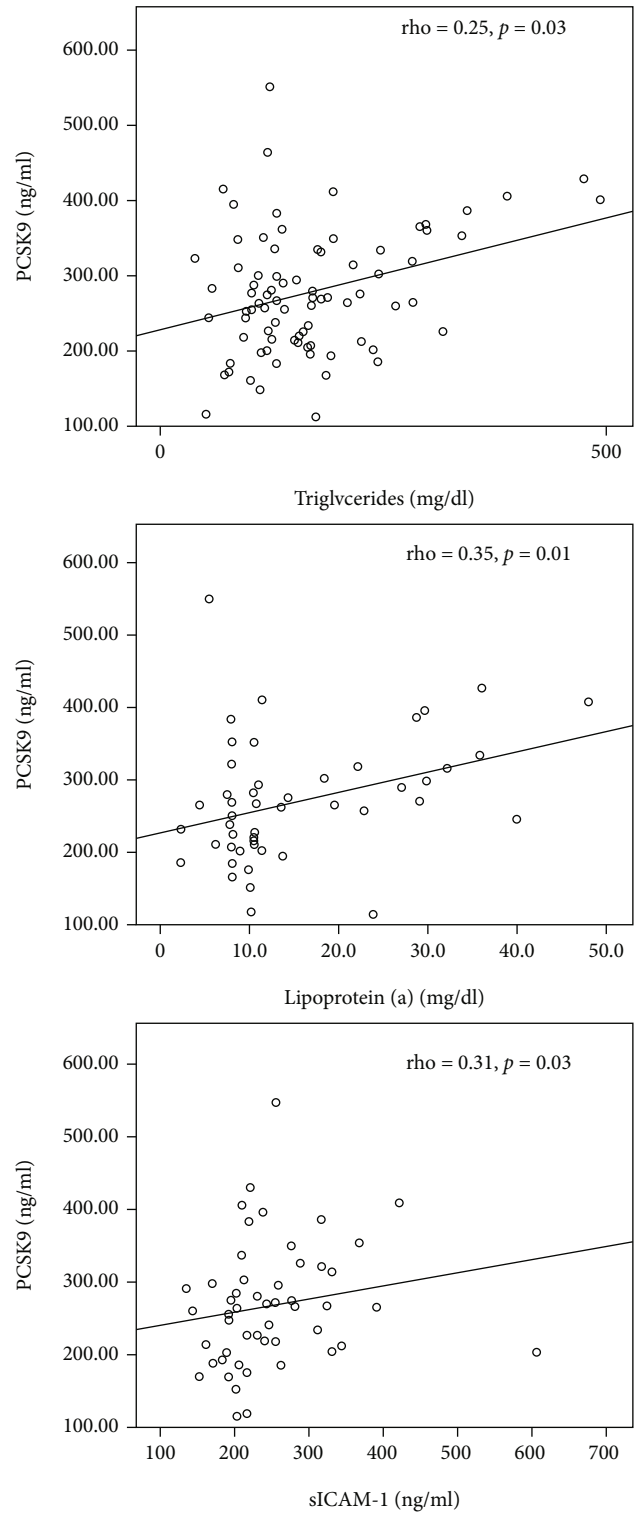

Figure 1: Significant associations of PCSK9 with triglycerides, Lp(a), and sICAM-1 in CKD patients.

Among studies estimating PCSK9 levels in nondialysis CKD patients, only two reported the association of a marker of inflammation hs-CRP with PCSK9 levels in this population. In the first one, with 44 CKD patients (eGFR 20.2 ml/min/1.73 m^2), the authors reported a rather weak direct

correlation between the two markers ($r = 0.26$, $p < 0.05$) [42]. In the largest one, Rogacev et al. assessed PCSK9 levels in two cohorts of CKD patients (CARE FOR HOMe cohort and LURIC cohort) and found no correlation between PCSK9 and hs-CRP levels nor with eGFR [13]. In accordance with Rogacev et al., we did not find any correlation between plasma PCSK9 levels and neither hs-CRP nor with kidney function markers, eGFR and UPR, in our patients. Most of the available studies showed no correlation between PCSK9 and eGFR. Interestingly, one small crossover study has shown that plasma PCSK9 can be manipulated in response to therapeutic interventions, which have other hemodynamic benefits such as endothelin antagonism [43]. Moreover, a recent experimental study in a LDL-R+/- mouse model investigated the potential effect of a vaccine targeting PCSK9 (PCSK9Qβ-003) on hypercholesterolemia and kidney fibrosis. According to their results, vaccination with PCSK9Qβ-003 had a positive effect on lipid accumulation and renal fibrosis through regulation of fatty acid β-oxidation [44]. PCSK9 is suggested to be involved in the dyslipidemia and proteinuria of nephrotic syndrome in CKD [45, 46]. The results of a very recent study of Molina-Jijon et al. showed that the kidney PCSK9 expression was enhanced in the collecting duct of nephrotic patients and animals, supporting the hypothesis that the kidney could be a major source for plasma PCSK9 in nephrotic syndrome. In our study, the vast majority of our patients were not nephrotic [47].

We found that among lipid metabolism parameters, plasma PCSK9 in our CKD patients correlated directly with TG and Lp(a) concentrations. Associations of PCSK9 with lipid profile markers are not consistent in published studies in the different high cardiovascular risk populations [11–13]. Dyslipidemia in CKD is characterized mainly by high TG levels and Lp(a), reduced levels of HDL-C, and normal or slightly reduced T-Chol and LDL-C [10, 11]. Pathogenetically, PCSK9 enhances the degradation of hepatic LDL-R, resulting in an increase in LDL cholesterol levels. We have found no association between plasma PCSK9 levels and T-Chol, HDL-C, or LDL-C in our patients. Rogacev et al. did not find either, while we both found a direct correlation with TG levels [13]. The absence of an association between PCSK9 and LDL-R might suggest that only a segment of circulating PCSK9 can mediate the degradation of LDL-R in addition to the fact that 36% of our patients were receiving statins. In our study, patients receiving statin had on average 63.8 ng/ml higher PCSK9 values compared to patients not receiving statins. It is known that statins inhibit 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGCoAR), a rate-limiting enzyme in cholesterol biosynthesis, and have been shown to significantly increase PCSK9 mRNA in HepG2 cells and primary human hepatocytes through activation of the sterol regulatory element-binding protein-2 (SREBP-2) pathway [48]. Increased levels of PCSK9 levels in patients on statin treatment could indicate a possible implication of PCSK9 in the lipoprotein TG contents by lipoprotein lipase regulation in CKD patients and an effect on high cardiovascular burden in this patient population. Nevertheless, more dedicated studies are required in order to elucidate the role of PCSK9 along with the role of PCSK9i in CKD.

Lipoprotein (a), a subtype of LDL-C, is an established risk factor for ASCVD in the general and CKD population [11, 49]. The metabolic pathways of Lp(a) production and clearance are not completely elucidated yet. Nevertheless, robust clinical evidence showed that PCSK9i reduced levels of Lp(a) by 20-30% and contributed to reduction of incident major cardiovascular event. Proposed mechanisms are either by increasing catabolism or by reducing production [49, 50]. In accordance to our results, a direct association between PCSK9 levels and Lp(a) was found by Bermudez-Lopez et al. in a cross-sectional study including 209 nondiabetic CKD patients not receiving statin treatment [51].

In this study, in line with our previous publications, we found significant associations between severity of CKD and inflammation (IL-6, TNF-α), oxidative stress (8-epiPGF2), and endothelial dysfunction (fibrinogen, sICAM-1, and sVCAM-1) markers and interesting correlations among these novel biomarkers as part of their well-recognized interplay in the uremic milieu [52, 53]. The results of this study did not demonstrate any association between PCSK9 with the majority of these biomarkers. On the other hand, a growing body of experimental evidence, as aforementioned, highlights the key role of PCSK9 in the pathogenesis of atherosclerosis by its implication in inflammation, apoptosis, oxidative stress, and endothelial damage [16, 54]. Clinical observational data are conflicting regarding the role of PCSK9 as a predictive risk factor for mortality in CKD patients. In a recent prospective study, Strålberg et al. included 265 patients starting dialysis and found a U shape association of PCSK9 levels with all-cause mortality independently of a number of confounders [55]. In contrast to this study, Rogacev et al. failed to demonstrate PCSK9 as a prognostic risk factor for cardiovascular outcomes in nondialysis CKD patients [13].

Our study has some strengths and limitations. To our knowledge, this is the only clinical observational study examining the possible association between PCSK9 with a panel of inflammation, oxidative stress, and endothelial dysfunction markers in nondialysis CKD patients. The main limitations of our study are rather small sample size and cross-sectional observational study design. Again, due to the observational nature of our findings, causality cannot be inferred from our data.

6. Conclusion

The emerging experimental data indicate that PCSK9 might have additional roles other than regulating blood LDL-C, while PCSK9i have emerged as a very promising category of hypolipidemic agents for the treatment of high-risk populations unable to achieve LDL-C target levels. Clinical data in CKD patients are scarce and not consistent regarding the role of PCSK9 in the inflammatory arm of atherosclerosis and on cardiovascular outcome. In our study, we showed that PCSK9 is possible to be a piece of the complex, unravel puzzle of ASCVD in CKD. As CKD patients remain a population with unmet needs in the management of dyslipidemia and cardiovascular morbidity and mortality, PCSK9 might be an interesting therapeutic target for the treatment of

atherosclerotic disease beyond LDL-C regulation. Further prospective research is warranted to elucidate the effects of PCSK9 and PCSK9i in patients with reduced renal function.

Data Availability

The demographic, clinical, and laboratory data of the patients used to support the findings of this study are available from the corresponding author upon request. In any case, personal data protection will be ensured.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Research Article

Association of rs11780592 Polymorphism in the Human Soluble Epoxide Hydrolase Gene (EPHX2) with Oxidized LDL and Mortality in Patients with Diabetic Chronic Kidney Disease

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Soluble epoxide hydrolase 2 (EPHX2) is an enzyme promoting increased cellular apoptosis through induction of oxidative stress (OS) and inflammation. The *EPHX2* gene which encodes soluble EPHX2 might be implicated in the pathogenesis and development of OS and atherosclerosis. We aimed to assess the possible association between two functional polymorphisms of the *EPHX2* gene (rs2741335 and rs11780592) with oxidized LDL (ox-LDL), carotid atherosclerosis, mortality, and cardiovascular (CV) disease in 118 patients with diabetic chronic kidney disease (CKD). At baseline, ox-LDL and carotid intima-media thickness (cIMT) were evaluated and all patients were followed for seven years with outcomes all-cause mortality and CV events. rs11780592 *EPHX2* polymorphism was associated with ox-LDL, cIMT, albuminuria, and hypertension. Compared to AG and GG, AA homozygotes had higher values of albuminuria, ox-LDL, and cIMT ($p = 0.046$, $p = 0.003$, and $p = 0.038$, respectively). These associations remained significant, even after grouping for the G allele. After the follow-up period, 42/118 patients died (30/60 with AA genotype, 11/42 with AG genotype, and 1/12 with GG genotype) and 49/118 experienced a new CV event (fatal or nonfatal). The Kaplan-Meier analysis revealed that patients with the AA genotype exhibited a significantly higher mortality risk, compared to patients with AG and GG genotypes ($p = 0.006$). This association became even stronger, when AG and GG genotypes were grouped (AA vs. AG/GG, $p = 0.002$). AA homozygotes were strongly associated with all-cause mortality in both univariate (hazard ratio (HR) = 2.74, confidence interval (CI) = 1.40 – 5.35, $p = 0.003$) and multivariate Cox regression analysis (HR = 2.61, CI = 1.32 – 5.17, $p = 0.006$). In conclusion, our study demonstrated that genetic variations of *EPHX2* gene were associated with increased circulating ox-LDL, increased cIMT, and all-cause mortality in diabetic CKD. Since *EPHX2* regulates the cholesterol efflux and the oxidation of LDL in foam cells and macrophages, our study suggests that a genetic basis to endothelial dysfunction and OS might be present in diabetic CKD.

1. Introduction

Chronic kidney disease (CKD) and type 2 diabetes mellitus (T2DM) are conditions with growing incidence and prevalence worldwide, characterized by high cardiovascular (CV) morbidity and increased mortality [1]. Epidemiologic data suggest that compared to T2DM patients with normal kidney function and nondiabetic patients with CKD, the risk of mortality and CV disease is much higher in patients with the combination of these two (diabetes and CKD) [2, 3]. Furthermore, in end-stage renal disease (ESRD) patients undergoing maintenance hemodialysis (HD), T2DM contributes to a 1.6-fold increase in the overall mortality risk [4]. This heavy atherogenic and CV burden of diabetic CKD and ESRD cannot be solely explained by traditional risk factors. During the past decade, oxidative stress (OS), defined as the disruption of balance between prooxidant and antioxidant molecules in favor of the former, has emerged as a novel risk factor for the onset and development of atherosclerosis and CV disease in CKD patients. OS is present even at the early stages of CKD, progresses along with disease severity, and is further exacerbated by dialysis procedures [5–7]. Increased OS status triggers inflammation resulting in a direct alteration of lipids, proteins, carbohydrates, and DNA. It is now known that the oxidative modification of low-density cholesterol (LDL) is the first crucial step leading to endothelial dysfunction, the hallmark of atherosclerosis [8].

Soluble epoxide hydrolase 2 (EPHX2) is an enzyme involved in the metabolic breakdown of anti-inflammatory, antiatherogenic arachidonic acid-induced eicosanoids to proinflammatory diol molecules. This enzyme increases cellular apoptosis, through promotion of OS and inflammation. Since EPHX2 is encoded by the *EPHX2* gene, it has been hypothesized that genetic variations of this gene might alter the enzyme's function and activity. In vitro and in vivo studies suggest that EPHX2 might be implicated in the pathogenesis and development of OS, endothelial dysfunction, and atherosclerosis [9]. Moreover, epidemiologic data have repeatedly reported that genetic variations of the *EPHX2* gene are associated with the onset of CV disease in several populations [10, 11], and thus, *EPHX2* might be a potential atherosclerosis-susceptibility gene. Since inhibition of the activity of soluble EPHX2 has been reported to improve several aspects of CV disease, including OS, hypercholesterolemia, inflammation, hypertension, endothelial dysfunction, and atherosclerosis, EPHX2 is now regarded as an emerging therapeutic target in the treatment of atherosclerosis and CV disease [12, 13]. Although several studies have examined the association between various polymorphisms of the *EPHX2* gene and CV outcomes, there are no data regarding the role of rs2741335 and rs11780592 EPHX2 polymorphisms in atherosclerosis and endothelial dysfunction. Having this background in mind, the aim of our study was to investigate the possible association between rs2741335 and rs11780592 *EPHX2* polymorphisms with oxidized LDL (ox-LDL), carotid atherosclerosis, mortality, and CV disease in a cohort of T2DM patients with various degrees of renal function.

2. Materials and Methods

2.1. Patients. We recruited a total of 118 Caucasian, unrelated, adult patients (54 male, 64 female) with mean age 67.8 ± 8.7 years and with established T2DM for at least 6 years that were regularly followed in the Diabetic CKD Clinic of the University General Hospital of Alexandroupolis, Greece. At baseline (first visit), clinical and anthropometric data and background information regarding the history of CV disease were recorded, blood and urine samples were obtained, and carotid intima-media thickness (cIMT) was assessed. CV events were defined as documented stroke, myocardial infraction, coronary heart disease, angina, or peripheral artery disease. At enrolment, we measured albuminuria and calculated estimated glomerular filtration rate (eGFR) using the CKD epidemiology collaboration (CKD-EPI) equation [14] and every patient was classified in CKD stages, according to the Clinical Practice Guidelines for Chronic Kidney Disease established by the National Kidney Foundation's Kidney Disease Outcomes Quality Initiative [15]. Based on UACR and eGFR, at baseline, our study population included 17 patients in G1A1 stage, 2 in G1A2, 16 in G2A1, 9 in G2A2, 1 in G2A3, 3 in G3aA1, 10 in G3aA2, 2 in G3aA3, 3 in G3bA1, 11 in G3bA2, 4 in G3bA3, 3 in G4A1, 3 in G4A2, 5 in G4A3, and 29 in G5 stage (maintenance HD). Sixty-six patients received antiplatelets and 108 were under antihypertensive medication (65 used diuretics, 49 calcium channel blockers, 44 angiotensin-converting-enzyme inhibitors, 48 angiotensin II receptor blockers, and 40 used b-blockers). Sixty-two patients received insulin, whereas the rest 56 received per os antidiabetic medication (48 sulfonylureas, 34 biguanides, 19 glitazones, 6 dipeptidyl peptidase-4 inhibitors, and 6 glinides). Our study protocol was in conformity with the Helsinki Declaration of Human Rights and was approved by the Ethics Committee of the Scientific Council of the Medical School of the University of Alexandroupolis, Greece. All participants gave their written, informed consent.

2.2. Follow-Up and Endpoints. After enrolment, patients were followed over a period of seven years or the occurrence of death. Secondary outcome of the study was the occurrence of CV events. We obtained follow-up information for our study cohort from regular follow-up visits, hospital medical records, **and** death certificates and through an integrated telephone interview.

2.3. cIMT Measurement. cIMT was assessed at baseline, by a single, well-trained physician, using real-time B-mode ultrasonography, as described before [16].

2.4. Laboratory Analyses. To obtain whole blood, serum, and plasma, we collected blood samples from all participants, after an overnight fasting of 8 hours. Total, LDL, and high-density lipoprotein (HDL) cholesterol, triglycerides, creatinine, c-reactive protein (CRP), and glycated hemoglobin (HBA1c) were measured, as described elsewhere [17]. To assess ox-LDL, samples were centrifuged immediately, and plasma was stored at -20°C, until analysis. The concentration of ox-LDL in plasma was quantitated by the enzyme-linked

immunosorbent assay (ELISA) method, following the manufacturer's instructions (human ox-LDL ELISA kit, Mercodia, Sweden), as was previously described [17]. Detection limit for ox-LDL assay was 0.3 U/L and intra/interassay coefficients of variation were <10%, according to the manufacturer. Albuminuria was evaluated as the urine albumin to creatinine ratio (UACR) in a morning, sterile, spot urine sample, as described before [18].

2.5. Genotyping of rs2741335 and rs11780592 EPHX2 Polymorphisms. DNA extraction and genotyping have been extensively described elsewhere [19–21]. We performed a GTEX analysis (Supplementary Table 1) showing that both SNPs (rs11780592 and rs2741335) are associated with the expression of the EPHX2 gene in various tissues. Moreover, it appears that the top hit SNP for this gene is rs11780592, since it is associated with the gene's expression in most tissues, including sites of endothelial dysfunction development, such as arteries, aorta, heart, ventricles, brain, adipose tissue, and circulation. Therefore, the results of the GTEX analysis indicate that by studying this top hit SNP, we can hypothesize that we are studying the expression of the EPHX2 gene.

2.6. Statistical Analysis. We used the Kolmogorov-Smirnov test to test our data for normality. Binary variables are expressed as percent frequency, nonnormally distributed variables are expressed as median with interquartile range, and normally distributed variables as mean ± SD. Patients' characteristics were compared among different genotypes of rs11780592 *EPHX2* using chi-square for categorical variables, Mann–Whitney for nonnormally distributed variables, and independent *t*-test for normally distributed variables, as appropriate. Comparison of ox-LDL plasma levels among stages of diabetic CKD was performed with the Mann–Whitney test. To evaluate overall survival and CV events (fatal and nonfatal) for the rs2741335 and rs11780592 *EPHX2* genotypes, we used the Kaplan-Meier method and log-rank tests to compare survival curves. For survival analyses, all patients were categorized in two groups, according to rs11780592 *EPHX2* polymorphism: AA versus grouped AG and GG genotypes. We used univariate and multivariate Cox proportional hazard models (enter selection) to calculate adjusted hazard ratios (HRs) and 95% confidence intervals (CIs) for the associations between rs11780592 and rs2741335 *EPHX2* polymorphism and all-cause mortality and CV events. Models for all-cause mortality and CV events were adjusted for age, sex, and previous history of CV disease. Statistical analyses were performed by the IBM Statistical Package for Social Sciences (SPSS) 18.0, for Windows, Chicago, Illinois, USA. Significance was set at $p < 0.05$.

3. Results

As shown in Supplementary Table 2 (submitted in supplementary materials), the allelic and genotypic frequencies found in our cohort of Greeks are similar to those of Tuscan Italians from Southern Europe, which are a good reference for the Greek population, as described before [22]. Regarding other non-European populations, it is interesting to mention that East Asians do not have this variant, whereas Ashkenazi Jewish are the population with the greatest allele frequency for the G allele (minor allele frequency = 18%, gnomAD data). The Mann–Whitney test showed that ox-LDL levels were significantly different among CKD stages ($p = 0.038$). Baseline clinical, anthropometric, and biochemical characteristics of diabetic CKD patients according to rs11780592 *EPHX2* genotypes are shown in Table 1 (AA, AG, and GG genotypes) and Table 2 (AA versus grouped AG/GG). Compared to AG and GG, AA homozygotes had significantly increased diastolic and mean blood pressure levels ($p = 0.007$ and 0.038, respectively). This association was even more pronounced when AG and GG genotypes were grouped (Table 2). The grouped genotypes of rs11780592 EPHX2 polymorphism differed significantly also, among stages of diabetic CKD -G1/G2 as mild CKD, G3a/G3b as moderate CKD, G4 as severe CKD, and G4 as ESRD ($p = 0.02$), Supplementary Table 3. Age, body mass index (BMI), systolic blood pressure, glycated hemoglobin, and CRP did not differ significantly among groups. Compared to the other genotypes, there was an excess of male participants in the AA group ($p = 0.04$). Although lipid profile parameters (including total, LDL, and HDL cholesterol and triglycerides) were not significantly different among genotypes, compared to AG and GG, AA homozygotes presented significantly increased levels of plasma ox-LDL ($p = 0.003$, Table 1). UACR and cIMT were significantly different between the rs11780592 EPHX2 genotypes. Compared to AG and GG, AA homozygotes had higher values of both UACR and cIMT ($p = 0.046$ and $p = 0.038$, respectively). ox-LDL differed significantly among rs11780592 genotypes in G1/G2 and G4 stages of diabetic CKD, whereas cIMT was significantly higher in AA homozygotes (compared to AG and GG) in stages G4 and G5 (Supplementary Table 4). All the baseline characteristics of the patients did not differ significantly among rs2741335 *EPHX2* genotypes (results not shown).

After the follow-up period, 42/118 patients died (30/60 with AA genotype, 11/42 with AG genotype, and 1/12 with GG genotype) and 49/118 experienced a new CV event (fatal or nonfatal). The Kaplan-Meier analysis revealed that patients with the AA genotype exhibited a significantly higher mortality risk, compared to patients with AG and GG genotypes (Figure 1(a), $p = 0.006$). This association became even stronger, when AG and GG genotypes were grouped (Figure 1(b), AA vs. AG/GG, $p = 0.002$). AA homozygotes were strongly associated with all-cause mortality in both univariate (HR = 2.74, CI = 1.40 – 5.35, $p = 0.003$, Table 3) and multivariate Cox regression analysis (HR = 2.61, CI = 1.32 – 5.17, $p = 0.006$, Table 3). However, both the Kaplan-Meier and Cox regression analysis failed to show any association between rs11780592 *EPHX2* polymorphism and the occurrence of CV events (Table 3). Moreover, rs2741335 *EPHX2* polymorphism was not associated with any of the study outcomes.

Table 1: Association of rs11780592 EPHX2 polymorphism with anthropometric, clinical, and biochemical characteristics of patients with diabetic chronic kidney disease.

	rs11780592 EPHX2 genotypes			p
$N = 118$	AA ($n = 60$)	AG ($n = 46$)	GG ($n = 12$)	
Age (years)	68.6 (8.1)	67.3 (9.4)	65.8 (8.8)	0.47
Gender (M/F)	34/26	17/29	3/9	**0.04**
BMI (kg/m^2)	31.4 (5.1)	31.1 (5.7)	29.7 (4.2)	0.45
SBP (mmHg)	140.9 (18.9)	136.2 (20.8)	135.4 (22.7)	0.35
DBP (mmHg)	79.5 (9.2)	75.3 (11.0)	71.4 (10.5)	**0.007**
Mean BP (mmHg)	99.9 (11.1)	95.6 (12.9)	92.7 (12.8)	**0.038**
Duration of T2DM (years)	16.3 (7.8)	13.1 (7.2)	14.8 (8.3)	0.10
HbA1c (%)	7.5 (1.2)	7.3 (0.9)	7.7 (1.3)	0.59
Total cholesterol (mg/dL)	183.7 (47.9)	168.2 (45.6)	159.6 (30.1)	0.15
LDL cholesterol (mg/dL)	104.0 (41.3)	93.1 (35.9)	81.6 (22.0)	0.22
HDL cholesterol (mg/dL)	44.8 (11.9)	44.5 (11.9)	52.1 (19.3)	0.45
Triglycerides (mg/dL)	157.5 (66-450)	141.5 (52-551)	93.0 (69-315)	0.12
History of CV disease (%)	70	74	58.3	0.57
CRP (mg/dL)	0.25 (0-11)	0.20 (0-4)	0.20 (0-4.5)	0.72
eGFR (mL/min/1.73 m^2)	43.6 (30.0)	49.5 (34.9)	60.9 (34.2)	0.27
UACR (mg/g)	66 (3-7000)	35.5 (7-2600)	20.3 (2.4-250)	**0.046**
Mean cIMT (mm)	0.94 (0.55-1.76)	0.89 (0.55-1.78)	0.83 (0.56-0.95)	**0.031**
ox-LDL (U/L)	68.3 (17.9-123.4)	53.1 (22.0-83.4)	52.9 (22.9-92.2)	**0.003**

p values of Mann–Whitney, t-test, or chi-square test for differences of variables among rs11780592 EPHX2 genotypes. BMI: body mass index; SBP: systolic blood pressure; DBP: diastolic blood pressure; mean BP: mean blood pressure; T2DM: type 2 diabetes mellitus; HbA1c: glycated hemoglobin A1c; LDL: low-density lipoprotein; HDL: high-density lipoprotein; CV: cardiovascular; CRP: C-reactive protein; eGFR: estimated glomerular filtration rate; UACR: urinary albumin-creatinine ratio; cIMT: carotid intima-media thickness; ox-LDL: oxidized low-density lipoprotein.

Table 2: Association of rs11780592 EPHX2 grouped genotypes with anthropometric, clinical, and biochemical characteristics of patients with diabetic chronic kidney disease.

	rs11780592 EPHX2 genotypes, grouped		p
$N = 118$	AA ($n = 60$)	AG & GG ($n = 58$)	
Age (years)	68.6 (8.1)	67.0 (9.2)	0.31
Gender (M/F)	34/26	20/38	**0.013**
BMI (kg/m^2)	31.4 (5.1)	30.8 (5.5)	0.34
SBP (mmHg)	140.9 (18.9)	136.1 (21.0)	0.15
DBP (mmHg)	79.5 (9.2)	74.5 (10.9)	**0.003**
Mean BP (mmHg)	99.9 (11.1)	95 (12.8)	**0.013**
Duration of T2DM (years)	16.3 (7.8)	13.5 (7.3)	**0.04**
HbA1c (%)	7.5 (1.2)	7.4 (1.0)	0.52
Total cholesterol (mg/dL)	183.7 (47.9)	166.4 (42.7)	0.06
LDL cholesterol (mg/dL)	104.0 (41.3)	90.7 (33.7)	0.14
HDL cholesterol (mg/dL)	44.8 (11.9)	46.1 (13.9)	0.52
Triglycerides (mg/dL)	157.5 (66-450)	137 (52-551)	**0.07**
History of CV disease (%)	70	70.6	0.55
CRP (mg/dL)	0.25 (0-11)	0.20 (0-4.5)	0.49
eGFR (mL/min/1.73 m^2)	43.6 (30.0)	51.9 (34.7)	0.22
UACR (mg/g)	66 (3-7000)	27.5 (2.4-2600)	0.055
Mean cIMT (mm)	0.94 (0.55-1.76)	0.86 (0.55-1.78)	**0.038**
ox-LDL (U/L)	68.3 (17.9-123.4)	53.1 (22-92.2)	**0.001**

p values of Mann–Whitney, t-test, or chi-square test for differences of variables among rs11780592 EPHX2 genotypes. BMI: body mass index; SBP: systolic blood pressure; DBP: diastolic blood pressure; mean BP: mean blood pressure; T2DM: type 2 diabetes mellitus; HbA1c: glycated hemoglobin A1c; LDL: low-density lipoprotein; HDL: high-density lipoprotein; CV: cardiovascular; CRP: C-reactive protein; eGFR: estimated glomerular filtration rate; UACR: urinary albumin-creatinine ratio; cIMT: carotid intima-media thickness; ox-LDL: oxidized low-density lipoprotein.

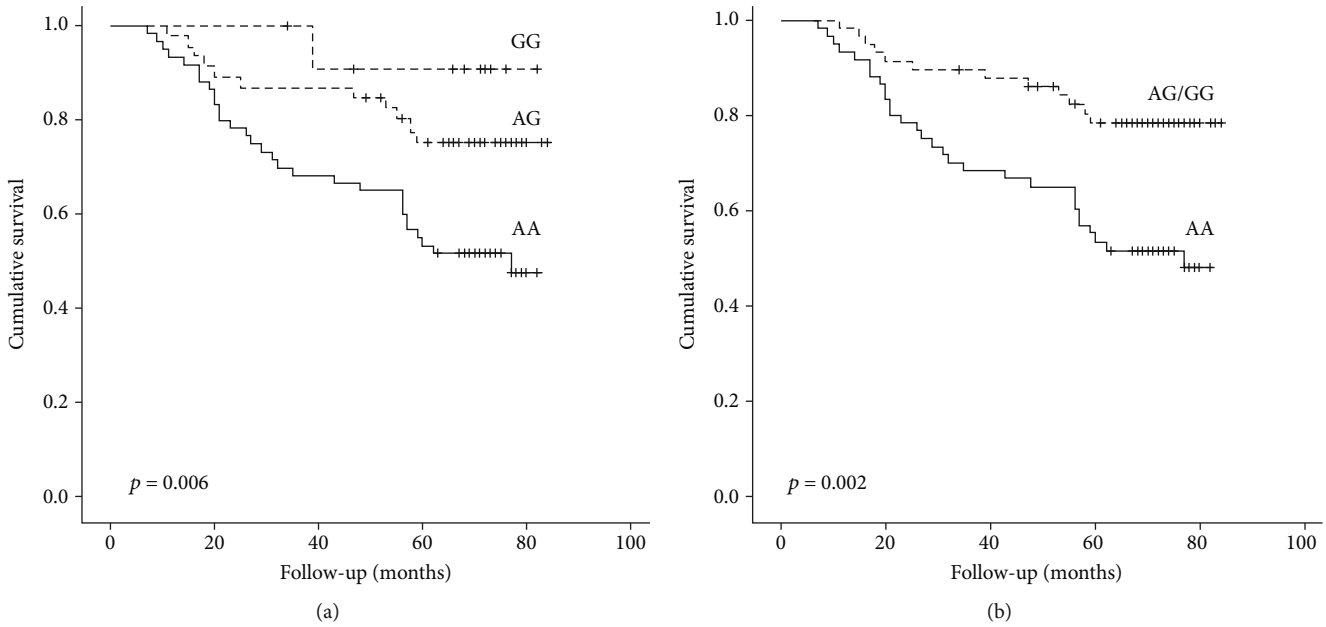

Figure 1: Kaplan-Meier curves for all-cause mortality according to rs11780592 EPHX2 polymorphism. (a) Comparison among patients with AA, AG, and GG genotypes. (b) Comparison of patients according to AA versus grouped AG/GG genotypes. Log-rank test, $p = 0.006$ and 0.002, respectively.

Table 3: Cox proportional hazard analysis (enter regression) showing predictors for all-cause mortality in univariate and multivariate models, in patients with rs11780592 EPHX2 and rs2741335 EPHX2 polymorphism.

	HR	CI	*p*
All-cause mortality			
Model 1[a]			
rs11780592 EPHX2	2.74	1.40-5.35	**0.003**
rs2741335 EPHX2	0.85	0.58-1.26	0.42
Model 2[b]			
rs11780592 EPHX2	2.61	1.32-5.17	**0.006**
CV events (fatal and nonfatal)			
Model 1[a]			
rs11780592 EPHX2	1.10	0.72-1.69	0.67
rs2741335 EPHX2	0.77	0.54-1.11	0.16

Model 1[a] = univariate model. Model 2[b] = multivariate model, adjusted for age, sex, and previous history of cardiovascular disease. HR = hazard ratio; CI 95% = confidence interval.

4. Discussion

Diabetic CKD is characterized by increased mortality and CV disease rates. This is partially attributed to the enhanced OS and inflammation that is triggered by diabetes and CKD. Oxidation of LDL cholesterol is the first crucial step towards endothelial dysfunction, the hallmark of atherosclerosis. Thus, it is hypothesized that ox-LDL might be not only a marker of OS but also a biomarker reflecting arterial health. Having these in mind, the identification of genetic biomarkers that might determine circulating ox-LDL levels and predict mortality might be of utmost clinical importance. Arachidonic acid-derived epoxyeicosatrienoic acids are anti-inflammatory, antioxidant agents with established beneficial effects on CV disease. Hydration of these molecules by soluble EPHX2 is the main pathway of their metabolic degradation to the less active, diol molecules. By catalyzing this reaction, soluble EPHX2 promotes inflammation, OS, and atherosclerosis. The EPHX2 gene is a protein-coding gene with genomic location in the short arm of chromosome 8 (8p21.2-p21.1). Genetic variance of the gene encoding EPHX2 has been repeatedly associated with CV outcomes; however, until to date, the data regarding the association between *EPHX2* gene polymorphisms, ox-LDL, and mortality remain extremely limited.

In a cohort of 118 patients with established diabetic CKD, we found a strong association between cIMT and rs11780592 *EPHX2* polymorphism. cIMT is a well-established surrogate marker for endothelial dysfunction and subclinical atherosclerosis in CKD patients and has been associated with mortality and CV disease in a population similar to ours [16] and patients undergoing maintenance HD [23]. In agreement with our results, in vivo data from animal and human cultured carotid artery smooth muscle cells suggest that soluble EPHX2 might promote atherosclerotic progression and arterial remodelling after acute injury [9]. The authors hypothesized that since EPHX2 is involved in the phenotypic transformation of vascular smooth muscle cells that leads to endothelial dysfunction and atherosclerosis, inhibition of its activity might be a potential therapeutic target for ameliorating carotid atherosclerosis and CV disease. Likewise, in T2DM patients, Duflot et al. showed that increased metabolic breakdown of epoxyeicosatrienoic acids by EPHX2 and impaired bioavailability of nitric oxide are tightly associated with impaired conduit artery endothelial function, independently of their blood pressure status [24]. Moreover, these

authors found that among hypertensive patients, only diabetics but not nondiabetics presented a significant elevation of circulating reactive oxygen species (a marker of OS) and decreased nitric oxide release. Furthermore, inhibition of EPHX2 might have pleiotropic beneficial effects on hypertension, endothelial dysfunction, OS, and CV disease. Recently, three novel agents acting as EPHX2 inhibitors were identified as potent vasodilators [25], whereas clinical data suggest that genetic variation in EPHX2 might be associated with forearm vasodilation, a marker of endothelial function [26]. Thus, in agreement with our study, there is a growing body of evidence suggesting a central role for EPHX2 in the regulation of endothelial function and the pathogenesis of atherosclerosis. In our study, the AA genotype of rs11780592 was associated with increased thickness of the carotid wall, when compared to AG and GG genotypes. The association remained significant after grouping for the G allele. Although several studies have examined other *EPHX2* polymorphisms, there are no existing data in the literature regarding rs11780592 and rs2741335 polymorphisms.

Another finding of our study was the association between rs11780592 *EPHX2* polymorphism and albuminuria. Silencing of the *EPHX2* gene in mice has been shown to attenuate renal inflammation and albuminuria [27], whereas experimental and clinical data have shown that epoxyeicosatrienoic acids and genetic variations of the *EPHX2* gene were directly associated with insulin sensitivity and glucose homeostasis [28]. Moreover, in diabetic, overweight animal models, inhibition of EPHX2 prevents albuminuria, independently from its glucose-lowering effect [29]. Diabetic CKD is characterized by a gradual reduction of eGFR and presence of albuminuria. In a case-control study, Ma et al. found that the coding rs751141 *EPHX2* polymorphism is associated with diabetic CKD in a cohort of Chinese T2DM population [30]. The association between genetic variation of the *EPHX2* gene and kidney outcomes has been also highlighted by two separate studies, in renal transplant recipients [31, 32]. These studies suggested that donor genetic variability in the *EPHX2* gene might be predictive of graft dysfunction and acute rejection in kidney transplant recipients. In our study, we also found that plasma ox-LDL levels were associated with the stage of diabetic CKD, as defined by albuminuria and eGFR. It is known that OS is present even at the early stages of CKD and progresses along with disease progression to ESRD. In agreement with our findings, Dounousi et al. reported that circulating 8-isoprostanes, a marker of lipid peroxidation status, increased significantly as CKD progressed and were strongly correlated with the degree of renal function [33].

The main finding of our study was that rs11780592 *EPHX2* polymorphism was associated with ox-LDL, cIMT, and mortality, in a cohort of patients with diabetic CKD. Compared to AG and GG, AA homozygotes presented significantly increased plasma ox-LDL levels, increased cIMT values, and increased mortality rates. We hypothesize that the association between *EPHX2* gene polymorphism and mortality might be attributed to the increased circulating ox-LDL levels. In agreement with our findings, in apolipoprotein E knockout mice, inhibition of EPHX2 significantly decreased the onset and development of atherosclerotic lesions. These antiatherogenic, beneficial effects of EPHX2 inhibitors were attributed to a significant decrease in LDL and elevation of HDL cholesterol [34]. Moreover, blocking EPHX2 was accompanied by a significant improvement of hypertension and endothelial function. Therefore, by reducing LDL cholesterol and OS status, EPHX2 inhibitors might decrease ox-LDL formation. In animal models with ischemic stroke, administration of selective EPHX2 inhibitors was found to improve clinical outcomes, by suppressing OS and inflammation [35]. The tight association between EPHX2 and ox-LDL was also highlighted in another animal study, where blocking of soluble EPHX2 was accompanied by a rise in β-oxidation of fatty acids [36]. In vitro, incubation of foam cells with a novel inhibitor of soluble EPHX2 caused a significant decrease in cholesterol accumulation in ox-LDL-loaded macrophages [37], possibly by increasing the circulating epoxyeicosatrienoic fatty acids. Inhibition of soluble EPHX2 promoted cholesterol efflux and prevented the internalization of ox-LDL cholesterol.

There is a growing body of evidence suggesting a direct association between soluble EPHX2 and CV disease. This association might be attributed to the increased degradation of the cardioprotective epoxyeicosatrienoic acids, to the promotion of OS and inflammation, and to the induction of endothelial dysfunction. Large epidemiologic studies (the Atherosclerosis Risk in Communities and the Diabetes Heart Study) reported that rs7837347, rs7003694, rs747276, and rs41507953 *EPHX2* gene polymorphisms were associated with subclinical CV disease and coronary heart disease [10, 11]. However, in our study, although only rs11780592 *EPHX2* polymorphism was associated with mortality, no association was found between the two polymorphisms and CV disease.

To the best of our knowledge, this is the first study evaluating the association of rs11780592 and rs2741335 *EPHX2* gene polymorphisms with ox-LDL, carotid atherosclerosis, and mortality in a cohort of patients with diabetic CKD. However, our study has certain limitations. First, the cross-sectional design of the study precludes establishing causality. Second, the relatively small sample size and the lack of data on soluble EPHX2 levels and activity are also recognized as limitations. Since there are no data regarding these two polymorphisms in the literature, further, larger epidemiologic studies are needed in order to fully elucidate the role of *EPHX2* gene variations. Identification of genetic markers might provide a deeper understanding of the molecular pathways involved in OS and atherosclerosis in diabetic CKD, which subsequently might lead to the recognition of novel therapeutic targets.

5. Conclusions

In conclusion, our study demonstrated that in a cohort of 118 T2DM patients with various degrees of CKD, including ESRD, rs11780592 *EPHX2* polymorphism was associated with increased circulating ox-LDL, increased cIMT, and all-cause mortality. The AA genotype of rs11780592 polymorphism was associated with OS, carotid atherosclerosis, and all-cause mortality in these patients. Since EPHX2 regulates

the cholesterol efflux and the oxidation of LDL in foam cells and macrophages, our study suggests that a genetic basis to endothelial dysfunction and OS might be present in diabetic CKD.

Data Availability

The data used to support the findings of this study are included within the article.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Supplementary Materials

Supplementary Materials include 4 tables. Supplementary Table 1: analysis of the associated variants on tissue expression. Supplementary Table 2: allelic and genotypic frequencies in the European populations of the 1000 Genomes dataset. Supplementary Table 3: distribution of rs11780592 EPHX2 polymorphism genotypes in different stages of diabetic CKD. Supplementary Table 4: association of rs11780592 EPHX2 polymorphism with ox-LDL and cIMT among different stages of diabetic CKD. *(Supplementary Materials)*

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Research Article

General Control of Amino Acid Synthesis 5-Like 1-Mediated Acetylation of Manganese Superoxide Dismutase Regulates Oxidative Stress in Diabetic Kidney Disease

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Diabetic kidney disease (DKD) is the major cause of end-stage renal disease (ESRD). In the past few decades, there has been a large amount of evidence to highlight the pivotal role of oxidative stress in the development and progression of DKD. However, the detailed molecular mechanisms are not fully elucidated. A new sight has been established that the mitochondrial acetyltransferase GCN5L1 participates in cellular redox homeostasis maintenance in DKD. Firstly, we found that the expression of GCN5L1 is significantly elevated both in human and mouse kidney tissues with DKD and in hyperglycemic renal tubular epithelial cells (TECs), while deletion of GCN5L1 could effectively ameliorate oxidative stress-induced renal injury in DKD. Furthermore, deletion of GCN5L1 could reduce MnSOD acetylation on lysine 68 and activate its activity, thereby scavenging excessive ROS and relieving oxidative stress-induced renal inflammation and fibrosis. In general, GCN5L1-mediated acetylation of MnSOD exacerbated oxidative stress-induced renal injury, suggesting that GCN5L1 might be a potential intervention target in DKD.

1. Introduction

Diabetic kidney disease (DKD) is one of the most common microvascular complications of diabetes mellitus and has become the leading cause of end-stage renal disease (ESRD), a worldwide public concern [1, 2]. The current clinical interventions for DKD are mainly to control risk factors such as hyperglycemia, hypertension, and proteinuria, which focus on relieving symptoms and delaying the progression of DKD, but their efficacy is limited [3–5]. Therefore, there is a strong clinical need to further explore the pathophysiological mechanism of DKD and find potential intervention targets.

Recently, hyperglycemia-induced oxidative stress is increasingly being seen as a major pathogenic mechanism that leads to kidney damage in DKD, which is caused by the overproduction of reactive oxygen species (ROS) induced by disturbance of mitochondrial respiration [6]. As a star molecule of antioxidant enzymes, manganese superoxide dismutase (MnSOD) could effectively scavenge the toxic ROS by

catalyzing the dismutation of superoxide anion radical ($O_2^{\cdot-}$) to hydrogen peroxide (H_2O_2) and molecular oxygen (O_2) [7, 8]. The conventional perspective is that the ability of MnSOD to scavenge ROS depends on its abundance, while a novel view believes that it depends more on the enzymatic activity [9]. It has been reported that the reversible lysine acetylation of MnSOD could regulate its detoxification activity [10]. However, the role of acetylated MnSOD in DKD and its upstream regulatory mechanisms have been largely unaddressed in the past.

General control of amino acid synthesis 5-like 1 (GCN5L1) is a novel molecule with sequence homology to the nuclear acetyltransferase GCN5. It predominantly locates in the mitochondria to promote mitochondrial protein acetylation and interacts with canonical substrates of SIRT3 to counter its activity [11]. Current studies indicate that GCN5L1 is involved in regulating multiple mitochondrial biological functions such as mitophagy, mitochondrial biogenesis, and fatty acid oxidation (FAO) [12–14]. In the present study, we supplemented the important role of GCN5L1 in the maintenance of cellular redox homeostasis in DKD. We found that the expression of GCN5L1 is significantly elevated both in vivo in kidney tissues from DKD patients and mouse models and in vitro renal tubular epithelial cells (TECs) treated with high glucose, while reducing GCN5L1 expression could effectively attenuate mitochondrial oxidative stress, epithelial-to-mesenchymal transition (EMT), and inflammation induced by high glucose. Furthermore, downregulated GCN5L1 in TECs stimulated with high glucose activates MnSOD by decreasing the acetylation level of its K68 site and alleviates hyperglycemia-induced renal damage. These findings shed new light on the pathophysiological mechanism of DKD and hint GCN5L1 might be a potential intervention target.

2. Materials and Methods

2.1. Cell Lines. Human renal proximal tubular epithelial cells (HK-2) were purchased from the American Type Culture Collection (ATCC) and sterilely cultured with 10% FBS and 1% penicillin/streptomycin in DMEM at 37°C and 5% CO_2.

2.2. Human Renal Biopsy Samples. Renal biopsy specimens from type 1 diabetes patients ($n = 6$) with pathological diagnosis of DKD were obtained from Department of Pathology, Qilu Hospital of Shandong University. The control samples ($n = 6$) were taken from healthy kidney poles of individuals who underwent cancer nephrectomy without other renal diseases. All procedures were approved by the Ethics Review Committee of Shandong University (ECSBMSSDU2018-1-045) and performed in accordance with the principles of the Helsinki Declaration after obtaining informed consent from the patients.

2.3. Animals. Male C57BL/6 mice (6-8 weeks old) were purchased from Shandong University Experimental Animal Centre. All animal experiments were performed according to the protocols approved by Animal Ethics Committee of Shandong University. The mice were randomly divided into the following experimental groups: normal control mice (non-DN group, $n = 6$), streptozotocin-induced DN mice (DN group, $n = 9$), AAV-empty vector DN mice (AAV-vector DN group, $n = 9$), and AAV-GCN5L1 DN mice (AAV-GCN5L1 DN group, $n = 9$). For adenovirus-associated virus experiments, mice were anaesthetized firstly and then were injected with AAV2/9-HU6-shGCN5L1 (titer: 1×10^{12} vg/ml) or AAV2/9-HU6-Scramble (titer: 1×10^{12} vg/ml) into renal cortex in situ. The target sequence of shGCN5L1 was 5′-GAAGAGGAGGAGAGAAGCTAT-3′. The sequence of negative control was 5′-TTCTCCGAACGTGTCACGT-3′. After a week recovery, the DN groups were given an intraperitoneal injection of streptozotocin (50 mg/kg) after 12 hours fasting for five consecutive days. The control group was treated with an equal volume of citric acid buffer solution. One week after STZ injection, mice with random blood glucose levels over 16.7 mM were considered diabetes [15–17]. Mice with random blood glucose levels below 16.7 mM were excluded from the experiment; the DN group excluded three mice ($n = 6$), the AAV-vector DN group excluded one mouse ($n = 8$), and the AAV-GCN5L1 DN group excluded one mouse ($n = 8$). The actual blood glucose data of mice is shown in Supplementary Table S1. Then, all mice were housed in temperature-controlled rooms with a 12-hour light-dark cycle and had free access to food and drinking water for sixteen weeks.

2.4. Histopathology and Immunohistochemistry. Kidney tissues were fixed in 4% buffered formalin and embedded in paraffin. Sections were then stained with hematoxylin-eosin (HE), periodic acid-Schiff (PAS), and Masson's trichrome. For immunohistochemistry staining, sections were retrieval antigens in sodium citrate buffer after deparaffinized and rehydrated and then incubated with the antibody at 4°C overnight. Afterwards, the kidney sections were incubated with HRP-conjugated secondary antibody for 1 h shaking at room temperature. Diaminobenzidine (DAB) was applied to visualize the signal. Images were captured by a Nikon light microscope. Image analysis was performed using ImageJ software.

2.5. Renal Function Measurements. Mice were placed in metabolic cages for 24 hours to collect urine samples. Blood samples were collected by cardiac puncture after mice was anesthetized at the end of the experiment. Serum creatinine and urinary protein were measured by using commercial determination kits (Nanjing Jiancheng Bioengineering Institute, China) according to the manufacturer's instructions.

2.6. Western Blotting Analysis. Proteins of cells or kidney tissues were extracted by using RIPA buffer supplemented with 1% protease inhibitor and phosphatase inhibitors. After diluted in 4× SDS-PAGE loading buffer and denatured in 95°C for 10 min, samples were separated by SDS-PAGE and subsequently transferred to PVDF membranes. The membranes were blocked with 5% skimmed milk and then incubated with rabbit anti-GCN5L1 antibody (Proteintech, 19687-1-AP), mouse anti-GCN5L1 antibody (Santa Cruz, sc515444), rabbit anti-SOD2/MnSOD (acetyl K68) antibody

(Abcam, ab137037), rabbit anti-MnSOD antibody (Proteintech, 24127-1-AP), rabbit anti-NLRP3 antibody (Abcam, ab210491), mouse anti-caspase-1 antibody (Santa Cruz, sc392736), rabbit anti-IL18 antibody (Proteintech, 10663-1-AP), rabbit anti-IL1beta antibody (ABclonal, A11370), rabbit anti-E-cadherin antibody (Proteintech, 20874-1-AP), and rabbit anti-αSMA antibody (Proteintech, 55135-1-AP). Then, the membranes were incubated with the corresponding secondary antibody (HRP-tagged goat anti-mouse or anti-rabbit IgG) and detected by enhanced chemiluminescence reagents (ECL, Millipore, USA). Quantitative analysis was performed using the ImageJ software.

2.7. Immunofluorescence Staining. Cells were fixed with 4% paraformaldehyde for 20 minutes, permeabilized in 0.5% Triton X-100 for 10 minutes, and then blocked with 1% goat serum for 1 hour at room temperature. Then, cells were incubated with mouse anti-GCN5L1 antibody (Santa Cruz, sc515444), rabbit anti-E-cadherin antibody (Proteintech, 20874-1-AP), and rabbit anti-αSMA antibody (Proteintech, 55135-1-AP) overnight at 4°C. Secondary antibody (Alexa Fluor 488 Goat Anti-Rabbit IgG H&L, ab150077, 1 : 500) was used to stain the cells, and DAPI nuclear stain was used to counterstain. Images were obtained using a Nikon microscope.

2.8. Intercellular ROS. In vitro, intercellular ROS was determined by the addition of MitoSOX (5 μM) to the cells which incubated at 37°C for 10 min in darkness. In vivo, superoxide production in renal tissues was measured in frozen kidney sections exposed to 6 μm dihydroethidium (DHE) at 37°C for 30 min and protected from light (DHE, Beyotime Institute of Biotechnology, Shanghai, China). The fluorescence intensity of intracellular ROS was captured using Nikon microscope imaging system (Nikon, Tokyo, Japan).

2.9. MnSOD Activity. The MnSOD activity was analysed in the kidney cortex and cell homogenates using commercial kits according to the manufacturer's instructions (WST-8; Beyotime Institute of Biotechnology, Shanghai, China).

2.10. Immunoprecipitation. The immunoprecipitation assay was performed as described previously [18]. In brief, 1 mg cell lysates was incubated with 10 μg antibody and 10 μl protein A/G resin at 4°C overnight. Next day, the protein was eluted from the resin with elution buffer and analysed by western blotting assay.

2.11. In Situ Proximity Ligation Assay (In Situ PLA). The in situ proximity ligation assay was carried out as described previously to detect the interaction between GCN5L1 with MnSOD using mouse anti-GCN5L1 antibody (Santa Cruz, sc515444) and anti-MnSOD antibody (Proteintech, 24127-1-AP) [19]. Representative images of the fluorescence were obtained using a Nikon microscope.

2.12. Quantitative Real-Time PCR. Total RNA was extracted from specimens using TRIzol reagent and converted to cDNA using reverse transcriptase kits (Takara, RR047A). qRT-PCR was carried out using SYBR Premix Ex Taq II (Takara, RR820L) as described previously [19]. Sequence-specific primers used were as follows: mouse-GCN5L1 Fwd: 5′-AGAACTGGGCTAGGAGCATC-3′, Rev: 5′-AGCTGC CCTTTGTAGACGTA-3′ and mouse-β-actin Fwd: 5′ -TGCGTGACATCAAAGAGAAG-3′, Rev: 5′-TCCATA CCCAAGAAGGAAGG-3′.

2.13. Statistical Analysis. Each experiment was performed at least for three times. All data were analysed using GraphPad Prism 6.0, with Student's t-test (comparison between two groups) or one-way ANOVA (comparison among multiple groups). $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. The Expression of GCN5L1 Is Significantly Elevated in Kidney Tissues from Diabetic Kidney Disease Patients and Mouse Models. To investigate whether GCN5L1 expression is associated with diabetic kidney disease, IHC staining was performed to evaluate the protein levels of GCN5L1 in renal tissues. Normal tissues from adjacent normal tissues of patients with renal cell carcinoma and kidney biopsy samples from patients with diabetic kidney disease were used in this study. The results showed that the protein level of GCN5L1 underwent significant increase in the human kidneys with DKD, which was measured by IHC (Figure 1(a)). To further study the role of GCN5L1 in DKD, we next established a DKD mouse model by STZ treatment. The renal injury of STZ-induced DKD mice was confirmed by enhanced urine albumin-to-creatinine ratio (UACR) (Figure 1(b)). Renal morphological examination by hematoxylin and eosin staining, Masson's trichrome staining, and periodic acid-Schiff staining indicated that mesangial matrix deposition and renal tubule vacuolation were aggravated in STZ-induced DN mice compared with control mice (Figure 1(c)). The GCN5L1 expression was also increased in kidney tissues of STZ-induced DKD mice by IHC and western blotting (Figures 1(d)–1(f)). These results showed that GCN5L1 was overexpressed in the DKD kidneys, which indicated the possible involvement of GCN5L1 in the pathogenesis and progression of DKD.

3.2. Reduction of GCN5L1 Ameliorates Renal Tubulointerstitial Injury and Proteinuria in DKD Mice. To further elucidate the importance of GCN5L1 in regulating renal injury, GCN5L1 shRNA-expressing adenovirus-associated virus was used to induce kidney-specific GCN5L1 knockdown (KD) mice. Firstly, GCN5L1 shRNA-expressing AAV was locally injected into the renal cortex of C57BL/6 mice, along with STZ treatment for about sixteen weeks to generate diabetic GCN5L1 KD mice (Figure 2(a)). The efficiency of GCN5L1 knockdown in the kidney was confirmed by western blotting, immunofluorescence staining, and IHC staining (Figures 2(b)–2(e), 2(g), and 2(h)). Then, we detected urinary albumin and creatinine to explore the effect of GCN5L1 in diabetic-induced renal injury. As shown in Figure 2(f), the levels of urine albumin-to-creatinine ratio (UACR) were reduced in diabetic GCN5L1 KD mice

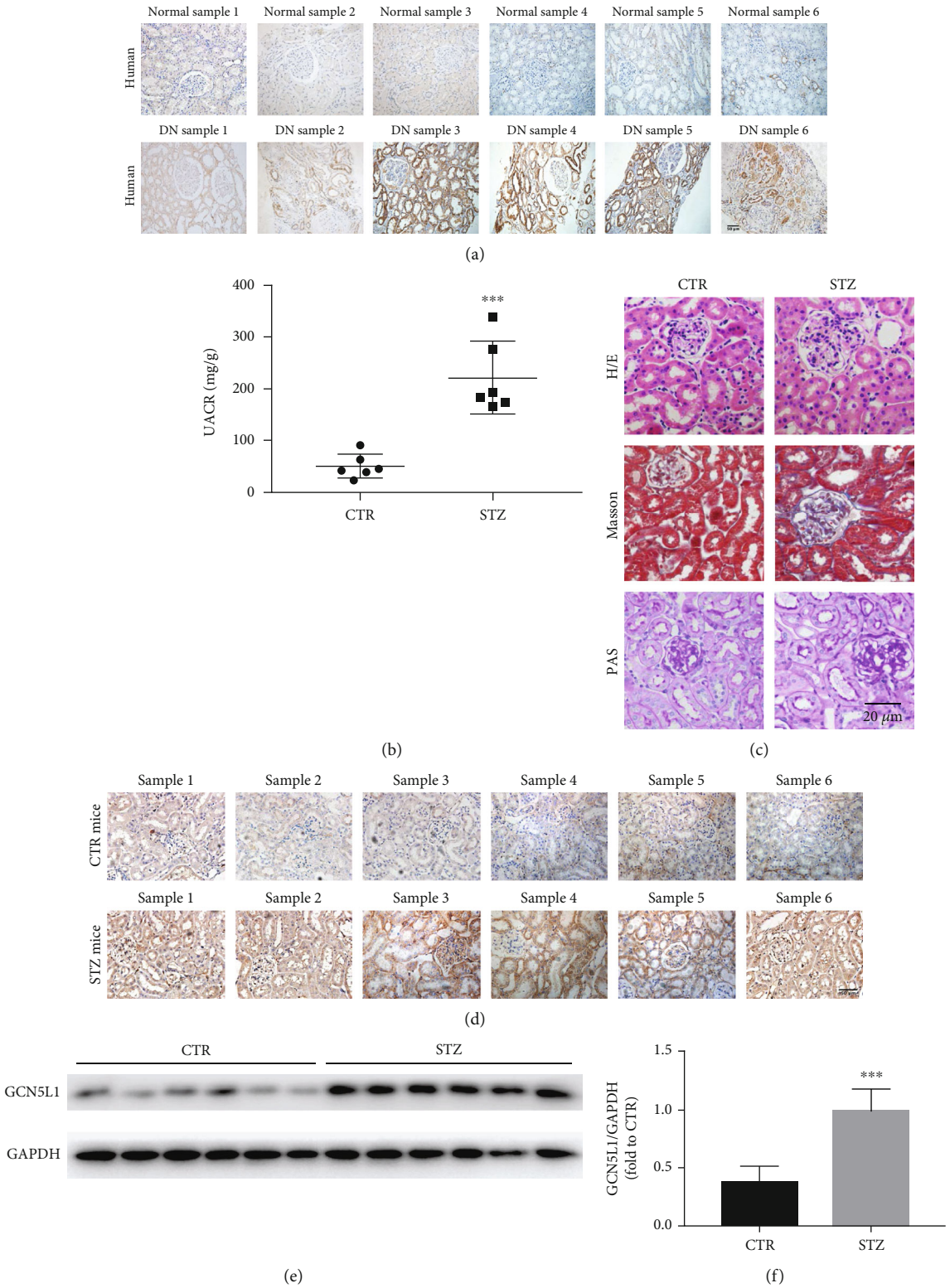
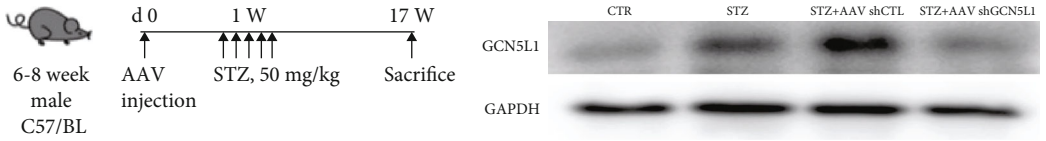
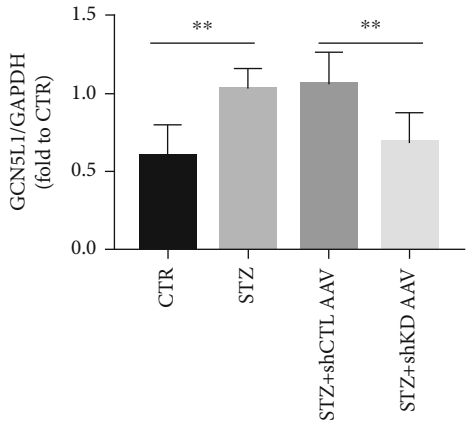


Figure 1: The expression of GCN5L1 is significantly elevated in kidney tissues from diabetic kidney disease patients and mouse models. (a) Immunohistochemistry staining for GCN5L1 expression in human renal biopsies. Scale bars = 50 μm. (b) Urine albumin-to-creatine ratio (UACR) in STZ-induced diabetic mice. Independent experiments were performed in triplicate. (c) Hematoxylin and eosin staining, Masson's trichrome staining, and periodic acid-Schiff staining in the kidneys from STZ-induced diabetic mice. Scale bars = 20 μm. (d) Immunohistochemistry staining for GCN5L1 expression in mouse kidney tissues. Scale bars = 50 μm. (e, f) Western blotting analysis of GCN5L1 protein expression in the kidneys from STZ-induced diabetic mice. Data are presented as the mean ± SD. $n = 6$; $^{***}P < 0.001$.



(a) (b)



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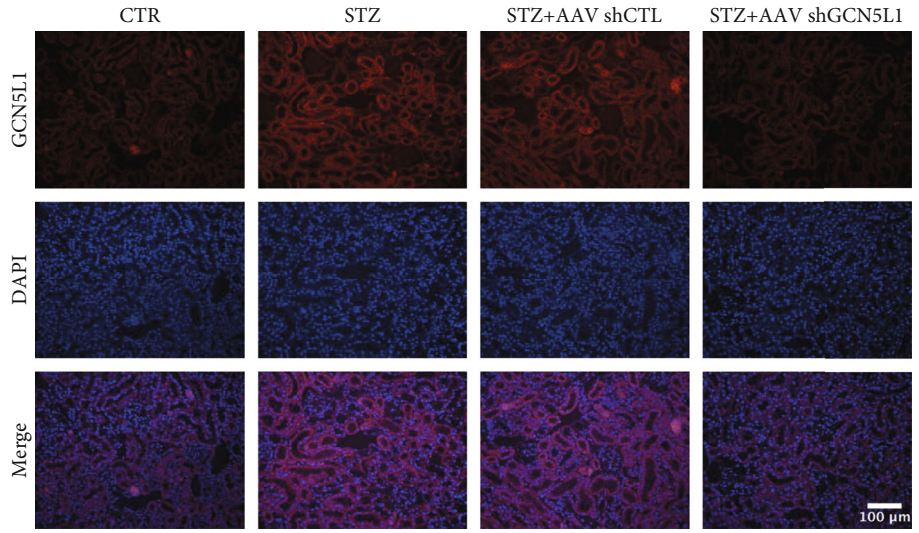

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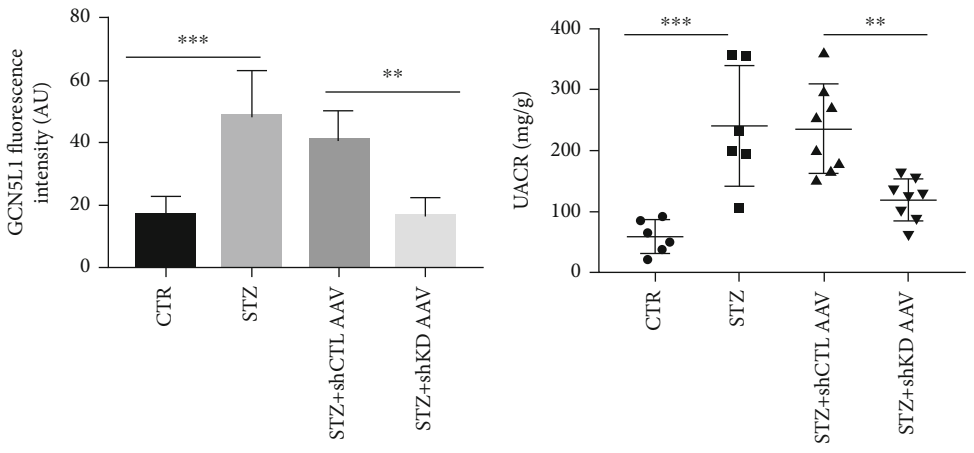

(e) (f)

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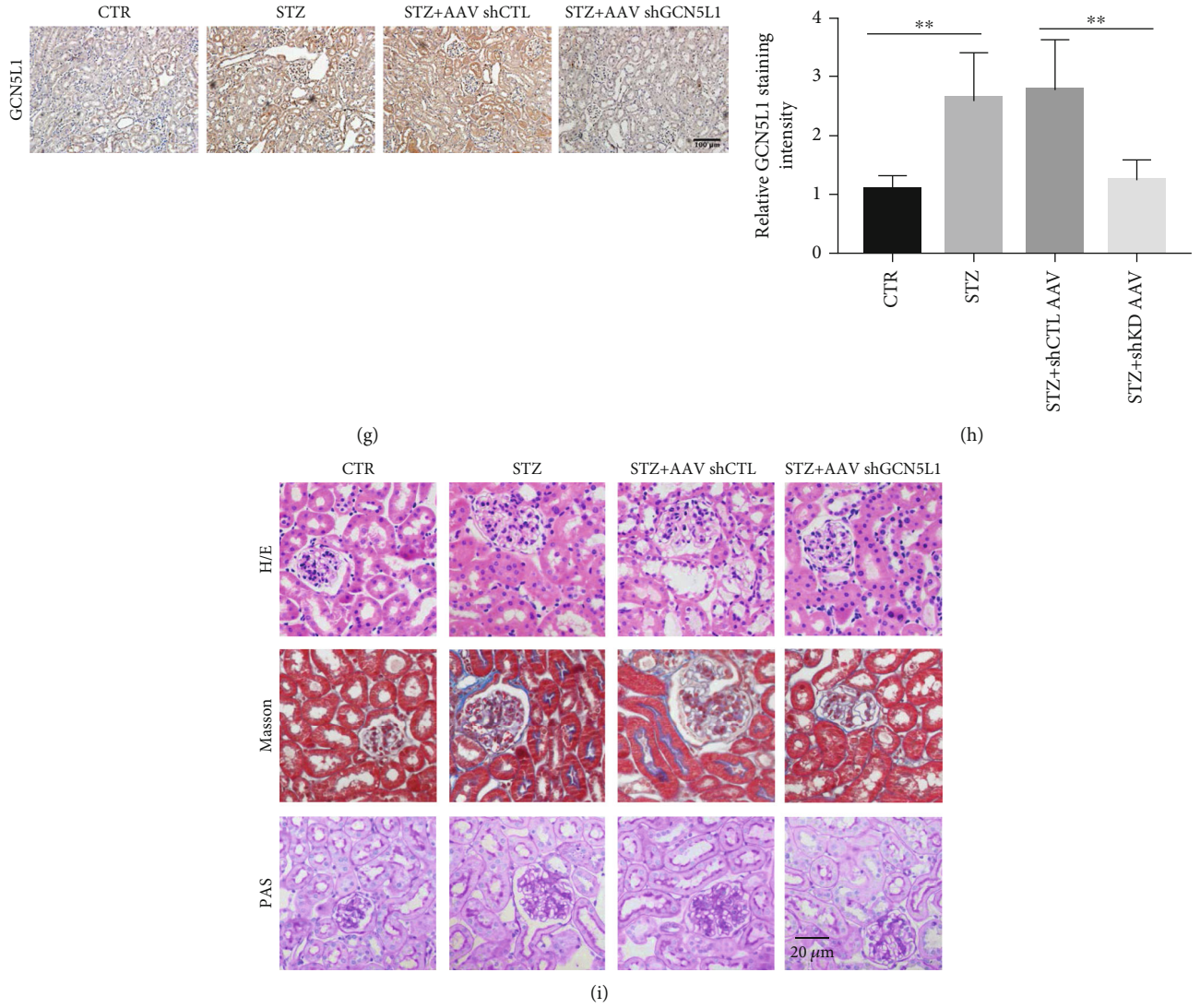
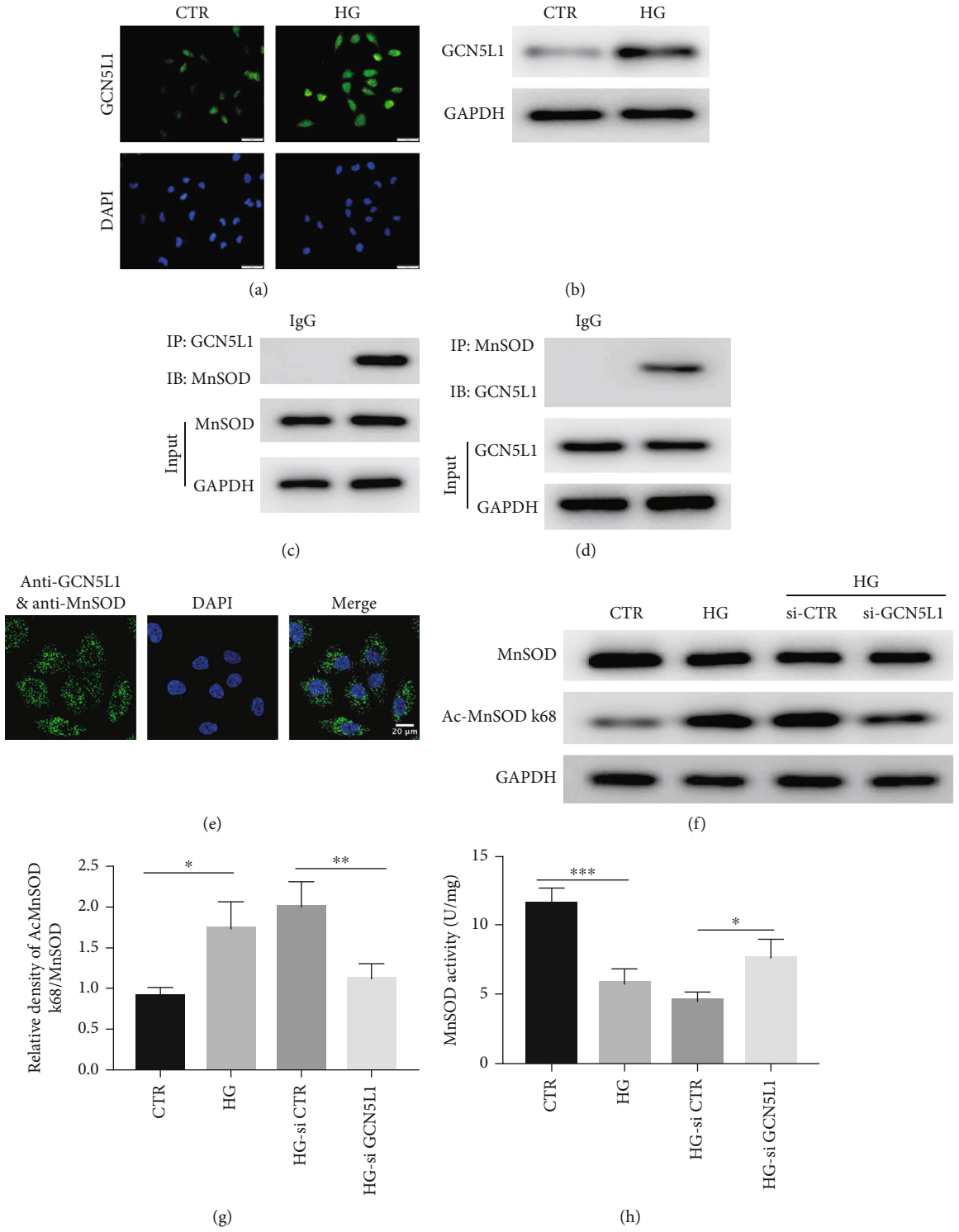


Figure 2: Reduction of GCN5L1 ameliorates renal tubulointerstitial injury and proteinuria in DKD mice. (a) Experimental design for STZ-induced diabetic GCN5L1 knockdown mice. (b, c) Western blotting analysis showing the expression of GCN5L1 in the kidneys from diabetic GCN5L1 knockdown mice. Data are presented as the mean ± SD. (d, e) Immunofluorescent staining and fluorescence intensity for GCN5L1 expression in kidney sections. Scale bars = 100 μm. (f) Urine albumin-to-creatinine ratio (UACR) in the kidneys from diabetic GCN5L1 knockdown mice. Independent experiments were performed in triplicate. (g, h) Immunohistochemistry staining and relative staining intensity for GCN5L1 expression in kidney sections. Scale bars = 100 μm. (i) Hematoxylin and eosin staining, Masson's trichrome staining, and periodic acid-Schiff staining in the kidneys from diabetic GCN5L1 knockdown mice. Scale bars = 20 μm. n = 6-8; $^{**}P < 0.01$ and $^{***}P < 0.001$.

compared with the control AAV-treated group, indicating that GCN5L1 KD alleviated STZ-induced renal injury. Consistently, in renal morphological analysis, diabetic GCN5L1 KD mice displayed decreased mesangial matrix expansion and renal tubule vacuolation in renal tissues compared with the diabetic control AAV group (Figure 2(i)). Together, these observations suggested that GCN5L1 plays a critical role in diabetic renal damage.

3.3. Downregulation of GCN5L1 Activates MnSOD to Scavenge ROS by Reducing MnSOD Acetylation on Lysine 68 in TECs Treated with High Glucose. In order to explore the specific mechanism of GCN5L1-mediated renal injury in DKD, we first applied tubular epithelial cells (TECs) to test the expression of GCN5L1 under high glucose in vitro. As shown in Figures 3(a) and 3(b), immunofluorescence staining and western blotting analysis revealed a significant elevation of GCN5L1 in TECs treated with high glucose. MnSOD, a major mitochondrial antioxidant enzyme, located in mitochondria, scavenged cellular ROS and was proved to exert an antioxidative stress effect in hyperglycemia-induced renal injury. Moreover, several studies have demonstrated that MnSOD is modified by acetylation and the acetylation level of MnSOD affects its enzyme activity and its ability of removing cellular ROS [10]. Mitochondria producing excessive ROS lead to oxidative stress in various energy excess conditions, including hyperglycemia [20]. We hypothesized that GCN5L1 may modulate the enzyme activity of MnSOD by



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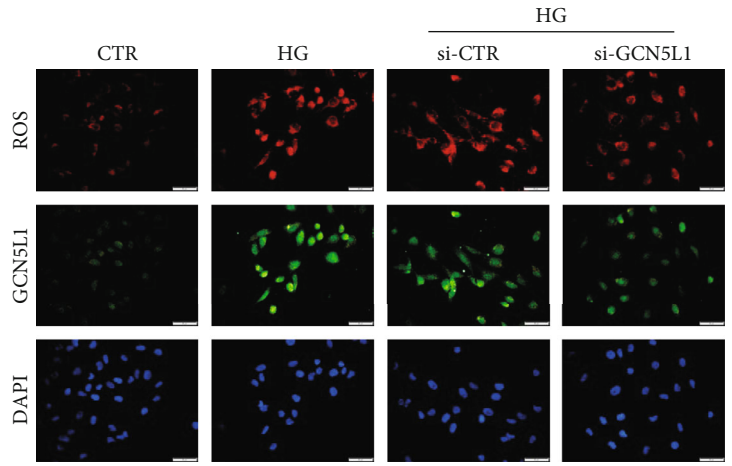


(i)

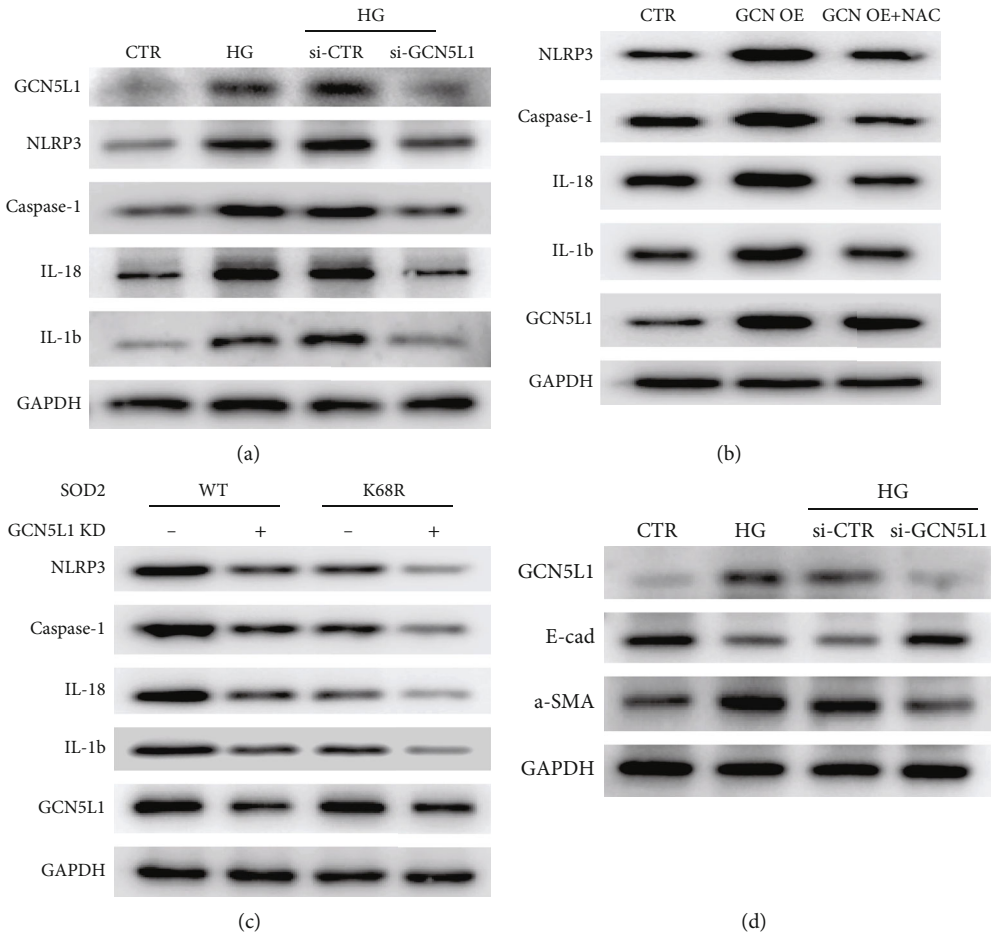
Figure 3: Downregulation of GCN5L1 activates MnSOD to scavenge ROS by reducing MnSOD acetylation on lysine 68 in TECs treated with high glucose. (a, b) Immunofluorescent staining and western blotting analysis of GCN5L1 protein expression in TECs exposed to high glucose. Scale bars = 50 μm. (c, d) Coimmunoprecipitation of MnSOD and GCN5L1 in TECs. (e) Interaction of MnSOD and GCN5L1 in TECs visualized by Duolink proximity ligation assay. (f, g) Western blotting analysis of the protein levels of MnSOD and Ac-MnSOD K68 in TECs exposed to high glucose following GCN5L1 knockdown. (h) MnSOD activity was assessed in TECs exposed to high glucose following GCN5L1 knockdown. Independent experiments were performed in triplicate. (i) Cellular ROS was detected by MitoSOX staining in GCN5L1 knockdown TECs treated with high glucose. Scale bars = 50 μm. Independent experiments were performed in triplicate. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

acetylation. First, we evidenced that MnSOD physically interacts with GCN5L1 by coimmunoprecipitation assay (Co-IP) and in situ proximity ligation assay (in situ PLA) (Figures 3(c)–3(e)). Then, the data showed that the acetylation level of MnSOD lysine 68 was upregulated after high-glucose treatment, while knockdown GCN5L1 could decrease the acetylation level of MnSOD^{K68} in high-glucose conditions (Figures 3(f) and 3(g)). Finally, to explore the further effect of GCN5L1 on MnSOD enzymatic detoxification activity, the MnSOD enzymatic activity and MitoSOX staining were determined. We found that GCN5L1 deletion rescued the enzymatic activity of MnSOD and diminished cellular ROS production under high-glucose conditions (Figures 3(h) and 3(i)).

3.4. Suppression of GCN5L1 Alleviates Inflammation and EMT through the MnSOD/ROS Pathway in TECs under High Glucose. Aberrant mitochondrial ROS production caused by diabetes is critical for NLRP3 inflammasome activation via the oxidation of mitochondrial DNA [21, 22]. Western blotting analysis revealed that the expression of NLRP3, caspase 1, IL18, and IL1b was enhanced in TECs treated with high glucose. In contrast, these protein expressions were completely reversed by GCN5L1 knockdown (Figure 4(a)). This suggested that GCN5L1 deletion may inhibit inflammation by eliminating cellular ROS production. To prove this hypothesis, N-acetyl-L-cysteine (NAC), a ROS inhibitor, was employed in GCN5L1-overexpressed TECs. Notably, NAC effectively blocked the GCN5L1 overexpression-mediated upregulation of NLRP3, caspase 1, IL18, and IL1b (Figure 4(b)). Then, transfection of the MnSOD$^{K68\text{-}R}$ mutant (lysine residue was replaced by arginine to mimic the deacetylated state) plasmid into GCN5L1 knockdown TECs significantly decreased the expression of NLRP3, caspase 1, IL18, and IL1b (Figure 4(c)). The results revealed that GCN5L1 acetylates MnSOD$^{K68\text{-}R}$ to affect inflammation through aberrant ROS-induced oxidative stress.

EMT could also be activated via reactive oxygen species and contributes to renal fibrosis related to diabetic nephropathy [23]. Under this context, whether GCN5L1 has an effect on EMT through the MnSOD/ROS pathway under high-glucose conditions has also aroused our attention. First, the epithelial marker E-cadherin and mesenchymal marker α-SMA were evaluated by both western blotting and immunofluorescence staining. As shown in Figures 5(d) and 5(e), E-cadherin was significantly decreased and α-SMA was obviously enhanced in TECs under high glucose, while knockdown of GCN5L1 could effectively reverse the expression of E-cadherin and α-SMA. In addition, the effects of ROS inhibitor NAC reversed the expression of E-cadherin and α-SMA mediated by GCN5L1 overexpression (Figure 4(f)). Then, we detected whether the effect of GCN5L1 on EMT was mediated by MnSOD acetylation. The roles of it were determined in TECs by cotransfection of siRNA of GCN5L1 and MnSOD$^{K68\text{-}R}$ mutant plasmid. Western blotting showed that GCN5L1 knockdown resulted in an increased expression of E-cadherin and a decreased expression of α-SMA, which was significantly reversed by additional MnSOD$^{K68\text{-}R}$ mutants (Figure 4(g)). All the results above suggested that GCN5L1 could acetylate MnSOD^{K68}, thereby mediating inflammation and EMT through mitochondrial ROS in vitro.

3.5. Downregulation of GCN5L1 Protects the Kidney from Hyperglycemia-Induced EMT and Inflammation via the MnSOD/ROS Pathway In Vivo. Then, we further explored



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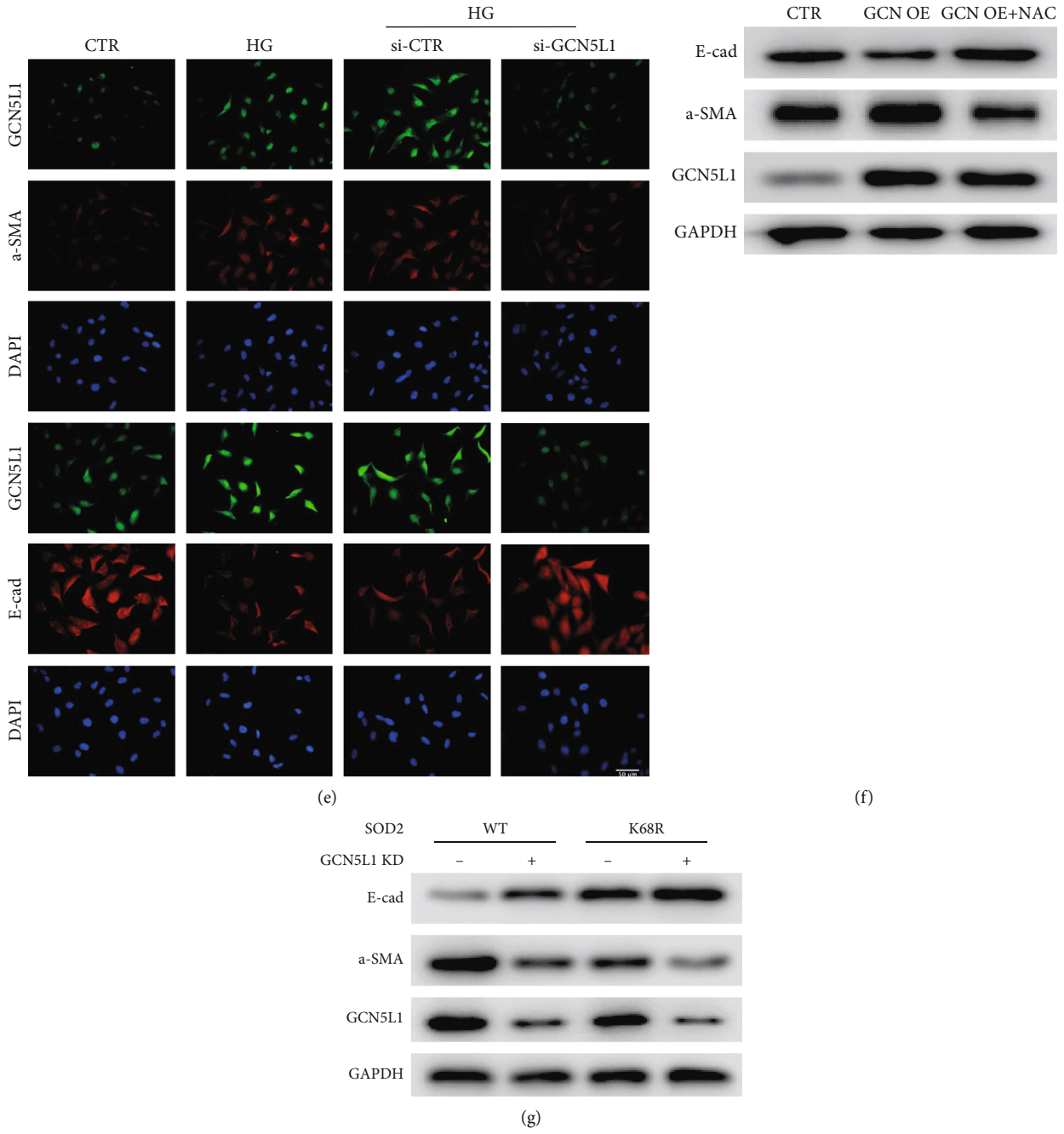
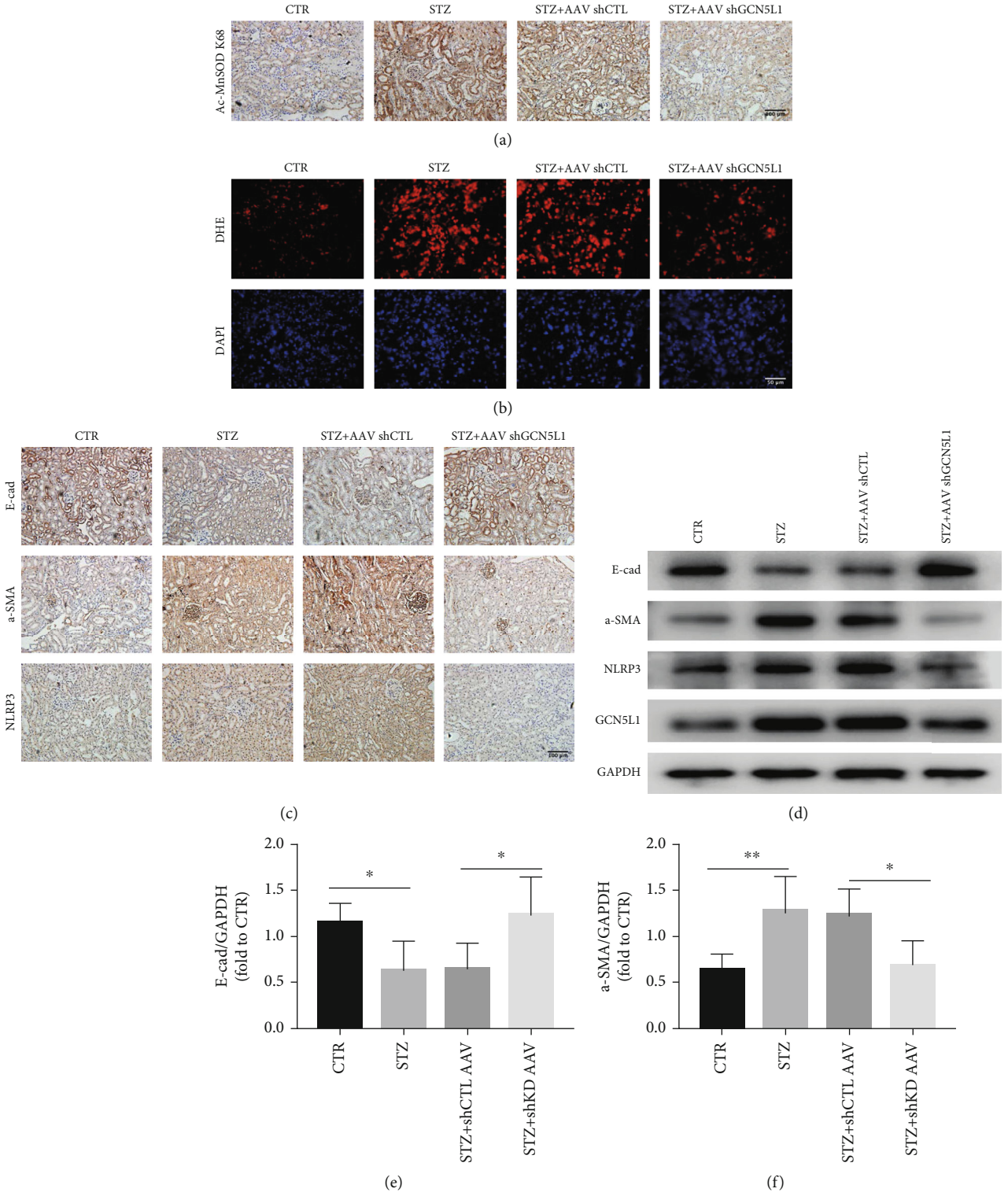


Figure 4: Suppression of GCN5L1 alleviates inflammation and EMT through the MnSOD/ROS pathway in TECs under high glucose. (a) Protein levels of NLRP3, caspase-1, IL18, and IL1beta were detected by western blotting in TECs exposed to high glucose following knockdown of GCN5L1. (b) Protein levels of NLRP3, caspase-1, IL18, and IL1beta were detected by western blotting in GCN5L1 overexpressed TECs treated with NAC. (c) Western blotting analysis of the protein levels of NLRP3, caspase-1, IL18, and IL1beta after cotransfected with MnSODK68-R mutant plasmid and GCN5L1 silencing siRNA. (d, e) Western blotting analysis and immunofluorescent staining for E-cadherin and α-SMA in TECs exposed to high glucose following knockdown of GCN5L1. Scale bars = 100 μm. (f) Protein levels of E-cadherin and α-SMA were detected by western blotting in GCN5L1 overexpressed TECs treated with NAC. (g) Western blotting analysis of the protein levels of E-cadherin and α-SMA after cotransfected with MnSODK68-R mutant plasmid and GCN5L1 silencing siRNA. Independent experiments were performed in triplicate.

the effect and mechanism of GCN5L1 on diabetic kidney injury in vivo. First, we determined the acetylation level of $MnSOD^{K68}$ in the mouse kidneys. Immunohistochemistry staining showed that GCN5L1 KD greatly diminished acetylation level of $MnSOD^{K68}$ induced by STZ (Figure 5(a)). Then, as stained with ROS-sensitive vital dye DHE, we found the enhanced ROS production induced by STZ in the mouse kidneys was abolished by silencing GCN5L1 (Figure 5(b)). Finally, immunohistochemistry assay revealed that diabetic mice exhibited a significant upregulation of NLRP3, which



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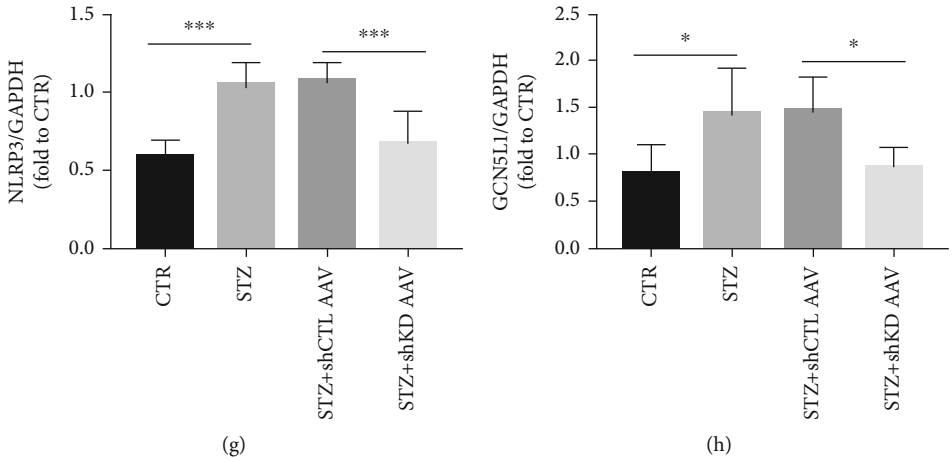

Figure 5: Downregulation of GCN5L1 protects the kidney from hyperglycemia-induced EMT and inflammation via the MnSOD/ROS pathway in vivo. (a) Immunohistochemistry staining for acetylation level of MnSODK68 in the kidneys from diabetic GCN5L1 knockdown mice. Scale bars = 100 μm. (b) DHE fluorescence to detect ROS in the kidneys from diabetic GCN5L1 knockdown mice. Scale bars = 50 μm. (c) Immunohistochemistry staining for E-cadherin, α-SMA, and NLRP3 expression in the kidneys of mice. Scale bars = 100 μm. (d–h) Western blotting analysis showing the expressions of E-cad, α-SMA, and NLRP3 in the kidneys of mice. Data are presented as the mean ± SD. n = 6-8; $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

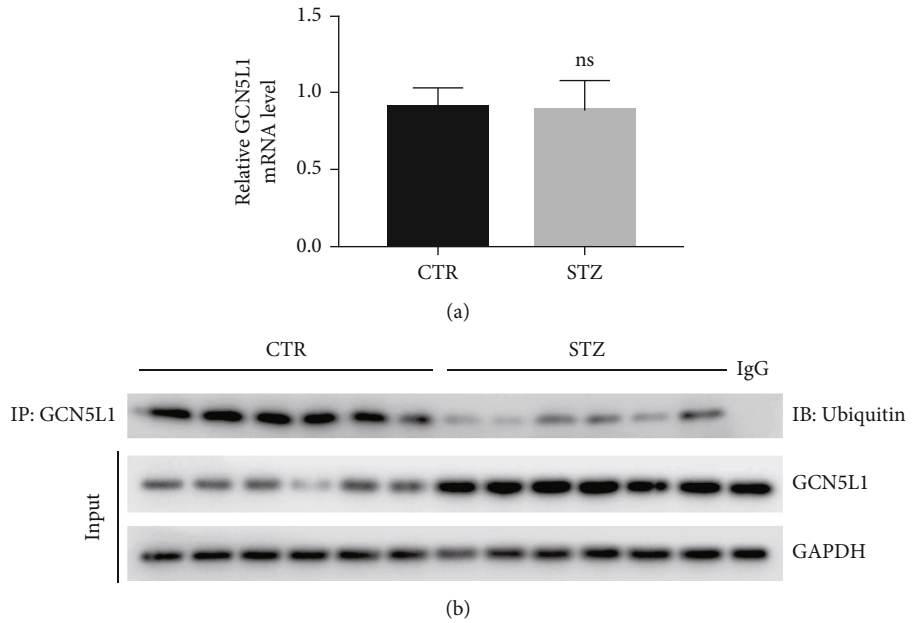

Figure 6: STZ treatment increases GCN5L1 expression by reducing its ubiquitination. (a) RNA levels of GCN5L1 were detected by RT-qPCR in the mouse kidneys. (b) The ubiquitination level of GCN5L1 was detected by immunoprecipitation assay. n = 6; ns: not significant.

were decreased by additional loss of GCN5L1 in the kidney compared with diabetic WT mice (Figure 5(c)). The same tendency was confirmed by western blotting analysis in the kidney (Figures 5(d) and 5(g)). Moreover, the renal fibrosis in diabetic mice was characterized by the loss of E-cadherin and the excess of α-SMA, which was almost completely reversed by GCN5L1 knockdown (Figures 5(c)–5(f)). Taken together, these results support that suppression of GCN5L1 protects the kidney from hyperglycemia-induced EMT and inflammation via the MnSOD/ROS pathway.

3.6. STZ Treatment Increases GCN5L1 Expression by Reducing Its Ubiquitination. In order to study the mechanism of increased GCN5L1 expression with STZ treatment, we first detected the mRNA expression of GCN5L1 under STZ treatment. According to the result of RT-qPCR, we found that this mRNA expression had no significant changes between the control and STZ groups (Figure 6(a)). Thus, we hypothesized that STZ treatment may increase GCN5L1 expression through a posttranslational mechanism. Ubiquitination is crucial for physiological processes which are

controlled by ubiquitin and deubiquitinating enzymes [24, 25]. One of the important cellular functions of the ubiquitin-proteasome system (UPS) is to selectively degrade damaged or abnormal proteins [26]. UPS has an important role in diabetic nephropathy [27]. Mitochondria possess about 1,000-1,500 proteins with multiple functions, and thus, the quality control of the mitochondrial proteome handled by UPS is very important for mitochondrial function. Thus, dysfunction of UPS may contribute to mitochondrial dysfunction and DN. Under these backgrounds, we used immunoprecipitation assay to detect the ubiquitination level of GCN5L1. The results showed that the ubiquitination level of GCN5L1 was decreased under the STZ treatment, which induced the GCN5L1 overexpression in protein level (Figure 6(b)). According to all of these results, we found that GCN5L1 overexpression was controlled by impaired ubiquitination.

4. Discussion

Accumulating evidences have identified that hyperglycemia-induced oxidative stress is of particular interest in the development and progression of DKD [2, 28], but its exact mechanisms involved remain largely unknown. A new sight has been established that the high expression of GCN5L1 contributes to oxidative stress-induced renal tubulointerstitial injury in DKD by mediating MnSOD hyperacetylation and inactivating its enzymatic detoxification activity. Collectively, we identified that GCN5L1 could participate in cellular redox homeostasis maintenance in DKD.

GCN5L1, given its sequence homology to the nuclear acetyltransferase Gcn5 and its mitochondrial enrichment, has recently been recognized as an indispensable element in the machinery of mitochondrial acetyltransferases and has been proved to counter the activity of SIRT3, a mitochondrial deacetylase protein [11]. The pathogenic role of GCN5L1 has been reported in the past few years [29]. Study conducted in mouse embryonic fibroblasts illustrated that knockout of GCN5L1 has the effects on inducing mitochondrial biogenesis and mitophagy via the activation of PGC-1α and TFEB [12]. Upregulation of cardiac GCN5L1 in response to high-fat diet participates to the fatty acid oxidation rate by increasing the level of acetylation and the activity of FAO enzyme [13]. Furthermore, another study reported that depletion of GCN5L1 mediated ERK activation and blunted hepatic gluconeogenesis [30]. In renal disease, we previously demonstrated that GCN5L1 could upregulate both the acetylation and activity of several mitochondrial fatty acid oxidation enzymes, thus regulating fatty acid oxidation in the kidney [18]. Nevertheless, the role of GCN5L1 in the initiation and progression of DKD is still unclear. In the present study, we concentrated on exploring the alteration of GCN5L1 in DKD and investigating the effect of downregulation of GCN5L1 on oxidative stress-induced renal tubulointerstitial injury in DKD. First, we found that GCN5L1 expression was significantly increased not only in the kidney tissues of DKD patients and mouse models but also in renal TECs treated with high glucose. Second, the renal tubulointerstitial injury was alleviated in STZ-DKD mice treated with sh-GCN5L1 AAV. Furthermore, the genetic ablation of GCN5L1 in TECs ameliorated oxidative stress-induced EMT and inflammation both in vivo and in vitro. Taken together, the results above suggest that GCN5L1 could affect oxidative stress-induced renal damage under hyperglycemic conditions and targeting GCN5L1 could be an attractive therapeutic target of DKD.

As one of the most energy-demanding organs, the kidney requires a great deal of mitochondria to produce sufficient adenosine triphosphate (ATP) through oxidative metabolism; at the same time, reactive oxygen species (ROS) were also generated as a by-product. It has been reported that hyperglycemia could induce excessive production of ROS, thereby leading to oxidative stress [31]. There are numerous evidences showing that enhanced oxidative stress (OS) is an important feature in CKD patients from early to late stage [32, 33]. Excessive OS is caused by the dysfunction of the balance between antioxidant defense mechanisms and oxidant products [34]. Therefore, the sources of excessive OS can be divided into two main parts. One is excessive OS produced like mitochondria dysfunction, disorder of metabolic networks, and impairment of mitophagy [35]. The other is the defect of the antioxidant system including the decrease of small-molecule antioxidants such as vitamins C and E and the tripeptide glutathione (GSH), dysfunction of enzymes whose specific role is the neutralization of ROS like superoxide dismutase (SOD), catalase, and GSH peroxidases (GPx) [36]. The mitochondrial antioxidant enzyme system is the major defense to eliminate superoxide free radicals, which is related to the pathophysiology of DKD.

As the key antioxidant enzyme in mitochondria, MnSOD could scavenge aberrant ROS to protect cells against oxidative stress. Several posttranslational modulation (PTM) patterns of MnSOD have been reported, such as nitration [37], acetylation [38], and ubiquitination [39], among which acetylation of MnSOD is one of the main drivers of its enzyme activity which has attracted widespread attention and been reported in various kinds of diseases, such as breast cancer [40] and cardiac diseases [41]. Current studies have revealed that elevation of lysine acetylation decreases MnSOD enzymatic activity, which is in accordance with our present study. The upstream mechanism of regulating MnSOD lysine acetylation is mostly concentrated on the mitochondrial deacetylase enzyme SIRT3, which deacetylated MnSOD on different reversible acetylated lysine residues. He and his colleagues have found that SIRT3-mediated deacetylation of lysine 68 regulates stem cell reprogramming in breast cancer [40]. Other researches have also reported that SIRT3 could also regulate the acetylation level of MnSOD lysine 53, 89, and 122 [10, 42]. In addition, Thapa and his colleagues reported that GCN5L1 is acetylating MnSOD at K122 in cardiomyocyte [43]. According to the published paper, acetylation of MnSOD K68 lysine residue can transform from a known homotetramer complex to a monomeric form. These monomers function as a peroxidase, distinct from the established MnSOD superoxide dismutase activity. Therefore, K68 lysine residue acetylation decreased the enzyme activity. Moreover, acetylation of other sites like K122 had no this effect [38]. According to all of these papers, we focused on K68 site

and hypothesized that this site acetylation can lower the activity of MnSOD. According to our results, we shed new light on the mitochondrial acetyltransferase GCN5L1 which could acetylate MnSOD K68 lysine residues, thereby diminishing MnSOD enzymatic detoxification activity. Furthermore, enhanced abundance of $MnSOD^{K68}$ acetylation and reduced MnSOD activity were displayed in the DKD mouse kidneys and hyperglycemic TECs. Moreover, silencing GCN5L1 declined $MnSOD^{K68}$ acetylation state but improved its ability to scavenge ROS to prevent oxidative stress and cellular damage in DKD. Moreover, we found that GCN5L1 overexpression under STZ treatment was induced by impaired ubiquitination. The exact molecules controlled this process and their roles in DN should be studied further. In general, we have discovered the acetyltransferase GCN5L1 could acetylate MnSOD, providing a new mechanism to improve renal injury in DKD.

5. Conclusions

In summary, our present study demonstrated that GCN5L1 mediates MnSOD acetylation on lysine 68 to regulate its detoxification activity, thereby affecting oxidative stress-induced renal tubulointerstitial injury in DKD. Hence, GCN5L1 might be a key regulator of renal oxidative stress and function as a potential therapeutic target in DKD.

6. Limitations of the Study

First, although we used adenovirus-associated virus to knock down GCN5L1 in the mouse kidneys to explore the role of GCN5L1 in DKD, it would be perfect to apply conditional TECs GCN5L1 knockout mice. Second, we explored that GCN5L1 abundance under STZ treatment was dependent on a posttranslational mechanism, but which molecules controlled this process and their roles in DN were unclear.

Data Availability

The data supporting the findings of this study could be obtained from the corresponding author.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

Acknowledgments

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Supplementary Materials

Supplementary Table S1: the blood glucose data of mice. Supplementary Figure S1: the actual blood glucose data of mice. Supplementary Figure S2: NAC impairs the NLRP3 activation and EMT induced by GCN5L1 overexpression. Three times of independent experiments were performed for detecting the effect of NAC on protein expression. Supplementary Figure S3: NAC has no significant effect on MnSOD activity and acetylation. (a) MnSOD activity was assessed in TECs treated with NAC. (b) Western blotting analysis of the protein levels of Ac-MnSOD K68 in TECs treated with NAC. *(Supplementary Materials)*

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Review Article

Plasma Protein Carbonyls as Biomarkers of Oxidative Stress in Chronic Kidney Disease, Dialysis, and Transplantation

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Accumulating evidence indicates that oxidative stress plays a role in the pathophysiology of chronic kidney disease (CKD) and its progression; during renal replacement therapy, oxidative stress-derived oxidative damage also contributes to the development of CKD systemic complications, such as cardiovascular disease, hypertension, atherosclerosis, inflammation, anaemia, and impaired host defence. The main mechanism underlying these events is the retention of uremic toxins, which act as a substrate for oxidative processes and elicit the activation of inflammatory pathways targeting endothelial and immune cells. Due to the growing worldwide spread of CKD, there is an overwhelming need to find oxidative damage biomarkers that are easy to measure in biological fluids of subjects with CKD and patients undergoing renal replacement therapy (haemodialysis, peritoneal dialysis, and kidney transplantation), in order to overcome limitations of invasive monitoring of CKD progression. Several studies investigated biomarkers of protein oxidative damage in CKD, including plasma protein carbonyls (PCO), the most frequently used biomarker of protein damage. This review provides an up-to-date overview on advances concerning the correlation between plasma protein carbonylation in CKD progression (from stage 1 to stage 5) and the possibility that haemodialysis, peritoneal dialysis, and kidney transplantation improve plasma PCO levels. Despite the fact that the role of plasma PCO in CKD is often underestimated in clinical practice, emerging evidence highlights that plasma PCO can serve as good biomarkers of oxidative stress in CKD and substitutive therapies. Whether plasma PCO levels merely serve as biomarkers of CKD-related oxidative stress or whether they are associated with the pathogenesis of CKD complications deserves further evaluation.

1. Introduction

Chronic kidney disease (CKD) has a worldwide prevalence of around 8-16%, and it is declared by the World Health Organization (WHO) as an ever-increasing public health problem [1]. CKD is usually characterized by albuminuria and/or decreased glomerular filtration rate (GFR), which is the volume of plasma filtered by the glomeruli per unit of time. A five-stage classification system for CKD has been established by the Kidney Disease Improving Global Outcomes (KDIGO) (Figure 1) [2]. Patients with stage 1-3 CKD are frequently asymptomatic. In CKD stages 1 and 2, GFR may be

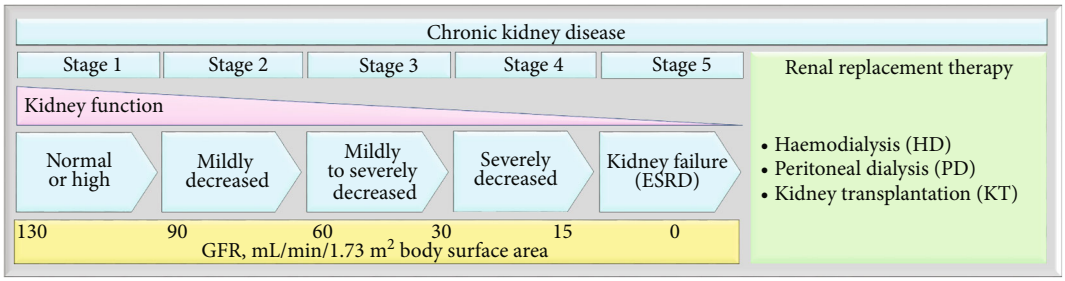

Figure 1: Five-stage classification system for CKD. During the progression of CKD, the decrease in kidney function, evaluated by the glomerular filtration rate (GFR), leads to a variety of disturbances in body homeostasis. The accumulation of uremic toxins, the increase in signs of volume overload, the worsening of hypertension, and the induction of metabolic and hormonal disturbances are typical of CKD patients. The progression of CKD often leads to a decline in residual renal function (RRF), eventually leading to renal replacement therapy (i.e., haemodialysis, peritoneal dialysis, and kidney transplantation).

normal or borderline normal; hence, reduced GFR alone does not clinch the diagnosis. Other elements due to tubular disorders, such as albuminuria, the presence of a pathological urine sediment, electrolyte unbalance, or histologic and structural abnormalities detected by imaging, can be useful to establish the diagnosis of CKD stages 1 and 2 [2]. Clinical manifestations from low kidney function typically appear in stages 4 and 5. Patients with CKD show a progressive decline in kidney function, and they develop end-stage renal disease (ESRD, i.e., CKD stage 5), where renal replacement therapy (RRT) is needed to ensure ESRD patient survival. RRT is achieved by haemodialysis (HD), peritoneal dialysis (PD), and/or kidney transplantation (KT). Compared to the general population, CKD patients have a higher risk of premature death, primarily because of cardiovascular diseases (CVDs) [3, 4]. Traditional risk factors (such as age, diabetes, left ventricular hypertrophy, dyslipidemia, and hypertension) are predictive of CVD mortality in CKD patients [5]. In addition, CVD can also arise from nontraditional risk factors, including inflammation and oxidative stress, which are highly prevalent in CKD patients [4, 6–9].

Inflammation and oxidative stress interplay in a self-perpetuating vicious circle and drive CKD progression, CVD, and other numerous complications such as malnutrition, atherosclerosis, coronary artery calcification, heart failure, anaemia, and mineral and bone disorders [10–13]. In fact, patients with CKD typically suffer from chronic inflammation and have severely impaired antioxidant systems, which worsen gradually with the progression of renal failure [10]. Inflammation is characterized by an increase in inflammatory markers, including cytokines (such as interleukin-6, interleukin-1, tumour necrosis factor-α, and adipokines), acute phase proteins (mainly C-reactive protein), and adhesion molecules, which are associated with many complications during CKD, as clinical studies have demonstrated [11]. Many factors contribute to chronic inflammation in CKD, including the increased production of proinflammatory cytokines, oxidative stress, acidosis, chronic and recurrent infections, intestinal dysbiosis, and altered adipose tissue metabolism [12]. Inflammation contributes to the progression of CKD, oxidative stress, insulin resistance, endothelial dysfunction, mineral and bone disease, anaemia, and resistance to erythropoietin [11]. High levels of oxidative stress have been found in the early stages of CKD, which increase in parallel with the progression to ESRD [13] and even more in patients undergoing HD [14, 15]. In particular, HD induces inflammation and oxidative stress due to loss of antioxidants during the dialysis session and activation of white blood cells, which generate ROS [16]. Compared to HD, PD is a more biocompatible dialysis modality that induces a lower level of oxidative stress, mainly due to the composition of PD solutions (low pH, high lactate content, increased osmolarity, high glucose concentration, and related degradation products) [17, 18]. In particular, HD induces inflammation and oxidative stress due to loss of antioxidants during the dialysis session and activation of white blood cells, which generate ROS [19]. Compared to HD, PD is a more biocompatible dialysis modality that induces a lower level of oxidative stress, mainly due to the composition of PD solutions (low pH, high lactate content, increased osmolarity, high glucose concentration and related degradation products) [20, 21].

Oxidative stress has also been associated with the production of highly reactive intermediates during inflammation; ROS, for their part, further enhance the inflammatory response by triggering proinflammatory mediators. In the kidneys, ROS are mainly produced by the mitochondrial respiratory chain and by the different isoforms of the enzyme NADPH oxidase. Oxidative stress is responsible for progressive renal damage, which can lead to renal ischemia, lesions to the glomeruli, cell death, and apoptosis, exacerbating the severe inflammatory processes. Further, oxidative stress is also responsible for several risk factors for CKD, such as diabetes, hypertension, and atherosclerosis [11]. Several biomarkers of oxidative stress, such as malondialdehyde, oxidized low-density lipoprotein, advanced glycation end products, and 7,8-dihydro-8-oxo-2′-deoxyguanosine, have increased levels in patients with CKD [11]. However, their specificity as a biomarker of oxidative stress can be questionable, as in the case of oxidized low-density lipoprotein, which is most commonly measured in plasma or isolated lipoprotein by immunological methods using one of three different antibodies, each of which has methodological limitations [22]. All methods available for the detection of malondialdehyde show pitfalls, including the numerous commercial kits

that lack specificity, making their significance for clinical practice dubious [22]. The thiobarbituric acid reactive substance assay to detect malondialdehyde reveals reproducibility and reliability when combined with HPLC, although it requires individual sample processing and its validity as a biomarker of *in vivo* oxidative stress remains uncertain, making it less suitable for routine clinical use [22].

CKD is also characterized by the accumulation of uremic toxins released from the intestinal tract, which have become clinically relevant in CKD progression and are tightly related to many CKD-associated systemic complications, including inflammation, oxidative stress, and decreased production of nitric oxide by endothelial cells [23]. The proinflammatory state, the enhanced oxidative stress, and the accumulation of uremic toxins also cause endothelial damage. Under uremia, endothelial cells produce danger-associated molecular patterns (molecules released by stressed, damaged, or necrotic cells that act as endogenous danger signals to promote and perpetuate a noninfectious inflammatory response), which induce the expression of adhesion molecules, the production of proinflammatory cytokines, and an enhanced production of ROS in endothelial cells [24]. Uremic toxins are involved in the inflammatory state in CKD and contribute to many uremia-associated dysfunctions [11]. In fact, several studies have shown that uremic toxins increase the levels of TNF-α and IL-6 and cause an exacerbation of the inflammatory state through the increase in ROS production [11].

Protein carbonyls (PCO) are among the most successful biomarkers of oxidative stress and are associated with disease state and treatment in multiple illnesses [22, 25–28]. The easy sampling of plasma proteins and the relatively long half-life of many of them make plasma PCO an attractive biomarker of oxidative stress in CKD [29, 30]. The most commonly used methods for quantifying PCO rely on derivatization with 2,4-dinitrophenylhydrazine (DNPH), which specifically reacts with PCO associated with aldehydes and ketones but does not react with other carbonyl-containing functional groups such as carboxylic acids and esters. DNPH generates the stable 2,4-dinitrophenylhydrazone (DNP) adduct (Figure 2) that absorbs UV light; therefore, PCO can be detected by a spectrophotometric assay [31]. DNPH-derivatized PCO can also be detected by specific anti-DNP antibodies by enzyme-linked immunosorbent assay (ELISA) and Western blot. The ELISA makes use of biotin-linked anti-DNP antibodies that bind DNP-derivatized proteins and allow detection with streptavidin-HRP [32, 33]. Carbonylation of specific plasma proteins is often detected by Western blot. After derivatization with DNPH, plasma proteins are separated by one-dimensional sodium dodecyl sulphate polyacrylamide gel electrophoresis (1D SDS-PAGE) or by two-dimensional gel electrophoresis (2D-GE), electrotransferred from the gel to a polyvinylidene fluoride (PVDF) membrane, and then, PCO can be immunodetected using primary anti-DNP antibodies and horseradish peroxidase- (HRP-) conjugated secondary antibodies [34, 35]. For mass spectrometry (MS) analysis, protein bands (or spots) can be excised from the gel, in-gel reduced, thiol-alkylated, digested with trypsin, and identified by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF)/MS mass fingerprinting [36–38]. PCO can also be detected by fluoresceinamine, a molecule that, unlike DNPH, labels PCO derived from metal-catalysed oxidative modification. Climent and colleagues demonstrated that fluoresceinamine labels specifically the γ-glutamyl semialdehyde group [39].

In this review, we summarize and discuss the main studies that have assessed plasma PCO levels in CKD, dialysis, and kidney transplantation and the potential role of protein carbonylation in driving CKD progression.

2. Plasma Protein Carbonyls in CKD

Although a high prevalence of oxidative stress in CKD is now well-established [4, 6, 30], few studies measured biomarkers of oxidative stress in people with CKD. Increased oxidative stress in patients with CKD stage 3 or higher, including ESRD, is demonstrated by an increase in plasma protein thiol oxidation, PCO, advanced oxidation protein products (AOPPs), and protein-bound di-tyrosines ([40–42] and citations therein). However, the properties of the oxidative modifications, e.g., the transience of cysteine modifications, their low abundance, e.g., protein-bound di-tyrosines, or methodological issues concerning the reproducibility, accuracy, and reliability of their detection, e.g., AOPPs, limit their applicability in clinical practice. Considering that PCO are chemically stable, their concentration is often higher than that of other biomarkers (since PCO formation can derive from different mechanisms), and validated detection methods are available [22, 34, 43], the plasma PCO assay has some advantages over other methods to assess oxidative stress and protein oxidative damage in clinical practice. In particular, the methods that seem to be most applicable in clinical settings are ELISA, as commercial kits are available, and HPLC, both of which allow for the rapid processing of many samples, the use of internal/external standards, and comparison of samples under constant conditions [22].

Eight studies examined the plasma PCO level in patients with CKD at various stages by spectrophotometric assay and ELISA (Table 1). Carbonylation of individual plasma proteins was measured by Western blot only in one study [44]. Four out of eight studies examined plasma PCO levels in patients with CKD compared with healthy subjects [45–47], while the other four examined plasma PCO levels in CKD patients at various stages [48–51].

Oberg et al. [45] compared 60 adult/elderly patients with CKD stages 3-5 (67 ± 14 years, 29 of whom with diabetes mellitus) and healthy control subjects (51.4 ± 1.7 years) (Table 1), showing that plasma PCO levels were significantly higher in patients with CKD than in healthy control subjects, as subsequently confirmed by other studies [46, 48]. As no significant correlation between GFR and plasma PCO content was observed [45], the authors suggested that PCO can undergo renal clearance primarily via renal tubular metabolism rather than glomerular filtration; plasma PCO content could therefore be largely regulated by proximal tubular function [45].

The plasma PCO level increased in parallel with decreased renal function (measured as creatinine clearance)

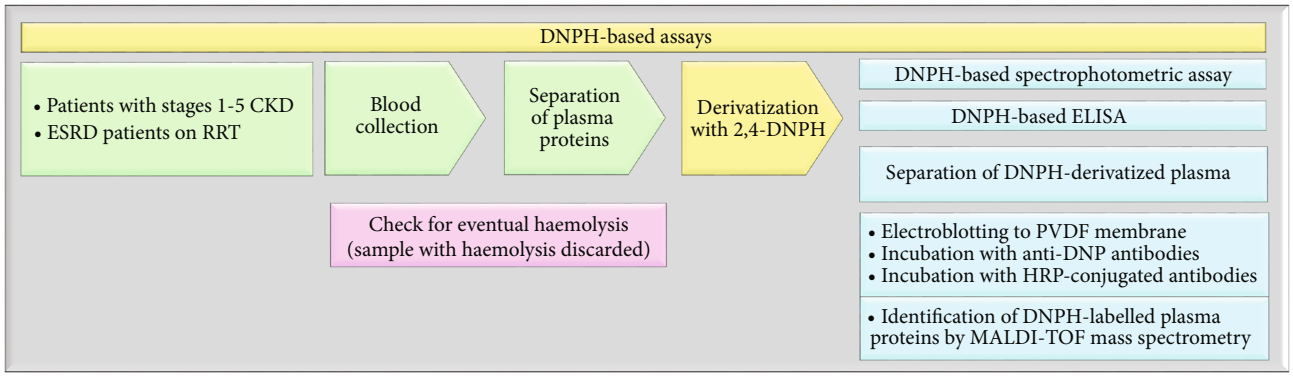

Figure 2: DNPH-based assays for PCO detection. Assays for the detection of PCO involve the derivatization with 2,4-DNPH, leading to the formation of a stable dinitrophenylhydrazone product. PVDF: polyvinylidene fluoride membrane; HRP: horseradish peroxidase; MALDI-TOF: matrix-assisted laser desorption/ionization time-of-flight; RRT: renal replacement therapy.

($R = -0.692$, $p < 0.0001$) in elderly patients (60.9 ± 15.2 years) with CKD at stages 1-5 [48]; in addition, a significantly positive correlation was observed between plasma PCO and blood urea nitrogen (BUN) ($R = 0.695$, $p < 0.0001$) [48]. Evaluation of plasma protein carbonylation in elderly patients with CKD at stages 1-4 showed that the PCO level in patients classified at stage 4 was higher than that of patients at stages 3, 2, and 1 ($p < 0.001$) [50]. A further study conducted in elderly patients with CKD stages 2-5 highlighted that plasma levels of PCO in CKD stage 5 were significantly higher than in stage 2 ($p = 0.003$), stage 3 ($p = 0.015$), and stage 4 ($p = 0.011$) [49]. On the contrary, the plasma PCO level in young patients (aged from 1.4 to 18.6 years) with CKD stages 1-5 did not depend on the degree of renal failure [51], probably as a consequence of the different diseases underlying kidney dysfunction in young people compared to the elderly. In fact, CKD is more commonly caused by diabetes mellitus and long-lasting hypertension in the elderly, while it is prevalently due to congenital abnormalities of the kidneys and urinary tract in young patients [51]. One study found significantly higher plasma PCO levels in CKD patients, both on conservative therapy (CT) and HD, than in healthy control adults [47]. This study also showed a negative correlation between plasma PCO levels and creatinine clearance [47] that was confirmed [49] or not [45] by other studies.

The plasma PCO level has been shown to have a negative correlation with GFR ($R = -0.26$, $p < 0.05$) and a positive correlation with C-reactive protein (CRP) ($R = 0.49$, $p < 0.0001$) and fibrinogen ($R = 0.30$, $p < 0.01$) levels [49]. Assessment of carbonylation of plasma albumin in elderly patients with CKD stages 2-5 and healthy control subjects by 1D Western blot analysis showed increasing carbonylation of albumin in parallel with the severity of CKD, which reached statistical significance at CKD stages 3 and 4 ($p < 0.01$, compared to healthy control subjects) [44].

3. Plasma Protein Carbonyls in Haemodialysis (HD)

Patients receiving HD, the most common type of dialysis, show a high prevalence of inflammation and oxidative stress [11, 13, 14, 52] and are exposed to additional health risk factors determined by the procedure itself (e.g., rapid changes in plasma electrolyte levels, haemodynamic stresses because of intra- and interdialytic changes in cardiac filling, and fluctuations of blood pressure). Inflammatory response can be caused by the use of synthetic membranes during HD [53] as well as by dialysate impurities, as biomarkers of inflammation and oxidative stress appear significantly lower in patients treated with ultrapure versus standard dialysate [54]. Various mechanisms have been proposed to account for the additional oxidative stress observed in patients following HD, including the activation of neutrophil NADPH oxidase, which provokes inflammation with the release of reactive oxygen species (ROS) [14, 55], and the depletion of circulating low-molecular-weight dialyzable antioxidants [56]. Some typical complications in patients undergoing HD can further exacerbate oxidative stress. For example, anaemia is a frequent and early complication of CKD and the treatment with iron can increase oxidative stress levels. Anaemia prevalence increases with worsening of renal function, involving over 50% of patients at stage 4 and virtually almost 100% of patients receiving HD [57]. Erythropoiesis is limited by the low iron availability [58], deriving from either absolute or functional deficiency and from the iron block due to underlying inflammatory status [59]. Iron deficiency is common in patients with ESRD on HD [60] overall; they lose on average 1-2 g of iron per year, and some of them as much as 4 to 5 g per year [61]. In the absence of concomitant iron supplementation, erythropoietin therapy does not affect oxidative stress [62]. Nevertheless, intravenous iron supplementation is one of the most used interventions in patients with CKD to correct anaemia [63], even if it further aggravates oxidative stress [64]. Recently, iron overload has been shown to increase plasma PCO levels in ESRD patients on HD [65]. Moreover, plasma PCO were positively associated with ferritin level ($R = 0.35$, $p = 0.01$) [65].

Biomarkers of inflammation are elevated in ESRD patients on HD [66–69]. The level of CRP increases in 30-60% of patients receiving HD, and it is closely associated with the progression of atherosclerosis, cardiovascular morbidity, and mortality [70]. Biomarkers of oxidative stress, such as *S*-thiolated proteins [71–73] and protein-bound di-

Table 1: Plasma PCO levels in CKD stages 1-5.

Study year [Ref.]	CKD stages	CKD group (age and sex)	Control group (age and sex)	Analytical methods	PCO in CKD group	PCO in control group	p value
Oberg et al. 2004 [45]	3 to 5	(A) 60 patients (age 67 ± 14 years, 38 M and 22 F)	(H) 53 healthy subjects (age 51.4 ± 1.7 years, sex unspecified)	ELISA after derivatization with DNPH (commercial kit)	(A) Stages 3-5 0.061 nmol/mg protein (0.020–0.134) (a)	(H) 0.029 nmol/mg protein (0–0.154) (a)	(A) vs. (H) $p < 0.001$
Puchades Montesa et al. 2009 [46]	4	(A) 32 patients (age 65.29 ± 15.6 years, 26 M and 6 F)	(H) 67 healthy subjects (age 48.08 ± 19.11 years, 29 M and 38 F)	ELISA after derivatization with DNPH [139]	(A) Stage 4 7.41 ± 0.84 nmol/mg protein	(H) 3.63 nmol/mg protein (1.12) (b)	(A) vs. (H) $p < 0.001$
Mitrogianni et al. 2009 [44]	2 to 4	(A) 25 patients on stage 2 (age range 35–79 years, 15 M and 10 F) (B) 29 patients on stage 3 (age range 29–77 years, 18 F and 11 F) (C) 27 patients on stage 4 (age range 30–81 years, 14 M and 13 F)	(H) 20 healthy subjects (age range 29–78 years, 12 M and 8 F)	Western blot analysis after derivatization with DNPH (commercial kit)	(A) Stage 2 58.88 ± 3.87 a.u. (B) Stage 3 49.48 ± 2.80 a.u. (C) Stage 4 73.34 ± 6.00 a.u	(H_A) Healthy subjects 49.26 ± 4.02 a.u. (H_B) Healthy subjects 34.16 ± 3.94 a.u. (H_C) Healthy subjects 40.24 ± 6.34 a.u	(A) vs. (H_A) No significant variations (B) vs. (H_B) $p < 0.05$ (C) vs. (H_C) $p < 0.05$
Matsuyama et al. 2009 [48]	1 to 5	(A) 7 patients on stage 1-2 (age 51.3 ± 7.6 years, 5 M and 2 F) (B) 7 patients on stage 3a (age 58.2 ± 12.2 years, 4 M and 3 F) (C) 6 patients on stage 3b (age 71.3 ± 8.2 years, 5 M and 1 F) (D) 12 patients on stages 4-5 (age 63.3 ± 19.4 years, 8 M and 4 F)	—	Spectrophotometric assay after derivatization with DNPH (commercial kit)	(A) Stages 1-2 0.7 ± 0.1 nmol/mg protein (B) Stage 3a 0.8 ± 0.1 nmol/mg protein (C) Stage 3b 1.0 ± 0.2 nmol/mg protein (D) Stages 4-5 1.1 ± 0.2 nmol/mg protein	—	(A) vs. (D) $p < 0.05$
Aveles et al. 2010 [49]	2 to 5	68 patients (age 57 ± 12.6 years, 31 M and 37 F)	—	Spectrophotometric assay after derivatization with DNPH [136]	(A) Stage 2 0.8 ± 1.3 nmol/mg albumin (B) Stage 3 1.2 ± 0.9 nmol/mg albumin (C) Stage 4 1.0 ± 0.7 nmol/mg albumin (D) Stage 5 2.2 ± 1.6 nmol/mg albumin	—	(A) vs. (D) $p = 0.003$ (A) vs. (C) $p = 0.015$ (A) vs. (B) $p = 0.011$
Caimi et al. 2013 [47]	2 to 5	(A) 27 patients at stages 2-5 on conservative therapy (CT) (age 58.2 ± 7.6 years, 15 M and 12 F)	(H) 26 healthy subjects (age 43.54 ± 6.92 years, 17 M and 9 F)	ELISA after derivatization with DNPH (commercial kit)	(A) CT stages 2-5 0.709 ± 0.107 nmol/mg protein	(H) 0.440 ± 0.134 nmol/mg protein	(A) vs. (H) $p < 0.001$
Tbahriti et al. 2013[50]	1 to 4	(A) 28 patients on stage 1 (age 37 ± 13 years, 10 M and 18 F)	—		(A) Stage 1 0.56 ± 0.15 nmol/mg albumin	—	(A-B-C-D) vs. (E)

Table 1: Continued.

Study year [Ref.]	CKD stages	CKD group (age and sex)	Control group (age and sex)	Analytical methods	PCO in CKD group	PCO in control group	p value
		(B) 28 patients on stage 2 (age 55 ± 11 years, 11 M and 17 F) (C) 28 patients on stage 3 (age 45 ± 15 years, 10 M and 18 F) (D) 18 patients on stage 4 (age 46 ± 14 years, 7 M and 11 F) (E) 40 HD patients (age 42 ± 11 years, 22 M and 18 F) (F) 25 PD patients (age 39 ± 15 years)		Spectrophotometric assay after derivatization with DNPH (commercial kit)	(B) Stage 2 0.95 ± 0.13 nmol/mg albumin (C) Stage 3 1.04 ± 0.33 nmol/mg albumin (D) Stage 4 1.37 ± 0.36 nmol/mg albumin (E) HD patients 1.85 ± 0.16 nmol/mg albumin (F) PD patients 1.92 ± 0.13 nmol/mg albumin		$p < 0.001$ (A-B-C-D) vs. (F) $p < 0.001$
Drożdż et al. 2016 [51]	1 to 4	(A) 11 patients on stages 1-2 (age 10.51 years; 5.04, 16.08) (B) 18 patients on stage 3 (age 11.33 years; 5.15, 16.33) (C) 14 patients on stage 4 (age 12.01 years; 8.70, 15.99) (D) 22 patients on stage 5 (age 11.61 years; 8.51, 15.20)	—	Spectrophotometric assay after derivatization with DNPH (commercial kit)	(A) Stage 1-2 1.15 nmol/mg protein (0.54, 1.32) (c) (B) Stage 3 1.24 nmol/mg protein (0.87, 1.69) (c) (C) Stage 4 1.64 nmol/mg protein (0.73, 2.41) (c) (D) Stage 5 1.23 nmol/mg protein (0.66, 2.05) (c)	—	No significant variations

Data are presented as the mean ± standard deviation (SD), in the reported studies, with the exception of (a) medians, with a range in parentheses, (b) median with interquartile range in parentheses, and (c) median with 25th–75th percentile. M: male; F: female; a.u.: arbitrary units; CKD: chronic kidney disease; PCO: protein carbonyls (carbonylated proteins); DNPH: 2,4-dinitrophenylhydrazine.

Table 2: Plasma PCO levels in HD.

Study year [Ref.]	HD patients (age and sex) and dialysis vintage	Healthy subjects (age and sex)	Analytical methods	Plasma PCO in HD patients	Plasma PCO in healthy subjects	p value
Himmelfarb at al. 2000 [131]	(A) 10 HD patients (sex and age unspecified) Dialysis vintage unspecified	(H) 10 healthy subjects (sex and age unspecified)	ELISA after derivatization with DNPH [32]	(A) 16.95 ± 2.62 μmol/L	(H) 0.76 ± 0.51 μmol/L	(A) vs. (H) $p < 0.05$
Himmelfarb and McMonagle 2001 [89]	(A) 25 HD patients (mean age 72.6 ± 2.0 years, 13 M and 12 F) Dialysis vintage unspecified	(H) 20 healthy subjects (age 62 ± 4 years, 17 M and 3 F)	ELISA after derivatization with DNPH (commercial kit) Western blot analysis after derivatization with DNPH [140]	(A) 1.22 ± 0.14 a.u.	(H) 0.60 ± 0.08 a.u.	(A) vs. (H) $p < 0.05$
Nguyen-Khoa et al. 2001 [132]	(A) 31 HD patients (mean age 64 ± 18 years, 15 M and 16 F) Dialysis vintage 6.0 ± 5.8 years	(H) 18 healthy subjects (age 45 ± 9 years, 8 M and 10 F)	Spectrophotometric assay after derivatization with DNPH [141]	(A) 0.55 ± 0.25 nmol/mg protein	(H) 0.37 ± 0.09 nmol/mg protein	(A) vs. (H) $p < 0.01$
Ward et al. 2003 [80]	22 HD patients divided into two groups depending on membrane composition (mean age 51 ± 5 years, 8 M and 4 F) Dialysis vintage 49 ± 11 months (A) Polysulfone membrane pre-HD (B) Polysulfone membrane post-HD (C) Cellulose triacetate membrane pre-HD (D) Cellulose triacetate membrane post-HD	(H) 17 healthy subjects (age range 23-54 years, both M and F)	ELISA after derivatization with DNPH (commercial kit)	(A) Pre-HD 0.144 ± 0.037 nmol/mg protein (B) Post-HD 0.175 ± 0.029 nmol/mg protein (C) Pre-HD 0.145 ± 0.030 nmol/mg protein (D) Post-HD 0.178 ± 0.035 nmol/mg protein	(H) 0.041 ± 0.008 nmol/mg protein	(A) vs. (H) $p < 0.05$ (C) vs. (H) $p < 0.05$
Danielski et al. 2003 [97]	36 HD patients divided into two groups: (A) 18 hypoalbuminemic patients (age 67.7 ± 13 years, 7 M and 11 F) (B) 18 normoalbuminemic patients (age 67.8 ± 11 years, 7 M and 1 F) Dialysis vintage unspecified	(H) 18 healthy subjects (age matched)	ELISA after derivatization with DNPH (commercial kit) Western blot analysis after derivatization with DNPH [140]	(A) Hypoalbuminemic patients 0.09 ± 0.02 nmol/mg protein (B) Normoalbuminemic patients 0.06 ± 0.01 nmol/mg protein	(H) 0.02 ± 0.01 nmol/mg protein	(A) vs. (H) $p < 0.05$ (B) vs. (H) $p < 0.05$
Pupim et al. 2004 [75]	(A) 50 HD patients (age 57.6 ± 17.2 years, 30 M and 20 F) Dialysis vintage unspecified	(H) 50 healthy subjects (age 49.7 ± 16.3 years, 18 M and 32 F)	ELISA after derivatization with DNPH (commercial kit)	(A) 0.154 ± 0.014 nmol/mg protein	(H) 0.029 ± 0.004 nmol/mg protein	(A) vs. (H) $p < 0.001$
Massy et al. 2003 [133]	(A) 22 HD patients (age 62 ± 19 years, 12 M and 10 F) Dialysis vintage 6.0 ± 5.8 years	(H) 12 healthy subjects (age 41 ± 8 years, 5 M and 7 F)	Spectrophotometric assay after derivatization with DNPH [141]	(A) 0.54 ± 0.17 nmol/mg protein	(H) 0.34 ± 0.09 nmol/mg protein	(A) vs. (H) $p < 0.001$
Köken et al. 2004 [77]	(A) 70 HD patients (age 49 ± 15 years, 33 M and 37 F) divided into six groups with	(H) 12 healthy subjects (age 50		(A) 1.10 ± 0.20 nmol/mg protein The plasma PCO values in the six	(H) 0.79 ± 0.01 nmol/mg protein	

Table 2: Continued.

Study year [Ref.]	HD patients (age and sex) and dialysis vintage	Healthy subjects (age and sex)	Analytical methods	Plasma PCO in HD patients	Plasma PCO in healthy subjects	p value
	different dialysis vintage: from 3-12 months (group 1) to 85–120 months (group 6)	± 5 years, 5 M and 7 F)	Spectrophotometric assay after derivatization with DNPH [141]	groups of ESRD patients on HD are shown in Figure 2 (no detailed values reported)		(A) vs. (H) $p < 0.001$
Anraku et al. 2004 [91]	22 HD patients (aged 25 to 87 years, 15 M and 7 F) Dialysis vintage 1-9 years (A) HD patients without intravenous iron administration (B) HD patients with intravenous iron administration	(H) 11 healthy subjects (age and gender matched)	Western blot analysis after derivatization with DNPH [140] Spectrophotometric assay after derivatization with fluoresceinamine [39]	No detailed values reported for WB analysis Spectrophotometric assay (A) 1.0 ± 0.1 nmol/mg protein (B) 2.2 ± 0.4 nmol/mg protein	(H) 0.40 ± 0.03 nmol/mg protein	(A) vs. (H) $p < 0.05$ (B) vs. (A) $p < 0.05$
Dursun et al. 2005 [81]	20 HD patients (age and sex unspecified) Dialysis vintage unspecified (A) Pre-HD (B) Post-HD	(H) 20 healthy subjects (age and sex unspecified)	Spectrophotometric assay after derivatization with DNPH [142]	(A) Pre-HD 0.889 ± 0.063 nmol/mg protein (B) Post-HD 0.997 ± 0.066 nmol/mg protein	(H) 0.417 ± 0.036 nmol/mg protein	(A) vs. (H) $p < 0.05$
Kalogerakis et al. 2005 [134]	(A) 22 HD patients (age 60.8 ± 17 years, 14 M and 8 F) Dialysis vintage unspecified	(H) 23 healthy subjects (age 42.5 ± 11.3 years, 12 M and 11 F)	ELISA after derivatization with DNPH [32]	(A) 0.15 ± 0.028 nmol/mg protein	(H) 0.093 ± 0.014 nmol/mg protein	(A) vs. (H) $p < 0.01$
Mera et al. 2005 [135]	(A) 20 HD patients (age 62.8 ± 12.7 years, 10 men and 10 women) Dialysis vintage 1-9 years	(H) 10 healthy subjects (67.8 ± 1.8 years, 6 M and 4 F)	Spectrophotometric assay after derivatization with fluoresceinamine [39]	(A) 3.12 ± 1.11 nmol/mg protein	(H) 2.10 ± 0.34 nmol/mg protein	(A) vs. (H) $p < 0.01$
Siems et al. 2005 [85]	107 HD patients divided into four groups with different Hb concentrations (A) Group 1: 13 patients with Hb < 8 g/dL (age 57 ± 12 years, 5 M and 8 F) (B) Group 2: 42 patients with Hb 8-10 g/dL (age 63 ± 14 years, 20 M and 22 F) (C) Group 3: 39 patients with Hb 10-12 g/dL (age 60 ± 15 years, 15 M and 19 F) (D) Group 4: 13 patients with Hb > 12 g/dL (age 61 ± 8 years, 6 M and 7 F)	(H) 80 healthy subjects (age 61 ± 14 years, 35 M and 45 F)	ELISA after derivatization with DNPH [143]	The plasma PCO values in the four groups of HD patients are shown in Figure 4 of Ref. [85] (nmol/mg protein) (no detailed values reported)	The plasma PCO values in the healthy control subjects are shown in Figure 4 of Ref. [85] (nmol/mg protein)	(A) vs. (H) $p < 0.001$ (B) vs. (H) $p < 0.01$ (C) vs. (H) $p < 0.01$ (D) vs. (H) $p < 0.05$
Lim et al. 2007 [90]	(A) 31 HD patients (age 57.2 ± 12.5 years, M) Dialysis vintage 4.6 ± 6.1 years	(H) 22 healthy subjects (age	Spectrophotometric assay after derivatization with DNPH [141]	(A) 10.5 ± 1.88 nmol/mg purified albumin	(H) 5.29 ± 1.21 nmol/mg purified albumin	(A) vs. (H) $p < 0.001$

Table 2: Continued.

Study year [Ref.]	HD patients (age and sex) and dialysis vintage	Healthy subjects (age and sex)	Analytical methods	Plasma PCO in HD patients	Plasma PCO in healthy subjects	p value
		53.4 ± 17.7 years, M)				
Pieniazek et al. 2009 [82]	10 HD patients (mean age 58 ± 11 years, sex unspecified) Dialysis vintage unspecified (A) Pre-HD (B) Post-HD	(H) 9 healthy subjects (age 46 ± 15 years, sex unspecified)	Spectrophotometric assay after derivatization with DNPH [141]	(A) Pre-HD 2.27 ± 0.2 μmol/L (B) Post-HD 2.94 ± 0.12 μmol/L	(H) 0.67 ± 0.07 μmol/L	(A) vs. (H) $p < 0.0002$ (B) vs. (H) $p < 0.0002$
Moradi et al. 2009 [124]	(A) 32 HD patients (mean age 51 ± 2.5 years, 22 M and 10 F) Dialysis vintage unspecified	(H) 13 healthy subjects (age-matched, 9 M and 4 F)	Spectrophotometric assay after derivatization with DNPH (commercial kit)	The plasma PCO values in HD patients are shown in Figure 1 (no detailed values reported) (A) ~ 11.00 ± 4.00 nmol/mg protein	The plasma PCO values in healthy control subjects are shown in Figure 1 (no detailed values reported) (H) ~ 0.50 ± 1.00 nmol/mg protein	(A) vs. (H) $p < 0.05$
Koca et al. 2010 [78]	111 HD patients divided into four groups according to HD duration: (A) Group 1: ($n = 31$, age 53 ± 14 y, 14 M and 17 F) Dialysis vintage 0-2 years (B) Group 2: ($n = 40$, age 55 ± 17 y, 19 M and 21 F) Dialysis vintage 3-5 years (C) Group 3: ($n = 27$, age 56 ± 14 y, 12 M and 15 F) Dialysis vintage 6-8 years (D) Group 4: ($n = 13$, age 47 ± 9 y, 6 M and 7 F) Dialysis vintage 9-11 years	(H) 24 healthy subjects (age 48 ± 10 years, 10 M and 14 F)	Spectrophotometric assay after derivatization with DNPH [141]	The plasma PCO values in HD patients are shown in Figure 2 (no detailed values reported) (μmol/L)	The plasma PCO values in healthy subjects are shown in Figure 2 (no detailed values reported) (μmol/L)	No significant variations
Terawaki et al. 2010 [86]	83 anuric HD patients divided into with or without CVD: (A) Patients with CVD pre-HD (B) Patients with CVD post-HD ($n = 66$), age 63.5 ± 12.5 years, 32 M and 34 F Dialysis vintage 85.0 ± 64.6 months (C) Patients without CVD pre-HD (D) Patients without CVD post-HD ($n = 20$), age 74.3 ± 12.8 years, 11 M and 9 F Dialysis vintage 58.3 ± 33.3 months	—	Spectrophotometric assay after derivatization with DNPH (commercial kit)	Patients with CVD (A) Pre-HD 0.81 ± 0.16 nmol/mg protein (B) Post-HD 0.53 ± 0.13 nmol/mg protein patients without CVD (C) Pre-HD 0.82 ± 0.17 nmol/mg protein (D) Post-HD 0.58 ± 0.16 nmol/mg protein	—	No significant variations
Pavone et al. 2011 [37]	14 HD patients (age 72 ± 10 years, 7 M and 7 F) Dialysis vintage 50 ± 25 months	—	Western blot analysis after derivatization with DNPH	The plasma PCO values before and after HD are shown in Figure 2 (no detailed values reported)	—	

Table 2: Continued.

Study year [Ref.]	HD patients (age and sex) and dialysis vintage	Healthy subjects (age and sex)	Analytical methods	Plasma PCO in HD patients	Plasma PCO in healthy subjects	p value
	(A) Pre-HD (B) Post-HD		Carbonylated protein identification was carried out by MALDI-TOF/MS mass fingerprinting	(A) ~ 5900 ± 1200 a.u. (B) ~ 7100 ± 2000 a.u.		
Albarello et al. 2012 [83]	23 HD patients (9 men and 14 women, mean age 50.8 ± 17.3 years) Dialysis vintage unspecified (A) Pre-HD (B) Post-HD	—	Spectrophotometric assay after derivatization with DNPH [141]	(A) Pre-HD 0.62 ± 0.14 nmol/mg protein (B) Post-HD 0.86 ± 0.16 nmol/mg protein	—	(A) vs. (B) $p < 0.001$
Almeida et al. 2013 [137]	(A) 35 HD patients (18 years old or older, 16 M and 19 F) Dialysis 26.0 (35.0-13.0) month (a)	(H) 35 healthy subjects paired to age and gender	Spectrophotometric assay after derivatization with DNPH [141]	(A) 1.9 (2.6-1.3) nmol/mg protein	(H) 0.9 (1.5-0.7) nmol/mg protein	(A) vs. (H) $p < 0.001$
Caimi et al. 2013 [47]	(A) 31 HD patients (61.5 ± 12.8 years, 16 men and 15 women) Dialysis vintage 48.5 ± 35.7 months	(H) 26 healthy subjects (age 43.54 ± 6.92 years, 17 M and 9 F)	ELISA after derivatization with DNPH (commercial kit)	(A) 1.230 ± 0.192 nmol/mg protein	(H) 0.440 ± 0.134 nmol/mg protein	(A) vs. (H) $p < 0.001$
Murillo-Ortiz et al. 2016 [65]	(A) 35 HD patients with ferritin levels < 500 ng/mL (age 45.4 ± 16.6 years, 17 M and 18 F) Dialysis vintage 40.87 ± 41.65 months (A) 35 HD patients with ferritin levels > 500 ng/mL (age 46.5 ± 16.9 years, 17 M and 18 F) Dialysis vintage 20.17 ± 29.00 months	—	Spectrophotometric assay after derivatization with DNPH [141]	(A) HD patients with ferritin < 500 ng/mL 22.5 ± 5.4 ng/μL (B) HD patients with ferritin > 500 ng/mL 27.2 ± 5.2 ng/μL	—	(A) vs. (B) $p < 0.0004$
Colombo et al. 2018 [41]	69 HD patients (mean 69 ± 1.5 years, 42 M and 24 F) Dialysis vintage 5.8 ± 0.46 years (A) M pre-HD (B) M post-HD (C) F pre-HD (D) F post-HD	—	ELISA after derivatization with DNPH (commercial kit)	(A) M pre-HD 0.118 ± 0.016 nmol/mg protein (B) M post-HD 0.118 ± 0.013 nmol/mg protein (C) F pre-HD 0.1348 ± 0.0267 nmol/mg protein (D) F post-HD 0.1604 ± 0.0313 nmol/mg protein	—	(C) vs. (D) $p < 0.01$

Data are presented as the mean ± standard deviation (SD), in the reported studies. Dialysis vintage (length of time on dialysis) is presented as months or years. M: male; F: female; CVD: cardiovascular disease; CKD: chronic kidney disease; ESRD: end-stage renal disease; HD: haemodialysis (haemodialysed); PCO: protein carbonyls (carbonylated proteins); DNPH: 2,4-dinitrophenylhydrazine; a.u.: arbitrary units.

tyrosines [40, 74], are also heightened in these patients. Additionally, 24 studies measured plasma PCO levels in ESRD patients on HD (Table 2), 18 of which determined that plasma PCO levels in haemodialysed patients are higher than in healthy subjects (Table 2).

Colombo et al. [74] were the first to point out significant differences in plasma PCO levels between healthy subjects and ESRD patients on HD. Many other studies later confirmed those findings (Table 2). Interestingly, Caimi et al. [47] also showed that patients receiving HD had higher plasma PCO levels not only when compared with healthy control subjects but also in comparison with CKD patients on CT.

A prospective cohort study (12-month period) showed that, at baseline, plasma PCO levels were significantly higher in patients with ESRD before starting the HD therapy than in healthy subjects [75]. After the initiation of HD, there were no significant changes both in plasma PCO content and in plasma concentration of inflammatory biomarkers, which remained stable over a 12-month period [75]. Other studies conducted in ESRD patients on HD reported an increase in IL-6 levels during a 3-year follow-up period [76] and a positive correlation between plasma PCO levels (higher than in healthy subjects) and the duration of HD (3 to 120 months) ($R = 0.364$, $p < 0.01$) [77]. Differently, another study showed that plasma PCO content was not significantly different in ESRD patients receiving HD for up to eleven years (Table 2) [78].

A study evaluated the within- and between-individual variability of plasma PCO levels in ESRD patients on HD, with PCO measurements every two weeks for ten weeks (six measures) [79]. Within-individual coefficients of variation (CVs) and between-individual CVs for PCO were, respectively, 16.3% (range 8.4–29.5%) and 19.5% (range 15.6–24.5%). PCO variability was not affected by various personal and external factors such as dietary antioxidant intake, medications, and clinical and demographic parameters. However, the higher number of males (10 men and 4 women) participating in the study may have influenced the ability to look at the effect of sex [79].

Some studies examined plasma PCO levels before (pre-HD) and after (post-HD) a single HD session (Table 2). Among these, Ward et al. [80] were the first to measure plasma PCO levels pre-HD and post-HD. They reported that plasma PCO levels increased slightly, but significantly over the course of dialysis [80]. Several other studies found that the post-HD plasma PCO content was higher than the pre-HD one [41, 47, 81–84]. Caimi et al. [84] detected increased levels of PCO in CKD patients on HD in comparison with normal controls before and, especially, after the HD session, but they did not find any difference in PCO after subdividing haemodialysed patients according to their dialysis vintage (i.e., length of time on dialysis) or the type of filter employed for HD. Interestingly, Colombo et al. [41] divided ESRD patients on HD into two groups according to sex, and they reported that pre-HD and post-HD plasma PCO levels in females were significantly different while in males were not. This finding suggests that female ESRD patients may be more susceptible to oxidative stress induced by the HD session than male ESRD patients [41]. As a consequence, the female sex could be considered a "risk factor" associated with HD-induced plasma protein carbonylation in ESRD patients. Two studies, on the other hand, reported that plasma PCO concentration did not change during the HD session [85] and that, in anuric haemodialysed patients with or without cardiovascular diseases, post-HD plasma PCO levels decreased [86]. However, it is not specified whether these differences are statistically significant [86].

In ESRD patients undergoing HD or PD, serum albumin is considered a biomarker of nutritional status and inflammation and a predictor of mortality [87, 88]. Some studies have shown that albumin is the major carbonylated protein in ESRD patients on HD (Table 2). In 2001, Himmelfarb and McMonagle [89] reported, for the first time, that the carbonylation of albumin accounts for almost all the excess plasma protein oxidation observed in haemodialysed patients when compared to healthy subjects. Later studies confirmed and reinforced this finding. The carbonyl content of purified albumin, detected by spectrophotometric analysis and immunoblot, was much higher in ESRD patients on HD than in healthy subjects [90]. Carbonylation of albumin increased in correlation with CKD stage severity, attaining significance at stages 3 and 4 ($p < 0.01$, compared to healthy controls), and reached even higher levels in patients undergoing HD [44]. As mentioned above, in haemodialysed patients, intravenous iron administration substantially increases carbonylation of plasma proteins [65], including albumin carbonylation [91]. Comparing healthy control subjects, ESRD patients undergoing HD without intravenous iron administration, and ESRD patients undergoing HD with intravenous iron administration, only albumin was found to be significantly carbonylated in haemodialysed patients, and intravenous iron administration increased the albumin carbonylation [91].

Advanced age (>65 years) has been associated with hypoalbuminemia (serum albumin level < 3.8 g/dL) in two large cross-sectional studies [92, 93]. Hypoalbuminemia in ESRD patients on HD is primarily associated with systemic inflammation [94] and confers a greater mortality risk [95, 96]. Danielski et al. [97] reported that both plasma PCO levels and albumin carbonyl content were significantly increased in normoalbuminemic and hypoalbuminemic ESRD patients on HD in comparison to healthy subjects, even if the difference between hypoalbuminemic and normoalbuminemic haemodialysed patients did not reach the statistical significance [97].

Albumin is not the only protein whose carbonylation increases in ESRD patients on HD [37]. Using Western blot with anti-DNP antibodies and MALDI-TOF/MS mass fingerprinting associated with nano-LC-MS/MS analysis, Pavone et al. [37] showed that post-HD plasma PCO levels were significantly increased compared to pre-HD levels and that carbonylation targets numerous plasma proteins. The same authors used MALDI-TOF/MS mass fingerprinting to identify carbonylated proteins in blood samples before and after the HD session carried out with ethylene vinyl alcohol and cellulose diacetate membranes. α2-Macroglobulin, chain A α1-antitrypsin, fibrinogen γ chain, immunoglobulin γ 1,

proapolipoprotein, transferrin, and albumin were found as the main carbonylated plasma proteins after HD [36].

4. Plasma Protein Carbonyls in Peritoneal Dialysis (PD)

PD is an alternative to HD and is used by approximately 200,000 ESRD patients worldwide, representing approximately 7% of the total dialysis population [98]. In PD, the peritoneal membrane acts as a dialyzing membrane. To achieve this, a dialysis solution (dialysate) is instilled in the peritoneal cavity through a peritoneal catheter. After a dwell time, the dialysate is drained out. How long the dialysate is present in the peritoneal cavity, how many times the dialysate is changed, and the duration of the dwell time depend on individual patient requirements. While the dialysate is present in the peritoneal cavity, across the peritoneal membrane there is a transport of solutes and water between the blood in the peritoneal capillaries and the dialysis solution, which is typically rendered hyperosmolar through the addition of glucose or other osmotic agents. Through this mechanism, the elimination of waste products and the correction of fluid and electrolyte imbalances are achieved [99]. Even though HD and PD can be viewed as equivalent therapies and used as primary therapy for ESRD patients [100], there are important differences between them. PD exposes patients daily to greater amounts of glucose loading, leading to a much higher prevalence of insulin resistance, dyslipidemia, and metabolic syndrome [101]. PD may also accelerate the development of atherosclerosis lesions through increased lipid oxidation and glycosylation [102]. Otherwise, patients with ESRD undergoing HD are exposed to a greater risk of CVDs because they show a more rapid decline of residual renal function (RRF) [103] and a more hyperdynamic status due to the arteriovenous fistula and the extracorporeal circulation [104]. Although PD is considered a less invasive therapy than HD, it produces chronic inflammation in the peritoneal cavity leading to an increased level of proinflammatory cytokines, which alters peritoneal membrane integrity [105]. Several lines of evidence indicated that oxidative metabolism in peripheral and peritoneal phagocytes is activated during PD with conventional dialysate, which is characterized by a high concentration of glucose, glucose degradation products, low pH, and high osmolality [106]. Bioincompatibility of PD solutions also seems to play a central role in the oxidative stress increase [107].

Five studies compared plasma PCO levels in patients with ESRD undergoing PD or HD, while one study examined plasma PCO content in ESRD patients on PD and healthy individuals (Table 3). Erdoğan et al. [108] compared plasma PCO content of patients with ESRD undergoing HD or PD with that of healthy individuals, showing that plasma PCO levels in ESRD patients on HD or PD are similar to those of healthy controls. Otherwise, another study showed that plasma PCO levels in ESRD patients on PD were lower than in haemodialysed patients, maybe because HD is associated with higher protein oxidation or because patients undergoing PD had greater RRF [109]. Conversely, two more recent studies showed that plasma PCO levels were higher in ESRD patients undergoing PD than in those undergoing HD [50, 110], although in one of the two studies it is not clear whether differences were statistically significant [50]. Another investigation proved that, in ESRD patients on PD, there was a highly significant positive correlation between copper/zinc ratio, the levels of CRP, and plasma PCO levels [111], whereas copper/zinc ratio was negatively correlated with the percentages of B- and T-lymphocytes and the ratio of CD4/CD8 antigens. Therefore, the authors suggested that, in ESRD patients on PD, elevated copper/zinc ratios are associated with increased oxidative stress and inflammation [111].

Mitrogianni et al. [44] estimated the carbonylation of plasma albumin in ESRD patients undergoing HD or PD compared to healthy control subjects by Western blot, showing that albumin carbonylation was higher in ESRD patients on HD, while it did not differ in ESRD patients on PD compared to controls. They suggested that PD may be more biocompatible, avoiding the generation of excess oxidative burden. Lack of contact of the blood with the dialysis membranes and less usage of intravenous iron administration might explain, at least in part, the low levels of plasma PCO observed in ESRD patients undergoing PD. The quite important albumin losses in ESRD patients on PD, replaced by newly synthesized albumin, may contribute to the lower albumin carbonylation [44].

5. Plasma Protein Carbonyls in Kidney Transplantation (KT)

KT is considered the best therapeutic option in ESRD, because it permits a higher quality of life compared to HD and PD. Moreover, KT presents the lowest mortality rates, around 1.5-7% per year [112]. In KT anaemia, in addition to hyperhomocysteinemia, it can induce oxidative stress [113]. Oxidative stress and inflammation can produce graft tissue damages because of fibrosis and nephron losses by necrosis or apoptosis [114].

Two studies examined plasma PCO levels before and after KT. A prospective cohort study evaluated time-dependent changes in biomarkers of inflammation and oxidative stress (plasma PCO levels) in 19 patients (mean age 38.3 ± 13.7 years, 11 men and 8 women), comparing them to 50 healthy control subjects (mean age 48.2 ± 16 years, 18 men and 32 women) [115]. This study reported that patients had substantial improvements in inflammatory biomarkers and plasma PCO levels after the restoration of kidney function by transplantation. Plasma PCO levels decreased rapidly, with significant changes notable within the first postoperative week ($p < 0.001$); final posttransplant levels of plasma PCO in recipients were not statistically different from those of healthy subjects ($p < 0.05$). This study also showed that CRP levels decreased significantly from baseline within two months after renal transplantation ($p < 0.001$) [115]. The second study investigated plasma protein carbonylation in 21 patients (mean age 36 ± 17 years, men 50%) who underwent a living donor KT and were evaluated before the transplantation and analyzed longitudinally after a mean follow-up time of nine months [35]. This study showed that

Table 3: Comparison of plasma PCO levels in PD and HD.

Study year	Number of HD or PD patients (age and sex) and dialysis vintage	Number of control healthy subjects (age and sex)	Analytical methods	Plasma PCO in HD or PD patients	Plasma PCO in control subjects	p value
Tbahriti et al. 2013 [50]	(A) 40 HD patients (age 42 ± 11 years, 22 M and 18 F) (B) 25 PD patients (age 39 ± 15 years)	—	Spectrophotometric assay after derivatization with DNPH (commercial kit)	(A) HD patients 1.85 ± 0.16 nmol/mg albumin (B) HD patients 1.92 ± 0.13 nmol/mg albumin	—	Statistical difference not specified
Erdoğan et al. 2002 [108]	(A) 7 HD patients (B) 9 PD patients (mean age 38.7 ± 12.9 years, 7 M and 9 F)	(H) 9 age-matched healthy subjects (2 M and 7 F)	Spectrophotometric assay after derivatization with DNPH [142]	(A) HD patients 1.1 ± 0.2 nmol/mg protein (B) PD patients 1.1 ± 0.3 nmol/mg protein	(H) 0.8 ± 0.3 nmol/mg protein	No significant variations
Doñate et al. 2002 [109]	(A) 21 HD patients (B) 42 PD patients	—	ELISA after derivatization with DNPH [32]	(A) HD patients 0.1665 ± 0.04 nmol/mg protein (B) PD patients 0.1452 ± 0.03 nmol/mg protein	—	(A) vs. (B) $p < 0.004$
Mitrogianni et al. 2009 [44]	(A) 25 HD patients (age 26–80 years, 16 M and 9 F) (B) 21 PD patients (age 18–77 years, 13 M and 8 F)	(H) 20 healthy subjects (age range 29–78 years, 12 M and 8 F)	Western blot analysis after derivatization with DNPH (commercial kit)	(A) HD patients densitometric units (B) PD patients densitometric units	Control densitometric units Control densitometric units	(A) vs. (H) $p < 0.05$
Mekki et al. 2010 [110]	(A) 20 HD patients (age 36 ± 12 years, 8 M and 12 F) HD vintage 12-60 months (B) 20 PD patients (age 40 ± 8 years, 10 M and 10 F) PD vintage 3-48 months	—	Spectrophotometric assay after derivatization with DNPH [141]	(A) HD patients 0.92 ± 0.15 μmol∗mL (B) PD patients 1.90 ± 0.10 μmol∗mL	—	(A) vs. (B) $p < 0.01$
Guo et al. 2011 [111]	(A) 45 PD patients (age 54 ± 9 years, 22 M and 23 F) PD vintage 2.4 ± 1.1 years	(H) 30 healthy subjects (age 52 ± 7 years, 12 M and 18 F)	Spectrophotometric assay after derivatization with DNPH [141]	(A) PD patients 0.42 ± 0.23 nmol/mg protein	(H) 0.16 ± 0.08 nmol/mg protein	(A) vs. (H) $p < 0.05$
Tbahriti et al. 2013 [50]	(A) 40 HD patients (age 42 ± 11 years, 22 M and 18 F) HD vintage 14-109 months (B) 25 PD patients (age 39 ± 15 years, 12 M and 13 F) PD vintage 5-49 months	—	Spectrophotometric assay after derivatization with DNPH (commercial kit)	(A) HD patients 1.85 ± 0.16 nmol/mg protein (B) PD patients 1.92 ± 0.13 nmol/mg protein	—	

Data are presented as the mean ± standard deviation (SD), in the reported studies. M: male: F: female; CKD: chronic kidney disease; ESRD: end-stage renal disease; HD: haemodialysis (haemodialysed); PCO: protein carbonyls (carbonylated proteins); DNPH: 2,4-dinitrophenylhydrazine; PD: peritoneal dialysis.

plasma PCO levels declined from seven to 11 months after KT. Plasma PCO content was significantly reduced after KT (1.4 ± 0.4 nmol/mg albumin) compared to pretransplantation (2.0 ± 1.4 nmol/mg albumin, $p < 0.05$). The study also revealed a significant correlation between CRP and plasma PCO levels after the transplantation ($R = 0.65$, $p < 0.005$) [49].

6. Conclusion and Perspectives

Plasma PCO levels are quite heterogeneous both in CKD patients (Table 1), in patients on RRT (Tables 2 and 3), and in healthy control individuals (Tables 1–3). A cause of PCO variability could be the use of different methods to measure plasma PCO levels due to the lack of a reference method. Nevertheless, even when the same methodology was used, a critical emerging aspect is the high variability of measurements (Table 1). The absolute value of plasma PCO content measured by ELISA in control and CKD subjects seems to spread over two orders of magnitude (e.g., 0.029 and 0.061 nmol/mg protein [45], 0.440 and 0.709 nmol/mg protein [47], and 3.63 and 7.41 nmol/mg protein [46]). This variability needs to be reduced by standardizing references or procedures to make comparable data obtained at different times and laboratories. The aim is to give solidity as well as diagnostic and prognostic value to PCO, an established biomarker of oxidative stress.

A problem with the DNPH-based spectrophotometric assay may be that its results are frequently displayed in different units, e.g., nmol/mg protein, nmol/mg albumin, nmol/L, mmol/L, ng/μL, and μmol$*$mL (Tables 1–3), making them particularly difficult to compare between different studies. In addition, a limit of the DNPH-based spectrophotometric assay is that absorbance wavelengths of haemoglobin are similar to those of DNPH and this can interfere with DNPH measurement, leading to inaccurate estimation of plasma PCO levels [116]. Therefore, reproducible results can arise only from meticulous sample preparation (i.e., during plasma separation from red blood cells, haemolysis should be strictly avoided) (Figure 2).

RRF can further contribute to variability in plasma PCO levels among patients with ESRD undergoing dialysis. Moreover, in various studies, the inclusion/exclusion criteria of CKD patients are quite heterogeneous or even unspecified (Table 4—Supplementary Material). Moreover, age, sex, ethnicity, and lifestyle can also potentially result in PCO variability. Therefore, the preliminary results of these small studies should be confirmed in the future with a larger number of CKD patients and/or patients on RRT with homogeneous (or at least well-specified) inclusion/exclusion criteria and healthy control subjects with different demographic characteristics (e.g., age, sex, and ethnicity) and lifestyle (e.g., physical activity level and smoking status).

However, despite these problems, some findings emerge clearly from the studies conducted so far. Firstly, the results of the studies reported in Table 1, taken together, emphasized the fact that plasma PCO levels are increased in adult or elderly patients with CKD compared to healthy subjects. Even in the early stages of CKD, plasma PCO levels are elevated, and they increase from one stage to the next one, as the kidney function declines over time. This supports the conclusion that systemic oxidative stress appears already at the initial stages of CKD and it gradually increases along with the severity of the disease. Few data are present in the literature about PCO levels in people younger than 18 years. The only study in this setting showed that, in children and young patients with CKD stages 1-5, the concentration of plasma PCO did not depend on the stage of disease [51]. In addition, plasma PCO levels from adult or elderly patients with CKD seem to be correlated negatively with GFR [47–49] and positively with BUN [48], even if caution is necessary to interpret these small studies.

Secondly, studies measuring plasma PCO levels in ESRD patients on PD *vs.* ESRD patients on HD have generated conflicting results [44, 50, 108–110] (Table 3). Maybe this could be due to interfering factors such as the different RRF between ESRD patients undergoing HD or PD. In fact, RRF decreases more slowly in people undergoing PD than in those undergoing HD [117, 118], probably because of sudden drops in blood pressure typical of HD, where fluid is removed much more quickly during the short and frequent HD sessions as compared to the longer PD cycles. In addition, other factors can influence RRF decline, such as gender (particularly female gender as being associated with a stronger decline), nonwhite race (associated with a stronger decline), and comorbidities [103, 119]. A further limitation of these studies was the relatively small number of patients.

Thirdly, after the restoration of kidney function by transplantation, plasma PCO content lowers to levels similar to those of healthy control subjects. Although the populations involved were limited in size, several studies support the conclusion that KT reduces oxidative stress [49, 115, 120, 121].

In conclusion, the studies presented in this review demonstrate that oxidative stress is higher in CKD. Western blot analysis with anti-DNP antibodies showed that not all proteins in the plasma of CKD patients are prone to carbonylation, supporting the view that protein carbonylation in CKD is a selective rather than a random process. In patients with various stages of CKD [44] and in ESRD patients on HD, carbonylation affects albumin [41, 44, 89–91] and other proteins present in the plasma in lower amounts [36, 37]. Direct, or primary, carbonylation is a protein irreversible damage, an oxidative modification that cannot be reversed by antioxidant defences [22, 25, 122, 123]. The increased carbonylation of proteins directly leads to the central unsolved question: does the carbonylation of proteins have a direct pathological impact or is it a secondary phenomenon? Albumin, along with ascorbate and urate, represents the most important antioxidants in the plasma [124]. As albumin is carbonylated in CKD patients, including ESRD patients on HD, it can be hypothesized that, in these subjects, the plasma antioxidant defences are lower and, consequently, the risk for oxidative tissue damage is higher [125]. Several studies conducted in ESRD patients on HD have indeed demonstrated that albumin carbonylation can adversely affect its vasculoprotective capabilities [89, 90], fibrinogen carbonylation can contribute to the impaired clotting activity [126], and

carbonylation of haptoglobin and ceruloplasmin [36, 37] can impair the antioxidant protective properties of these proteins.

Overall, all these studies point out that plasma protein carbonylation in CKD, and especially in ESRD patients undergoing HD, is not solely a secondary phenomenon. Despite the fact that the role of plasma PCO in CKD is often underestimated in clinical practice, emerging evidence continues to highlight that plasma PCO can serve as good biomarkers of oxidative stress in CKD and substitutive therapies, HD, PD, and KT. Whether plasma PCO levels merely serve as biomarkers of CKD- and RRT-related oxidative stress or whether they are associated with the pathogenesis of CKD complications deserves further evaluation. In this regard, it is interesting to note that advanced glycation end products (AGEs), i.e., glycated amino acid residues of proteins, contribute to the development of CKD [127]. AGEs are stable posttranslational modified proteins derived by the nonenzymatic reaction of reducing sugars and related metabolites with Arg and Lys residues, giving rise to indirect, or secondary, protein carbonylation. Proteolysis of AGEs produces glycated amino acids, or AGE-free adducts, which are cleared by the kidneys under healthy conditions but accumulate in plasma with the decline in GFR during CKD [127, 128]. AGEs also result from dicarbonyls derived from glucose degradation and absorbed from thermally processed dialysis fluids in RRT and from the so-called dicarbonyl stress, i.e., the accumulation of various dicarbonyl compounds that causes increased AGE formation in people with CKD [127, 129]. In patients with ESRD, plasma AGE-free adducts increased up to 18-fold on PD and up to 40-fold on HD, whereas the increase in AGE residues of plasma proteins was 2- to 5-fold [128]. Protein dysfunction and inactivation caused by AGE formation contribute to CKD development [127]. Indeed, several studies that investigated dysfunction of proteins modified by dicarbonyl compounds—the so-called dicarbonyl proteome—suggest that dicarbonyl stress is a key factor for the development of vascular renal inflammation, kidney and muscle fibrosis, which are critical to CKD progression and comorbidities, CVD, and muscle wasting [127, 130].

Abbreviations

BUN: Blood urea nitrogen
CKD: Chronic kidney disease
CRP: C-reactive protein
CT: Conservative therapy
CVD: Cardiovascular disease
DNP: 2,4-Dinitrophenylhydrazone
DNPH: 2,4-Dinitrophenylhydrazine
ELISA: Enzyme-linked immunosorbent assay
ESRD: End-stage renal disease
GFR: Glomerular filtration rate
HD: Haemodialysis
HRP: Horseradish peroxidase
KDIGO: Kidney Disease Improving Global Outcomes
KT: Kidney transplantation
MALDI-TOF: Matrix-assisted laser desorption/ionization time-of-flight
MS: Mass spectrometry
PCO: Protein carbonyls (carbonylated proteins)
PD: Peritoneal dialysis
PVDF: Polyvinylidene fluoride membrane
ROS: Reactive oxygen species
RRF: Residual renal function
RRT: Renal replacement therapy.

Conflicts of Interest

We wish to confirm that there are no known conflicts of interest associated with this publication.

Authors' Contributions

We further confirm that the manuscript has been read and approved by all named authors and that the order of authors listed in the manuscript has been approved by all of us.

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Supplementary Materials

Table 4: patient selection. CKD or RRT inclusion and/or exclusion criteria in various studies [37, 41, 45–52, 66, 76, 78, 79, 81–84, 86, 87, 90–92, 98, 109–112, 116, 132–139]. *(Supplementary Materials)*

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Research Article

Local and Systemic Oxidative Stress in Balkan Endemic Nephropathy Is Not Associated with Xanthine Oxidase Activity

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Balkan endemic nephropathy (BEN) represents a chronic tubulointerstitial nephropathy which is followed by the progression of kidney fibrosis to end-stage kidney failure. The critical involvement of poisons in food (aristolochic acid (AA), ochratoxin, and heavy metals) and selenium deficiency are among nutritive factors which contribute to the pathogenesis of BEN, due to reactive oxygen species (ROS) liberation and/or decreased antioxidative defence system. The aim of the study is to distinguish a possible systemic and local origin of ROS through the measurement of xanthine oxidase (XO) activity in urine and plasma, along with the determination of the oxidative changes in lipids and proteins. The study included 50 patients with BEN and 38 control healthy subjects. We noted increased levels of both thiobarbituric acid-reactive substances (TBARS) and advanced oxidation protein products (AOPPs) in the plasma of patients with BEN, compared to the control group ($p < 0.001$). The urinary levels of AOPPs were higher in patients with BEN in comparison to the control ($p < 0.001$). The specific activity of XO was significantly lower in plasma and urine in BEN samples, compared to controls ($p < 0.005$). Based on these results, we hypothesize that XO might not be considered a direct systemic or local contributor to ROS production in BEN, most probably because of the diminished kidney functional tissue mass and/or AA-induced changes in purine nucleotide conformation. The increased AOPP and TBARS level in both plasma and urine in BEN may predict ROS systemic liberation with toxic local effects.

1. Introduction

Balkan endemic nephropathy (BEN) is a chronic tubulointerstitial nephropathy characterized by an unpredictable onset and a gradual progression to end-stage renal disease. Upper urothelial cancer (UUC) of the pelvis and ureter has increased prevalence in BEN patients [1–3] and predisposes to increased mortality risk [4]. Moreover, it has been reported that kidney transplantation in BEN patients, although partially restores renal function, does not reduce the risk of developing UUC [5]. This disease was first discussed over sixty years ago [6, 7]. Typical of the disease is that it affects residents of villages of certain areas of the Danube tributaries within the region of the Balkans [8, 9].

The pathophysiology of BEN has been elaborated in a number of reports [9–11]. It is similar to all tubulointerstitial nephritic diseases and is characterized by progressive kidney atrophy and sclerosis [9]. Histopathological findings indicate a hypocellular interstitial fibrosis accompanied with tubular atrophy. Glomerular and vascular lesions are associated with periglomerular fibrosis, ischaemic, microcystic glomeruli, thrombotic microangiopathy-like lesions, and focal segmental sclerosis-like lesions [9]. To date, the aetiology of BEN is not yet fully elucidated. Some suggest that environmental

factors might play a pivotal role in its pathophysiology, while others support that confounding factors might also be involved. Among these, the chronic food poisoning by aristolochic acid (AA), a toxin present in plants of the genus Aristolochia, seems to be the most acceptable theory. AA promotes kidney damage in BEN, associated with the hepatic enzymes metabolizing AA, which may have high cancerogenic potential [12–14]. A prolonged exposure to AA-contaminated food grown in polluted soil could be one of the main aetiological mechanisms of BEN observed in the Balkan area. Since AAs are resistant to degradation, it is possible that they could be absorbed by the root and transported to the edible part of food crops [15]. Metabolic activation of AAs leads to a reduction of the nitro group to produce N-hydroxylaristolactams (N-hydroxyl-ALs) [16–20]. Hydrolysed lactams create reactive nitrenium ions which yield large AL-purine deoxyribonucleic acid adducts (addition products) at the exocyclic amino groups of the purines. The foremost overabundant deoxyribonucleic acid adduct, 7-(deoxyadenosine-N6-yl) aristolactam I, causes characteristic AT→TA transverse found in the gene responsible for tumour-suppressive protein p53 transcription [19, 20]. Furthermore, mycotoxins such as ochratoxin (OTA) and citrinin, which are mainly produced by some species of Penicillium, Aspergillus, and Monascus genera, are implicated to be a potential cause of BEN [21]. The concentrations and the extent of selenium deficiency are documented in rock, soil, water, foodstuff, and blood samples collected from endemic and nonendemic regions of BEN in the geographical area of Serbia [22–24]. Some findings suggest that heavy metals and metalloids may also contribute to BEN or similar diseases in the same geographic area [25].

Increased oxidative stress (OS) caused by overproduction of reactive oxygen species (ROS) and deficiency of antioxidative defence systems may be possible common mechanisms for all of the factors mentioned above, including the progressive renal injury of the disease [26]. Moreover, OS might also contribute to the increased cardiovascular burden seen in CKD patients [27]. Accumulation of ROS starts at early CKD stages and is gradually elevated, parallel to progression to end-stage renal disease [28]. Also, all tubulointerstitial injuries are characterized by the generation of free oxygen radicals, highly nephrotoxic and very reactive metabolites, released throughout the oxidative burst [29]. ROS may indirectly cause the formation of DNA adducts by initiating autocatalytic lipid peroxidation, which generates a large variety of potential genotoxic breakdown products, including alkoxy radicals, peroxyl radicals, and aldehydes, such as thiobarbituric acid-reactive substances (TBARS) [30]. Lipid peroxidation is one of the most significant implications of ROS generation, which causes irreversible damage to the function and structure of cell membranes. TBARS, the final products of OS, might also serve as indicators of lipid peroxidation status [31]. Moreover, ROS can also cause irreversible damage in the morphology and function of proteins, carbohydrates, and DNA. Earlier studies have found that plasma concentration of advanced oxidation protein products (AOPPs) significantly increased with the progression of nephritic pathology in CKD patients [32] and in patients with diabetes [33]. To date, the exact pathophysiologic mechanism underlying the overproduction of ROS has not yet been elucidated. However, accelerated inflammation and impaired antioxidative defence mechanisms have been in the centre of scientific attention. OS might also mediate urinary tract injury through direct cytotoxic effects on proximal tubular cells and altered renovascular responses or even as a configuration of many factors.

One of the hypothetical mechanisms for ROS production in BEN may be the xanthine oxidase (XO) reaction. XO is the oxidative radical-forming isoform of xanthine oxidoreductase. XO is the main enzyme involved in uric acid production, acting as the final metabolite of the adenine nucleotides. Simultaneously with the production of uric acid, XO activity liberates hydrogen peroxide and superoxide anion, well-established prooxidant molecules [34]. Increased plasma XO activity has been reported in several disease states, such as cholecystitis, shock, ischaemia-reperfusion injury, acute virus infection, adult respiratory distress syndrome, and carcinogenesis [28]. However, it remains debatable whether XO activity is involved in the pathogenesis of BEN-associated kidney injury and cancerogenesis of the upper urothelial tract.

The aim of the present study is to determine whether OS is involved in the pathogenesis of BEN, observed via oxidative changes in lipids and proteins in plasma and urine.

2. Materials and Methods

All the reagents were purchased from Sigma (St. Louis, MO, USA). All chemicals used were of analytical grade.

2.1. Patients. We recruited patients with BEN from the Institute of Nephrology, Clinical Centre of Nis, Serbia, in accordance with a standard diagnostic protocol [35]. All subjects gave their informed consent before they participated in the study. The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the Ethics Committee of Clinical Centre in Niš (Decision No. 17776/8). Regarding the tubulointerstitial character of the disease, the clinical-biochemical diagnostic set used the following: (1) a history of the birth place and their place of residence; (2) laboratory urine analysis: glomerular filtration rate (GFR) reduction, which was less than 60 ml/min/1.73 m^2 in these patients; microalbuminuria (proteinuria < 1 g/24 h) associated with urinary marker tubular lesions (β2-microglobulin in urine), laboratory blood test: urea and creatinine; (3) radiological findings; and (4) kidney ultrasound. The estimates included in the study [36] were selected from the endemic areas around South Morava. Patients with BEN who had other chronic diseases were excluded from the study. The control group [37] involved the corresponding non-BEN rural areas. The control patients did not have any acute or chronic illness or high blood pressure, since hypertension is not the characteristic of BEN. The respondents were matched for gender and age. The demographic characteristics of BEN patients and the control group are shown in Table 1. Blood and urine of BEN patients and the control groups were collected, centrifuged to separate plasma at 3000 rpm, and stored at -20°C prior to the analysis.

Table 1: Baseline characteristics of BEN study cases and control group.

Characteristics	BEN	Controls	p value
Men	30 (60%)	22 (57.5%)	0.866
Women	20 (40%)	16 (42.5%)	
Age (years)	72 (52.6-86.7)	73 (65.05-83.95)	0.377
SCr (mol/l)	120.2 (70.22-606.4)	80.2 (68.63-125.15)	*0.001*
CCr (ml/min)	35.61 (7.93-87.41)	65.9 (23.09-108.7)	*0.001*
UCr (mmol/l)	7.02 (1.77-22.75)	10.23 (5.2-23.53)	*0.020*
UPCr (mg/mmol)	20.61 (5.43-423.47)	10.42 (5.22-24.24)	*0.001*
UACr (mg/mmol)	1.22 (0.18-60.72)	0.87 (0.2-12.07)	*0.043*
Hgb (g/l)	13.1 (8.7-131.6)	122 (12.71-152.9)	*0.001*
Glucose (mmol/l)	4.66 (3.8-6.4)	5.3 (4.31-6.89)	*0.022*
β2-Microglobulin (g/l)	94.81 (5.85-4754)	—	—
U protein (mg/l)	215 (36.5-1510.5)	107 (50-496)	*0.005*
U albumin (mg/l)	16.48 (1.44-434.24)	8.67 (2.14-234.65)	0.197

Values are expressed as mean value (or percent) and median value (5th-95th percentiles). SCr: serum creatinine; CCr: creatinine clearance; UCr: urea : creatinine ratio; UPCr: urine protein : creatinine ratio; UACr: urine albumin : creatinine ratio; Hgb: hemoglobin.

2.2. Methods. We analysed plasma and urine for biochemical parameters on an A24 automatic analyser for in vitro diagnostics (Biosystems SA).

2.2.1. Lipid Peroxidation Products. Lipid peroxidation in urine and plasma in terms of TBARS formation was determined using a slightly modified method of Nabavi et al. [38]. We added TCA and TBA into the serum. Subsequently, it was incubated at 100°C for one hour. After cooling, the samples were centrifuged to remove the precipitate, the supernatant is separated, and the quantity of TBA-reactive lipid peroxidation products was measured at 532 nm against a blank which contained all the reagents except the serum. The concentration of TBARS is expressed in μmol/l.

2.2.2. AOPP Concentration. The concentration of AOPPs in plasma and urine was determined by spectrophotometric technique according to the method of Witko-Sarsat et al. [32]. We diluted 200 microliters of plasma in 1 : 5 in PBS or chloramine-T standard solutions, which were placed in wells of a 96-well microtiter plate, to which 20 μl of acetic acid was added. Ten microliters of 1.16 M potassium iodide was then added, followed by 20 μl of acetic acid. The absorbance of the mixture was immediately read at 340 nm in a microplate reader against a blank containing all reagents only. The AOPP concentration is expressed in μmol/l chloramine-T.

2.2.3. XO Activity. The specific activity of XO was evaluated in plasma, spectrophotometrically, according to the liberation of uric acid by using xanthine as substrate, in the absence of NADH in cases in which only molecular oxygen was the acceptor of electrons. Uric acid was stoichiometrically formed from xanthine, and it was measured at 293 nm. XO activity was expressed in IU/l [37].

2.3. Statistical Analysis. Quantitative variables were presented as arithmetic means with standard deviation (mean ± SD). Differences between the group means were analysed using one-way analysis of variance. p value < 0.05 was accepted as statistically significant. We performed the statistical analysis with Statistical Package for the Social Sciences for Windows (SPSS, version 11.0, Chicago, Illinois, USA).

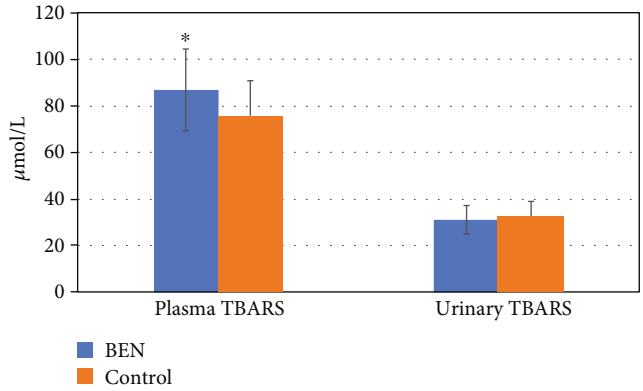

Figure 1: TBARS values in patients with BEN and controls. Data are mean ± SD values. $*p < 0.05$ versus control.

3. Results

The concentration of TBARS in plasma and urine is shown on Figure 1. Compared to controls, BEN patients exhibited significantly increased plasma TBARS levels ($p < 0.001$). However, urine TBARS levels were not significantly different among groups. Therefore, compared to healthy controls, BEN patients present an increased systemic lipid peroxidation status. However, the local, urine lipid oxidation status did not differ among groups, presumably due to the low lipid content in the urine. The level of AOPP in plasma and urine is shown in Figure 2, whereas Figure 3 shows the difference in plasma/urine AOPP ratio among groups. Compared to those in controls, plasma and urine levels of AOPPs and plasma/urine AOPP ratio were significantly increased in BEN patients ($p < 0.05$, $p < 0.001$, and $p < 0.001$, respectively). Therefore, the damaging effect of OS on proteins is

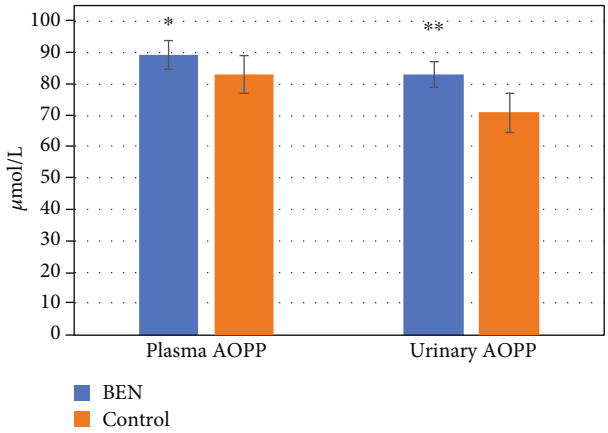

Figure 2: AOPP values in BEN patients and controls. Data are mean ± SD values. $*p < 0.05$ versus control; $**p < 0.001$ versus control.

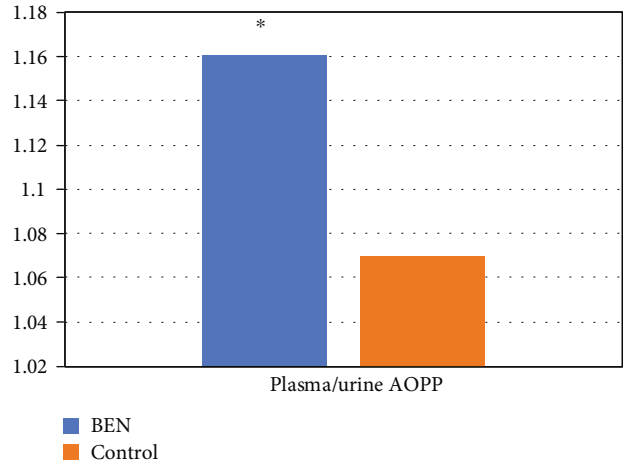

Figure 3: AOPP values in patients with BEN and controls: plasma/urine ratio. $*p < 0.001$ versus control.

significantly pronounced in BEN patients both systemically (in plasma) and locally (in urine), compared to healthy individuals. The activity of XO in plasma and urine is shown in Figure 4. The decrease in plasma XO activity of BEN patients was statistically significant when compared to that of controls ($p < 0.05$). We can assume that there is a systemic lower activity of XO. The urinary level of XO activity was significantly lower in patients with BEN when compared to the control group ($p < 0.001$), which suggests a local low XO activity. Moreover, we found statistically significant lower plasma/urine XO ratio in BEN patients when compared to controls ($p < 0.001$) (Figure 5). We hypothesize that there is a low systemic level of XO activity in patients with BEN.

4. Discussion

BEN affects a large number of inhabitants of endemic areas. In the last decades, epidemiologic findings have revealed that BEN is an environmentally induced disease. Aristolochia, ochratoxin, selenium deficiency, and heavy metals are among the most important factors contributing to the onset and

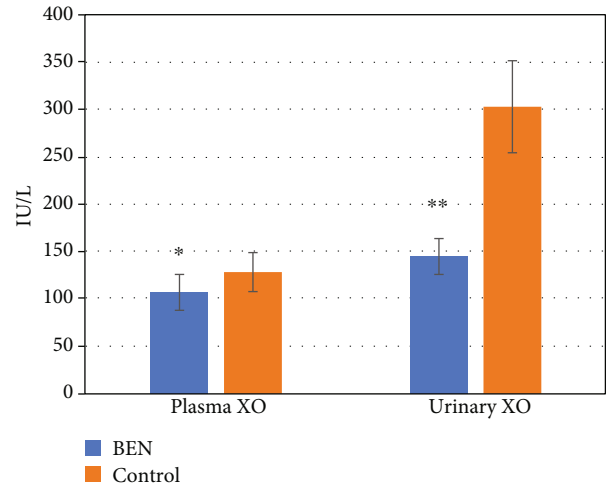

Figure 4: Xanthine oxidase activity in patients with BEN and controls. Data are mean ± SD values. $*p < 0.05$ versus control; $**p < 0.001$ versus control.

development of this disorder. However, the influence of these factors in association with chronic tubulointerstitial nephropathy and end-stage renal disease remains undetermined.

The association of food containing AA with BEN and UUC was initially documented in 1969 by Ivić [39]. Also, the inhabitants in regions characterized by BEN are exposed to relatively high concentrations of OTA [40].

Heavy metals are also implicated in the pathophysiology of BEN. Among them, increased levels of silica, lead, uranium, copper, cobalt, zinc, manganese, arsenic, titanium, barium, aluminium, chromium, strontium, cadmium, bismuth, molybdenum, nickel, tungsten, and antimony in water and soil have been documented in areas with increased incidence and prevalence of BEN [13]. It is well known that metal ions can produce free radicals via Fenton reaction. We documented the influence of a heavy metal intake and ROS development in our previous study [41]. The concentrations and the extent of selenium deficiency (a well-known antioxidant) have also been documented in rock, soil, water, foodstuff, and blood serum samples collected from endemic and nonendemic regions of BEN in the geographic area [22–24].

The deoxyribonucleic acid DNA-AA adducts induced by AA produce a particular molecular signature in the kidneys with BEN nephropathy. However, the pathophysiological mechanism whereby AA results in renal injury is still unclear. Declèves et al. [42] showed that nitric oxide (NO) plays an important role in mediating AA-induced kidney injury, leading to enhanced OS and tubular cell programmed cell death. AA-induced OS could mediate a peritubular capillary loss and additional vascular transformation, the vital pathophysiological processes within the development of chronic nephropathy from acute kidney injury in different injury models.

Among mycotoxins, OTA was documented to be an OS inducer [40]. Taking into consideration all the information, the EU Food Safety Authority (EFSA) scientific panel on contaminants within the food chain concluded that there was no proof for the existence of specific OTA-DNA adducts and

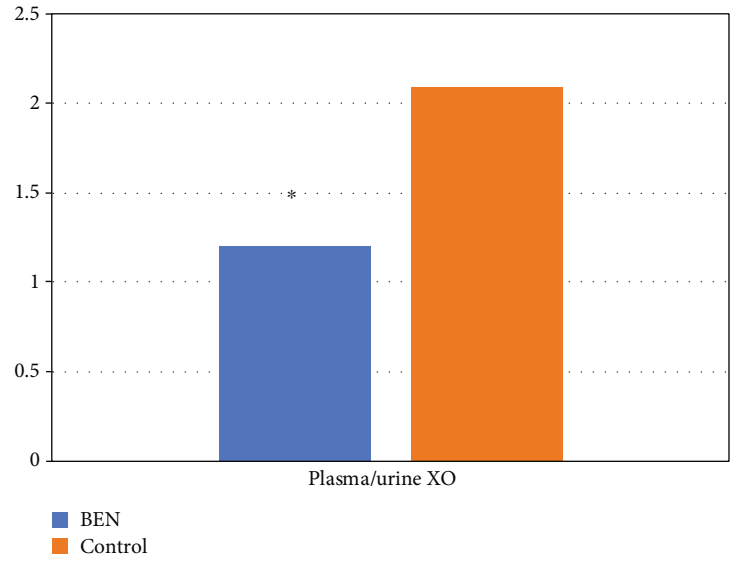

Figure 5: XO values in patients with BEN and controls: plasma/urine. $*p < 0.001$ versus control.

that the genotoxic effects of OTA were presumably the result of OS injury [43].

The kidneys are extremely vulnerable to ROS damage, because of long-chain-polyunsaturated fatty acids present in cell membranes and higher local concentration of excretory toxic products. The results of our study demonstrated a higher plasma level of TBARS in patients with BEN compared to controls. One of the first events in oxidative cellular injury is the oxidization of membrane lipids. Lipid hydroperoxides are nonradical intermediates derived from unsaturated fatty acids, phospholipids, glycolipids, cholesterol esters, and cholesterol itself. Their formation occurs in an enzymatic or nonenzymatic reaction involving ROS. Measuring a breakdown product like TBARS, a nephrotoxic molecule which is a biological marker of OS, is the most frequently performed method for determination of lipid peroxidation [44]. In our study, the increased level of lipid peroxidation was statistically significant in plasma, but not in urine, which may imply that systemic OS may arise due to kidney dysfunction and accumulation of different uremic toxins. A recently published article by Kim et al. [45] has shown that the administration of melatonin as a well-known antioxidant interfered with AA-induced renal dysfunction, suppressed AA-induced OS, as evidenced by the downregulation of 4-hydroxynonenal and reduced level of malondialdehyde and a modulated expression of prooxidant and antioxidant enzymes.

Circulating AOPPs serve as a marker of protein oxidation status. Our study showed that AOPPs were significantly elevated both in the plasma and urine of patients with BEN. Li et al. [46] reported that in the remnant kidney model, a higher AOPP level resulted in accelerated progression of renal injury, as proved by a marked increase of tubular fibrosis and glomerulosclerosis, additionally higher level of albuminuria, and deteriorated renal dysfunction. Moreover, experimental AOPP administration raised the urinary protein excretion in sham-operated rats thus confirming their direct toxic effect [46]. Our results also suggest that AOPPs are involved in the process of renal recast, because of the subsequent fibrogenic process in the kidney. Furthermore, the increase in the plasma/urine ratio in BEN patients might indicate their systemic origin as well. Li et al. [46] reported that chronic administration of AOPPs during this remnant kidney model raised the renal levels of AOPPs considerably, followed by magnified levels of TBARS and a reduced Glutathione Peroxidase (GSHPx) activity. Moreover, exogenous AOPP administration evoked an imbalance of oxidation-reduction reaction in rats with intact kidney and normal renal function, suggesting that a more severe OS could not *per se* promote the progression of kidney dysfunction. In sum, these results suggest that AOPPs could be one of the inducers of OS in BEN. A close relationship between AOPP and TBARS levels or GSHPx activity and the in vitro studies demonstrating a respiratory burst of human neutrophils which were exposed to AOPPs give additional proof to support the notions [47]. Since chronic AOPP administration raised the expression of transforming growth factor beta 1 (TGF-β1), a well-documented fibrogenic growth factor, there was a direct connection between the AOPP and the pathogenesis of nephritic inflammation and fibrosis, which is one of the key mechanisms of kidney deterioration in patients with BEN [48].

ROS can directly damage the deoxyribonucleic acid of proximal tubular cells and accelerate programmed cell death. To support this hypothesis, it was reported in a previous study that AA depleted the antioxidant glutathione in human renal tubular cells (HK-2) leading to tubular cell death [36]. Tubular cell death may be related to the OS-induced vascular effects of AA. Intrarenal OS exacerbates smooth muscle cell proliferation of the afferent arterioles and promotes renin-angiotensin system activation. It was documented that ischaemic injury as a part of AA-induced acute kidney injury is related to reduced NO levels; such OS-induced vascular changes may also lead to tubular cell death. AA can directly

reduce the concentration of NO and can additionally cause the peritubular capillary loss and vascular transformation [49]. Another important question is which is the most probable source of ROS overproduction. AA may be a direct inducer of OS, but that is certainly not the main cause of it. In the literature, there is extensive evidence regarding the role of enhanced ROS within the kidney and protective role of antioxidants and ROS scavengers in ischaemia-reperfusion injury [49–52]. Indeed, fibrotic kidney is under ischaemia. However, this mechanism occurs only in the last stages of the disease.

Another potential source of ROS could be uremic toxins in patients with CKD. A higher level of those toxins promotes systemic inflammation via priming polymorphonuclear leukocytes and stimulating CD-8^+ cells [53]. Increased OS occurs even in early stages of the disease, progresses with deterioration of renal function, and is further aggravated by hemodialysis (HD), due to the bioincompatibility of HD systems [54, 55]. In general, two major parts of a HD system will contribute to OS: the dialyzer membrane and trace endotoxins within the dialysate [56]. However, it is an unlikely explanation in this disease because not all patients with BEN were uremic.

Our hypothesis was that the source of free radicals could be XO activity, since the liver, gut, and kidney represent XO-rich organs. XO-induced ROS production is known to be elevated in septic development by significant liver and kidney injuries, and XO inhibitors are one of the protectors from kidney injuries [57]. Additionally, the long-term treatment with XO inhibitors achieved renoprotective advantages in individuals with a nonadvanced chronic renal disorder [58]. A large body of clinical proof has cited uric acid as a possible therapeutic target for slowing down CKD progression [59]. Increased serum levels of uric acid have been associated with the onset and development of chronic kidney disease (CKD), cardiovascular disease, and mortality, through several molecular pathogenetic mechanisms, such as inflammation and OS [60]. Gouty patients and even people with symptomless hyperuricemia have a sustained risk of developing renal damage; equally, in patients with obvious CKD, steady elevated uric acid levels might contribute to the deterioration of renal function [61]. The deterioration of renal function which leads to end-stage disease such as BEN might be the result of coexisting conditions such as vascular calcification, obesity, and hypertension and not only elevated serum uric acid [62]. Since ROS are closely related to kidney pathology [63], it has been reported that XOR redox, i.e., the quantitative relation of XO to total XOR (XO and XDH), changes the oxidative state and is in correlation with kidney pathology [34]. We find XOR not only as a xanthine dehydrogenase (XDH) type but also as an XO form, which uses an oxygen molecule as an electron acceptor and generates ROS as superoxide anion radicals and hydrogen peroxide [34]. We have already proven that XO activity is one of the possible reasons for ROS liberation in experimental kidney damage [41]. It is also demonstrated that XO is responsible for ischaemia injury and fibrosis [64].

All findings mentioned above led to the expectancy of a higher activity of XO in BEN patients since there were many causes which would raise its activity, but this was not the case. Our results showed a lower XO activity in patients with BEN when compared to healthy control subjects. Plasma/urine ratio showed even more significant differences, which may point to a systemic effect of the enzyme. There are only a few patients with BEN who suffered from gout. One of the possible reasons would be the lack of actual substrates for XO, like urine nucleotides. Lower kidney mass, accompanied with diminished functional tissue, may be responsible for a lower substrate level. Furthermore, studies showed the importance of AA in the formation of DNA-AA adducts [16]. The resulting adenine (AL-N6-dA) and guanine (ALN2-dg) adducts misincorporate adenine throughout replication [20, 21]. Specifically, the intrinsic twisted conformation of ALII-N2-dG induces destabilizing distortions to deoxyribonucleic acid at the lesion site, reduces van der Waals (stacking) interactions with the neighbouring base pairs, and enhances the helical dynamics at the damaged site [65]. The abovementioned changes affect the purine nucleotide structure in patients with BEN, probably making them conformationally not recognizable for XO, because AA binds covalently with the exocyclic amino group of purine nucleotides.

5. Conclusions

The level of lipid peroxides and AOPPs is significantly increased in BEN patients compared to controls. We hypothesize that both local and systemic overproduction of ROS might play a pivotal role in the pathogenesis of BEN which leads to end-stage renal disease and even cancerogenesis. Our results may for the first time demonstrate that XO would not be considered a direct systemic or local contributor to ROS production in BEN, most probably because of the diminished kidney functional tissue mass and AA-induced changes in purine nucleotide conformation. A low XO activity might also prevent gout in patients with BEN.

Data Availability

The data used to support the findings of this study are included within the article.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

Acknowledgments

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Research Article

Short-Chain Fatty Acids Ameliorate Diabetic Nephropathy via GPR43-Mediated Inhibition of Oxidative Stress and NF-κB Signaling

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Diabetic nephropathy (DN) is a chronic low-grade inflammatory disease. Oxidative stress and nuclear factor kappa B (NF-κB) signaling play an important role in the pathogenesis of DN. Short-chain fatty acids (SCFAs) produced from carbohydrate fermentation in the gastrointestinal tract exert positive regulatory effects on inflammation and kidney injuries. However, it is unclear whether SCFAs can prevent and ameliorate DN. In the present study, we evaluated the role and mechanism of the three main SCFAs (acetate, propionate, and butyrate) in high-fat diet (HFD) and streptozotocin- (STZ-) induced type2 diabetes (T2D) and DN mouse models and in high glucose-induced mouse glomerular mesangial cells (GMCs), to explore novel therapeutic strategies and molecular targets for DN. We found that exogenous SCFAs, especially butyrate, improved hyperglycemia and insulin resistance; prevented the formation of proteinuria and an increase in serum creatinine, urea nitrogen, and cystatin C; inhibited mesangial matrix accumulation and renal fibrosis; and blocked NF-κB activation in mice. SCFAs also inhibited high glucose-induced oxidative stress and NF-κB activation and enhanced the interaction between β-arrestin-2 and I-κBα in GMCs. Specifically, the beneficial effects of SCFAs were significantly facilitated by the overexpression GPR43 or imitated by a GPR43 agonist but were inhibited by siRNA-GPR43 in GMCs. These results support the conclusion that SCFAs, especially butyrate, partially improve T2D-induced kidney injury via GPR43-mediated inhibition of oxidative stress and NF-κB signaling, suggesting SCFAs may be potential therapeutic agents in the prevention and treatment of DN.

1. Introduction

Diabetic nephropathy (DN) is a serious microvascular complication of diabetes and a major cause of end-stage renal disease (ESRD) [1]. Oxidative stress, NF-κB signaling activation, and overexpression of various inflammatory cytokines caused by a persistent hyperglycemic state as well as hemodynamic changes play an important role in the pathogenesis of DN. These events cause glomerulosclerosis, tubular atrophy, and fibrosis, eventually leading to irreversible renal damage [2]. However, as clinical strategies based on antioxidant stress and anti-inflammation are limited in their effectiveness, it is becoming increasingly important to explore new methods for the prevention and treatment of DN [3].

The gut microbiota and its metabolites play pivotal roles in host physiology and pathology [4]. Short-chain fatty acids (SCFAs), mainly acetate, propionate, and butyrate, with their ratio in the colon being 60 : 25 : 15, respectively, are produced predominantly by gut microbiota in the fermentation of dietary fiber and undigested carbohydrates [5, 6]. SCFAs are involved in the maintenance of a gastrointestinal epithelial barrier, the regulation of hormone secretion, and the inhibition of enteric endotoxemia and inflammation via the combination of a cell surface G-protein-coupled receptor (GPCR) pair of GPR41 and GPR43 or by inhibiting histone deacetylation (HDAC) [7, 8]. In clinical investigations and animal models, the increased intake of dietary fibers or SCFA administration has also been shown to possess protective effects in inflammatory bowel conditions, allergic airway disease, obesity, type 1 diabetes (T1D), and type 2 diabetes (T2D) due to their inhibitory effects on proinflammatory cytokines and reactive oxygen species (ROS) [9, 10]. These studies show oxidative stress and NF-κB signaling as effector mechanisms of SCFAs, suggesting a promising therapeutic potential in the treatment of chronic low-grade inflammatory diseases [11, 12].

Recent, albeit limited, studies have attempted to use SCFAs therapeutically in both animal and cell models of kidney injuries, such as ischemia-reperfusion-induced acute kidney injury (AKI) [13], contrast-induced nephropathy [14], and gentamicin-induced nephrotoxicity [15]. However, it is unclear whether exogenous SCFAs may directly prevent and ameliorate T2D-induced DN and how SCFAs may regulate this process. In this study, we first evaluated the effect of the three main SCFAs (acetate, propionate, and butyrate) on high-fat diet (HFD) and streptozotocin- (STZ-) induced T2D and DN mouse models *in vivo*. Next, we evaluated the effect of SCFAs on high glucose-induced oxidative stress and NF-κB signaling in mouse glomerular mesangial cells (GMCs) *in vitro*. Finally, we investigated whether GPR43-β-arrestin-2 signaling could be involved in these positive effects of SCFAs by transfection with a GPR43 overexpressing vector or siRNA-GPR43. Our present study and findings may provide new insights on the role of microbiota metabolites in the intervention of DN.

2. Materials and Methods

2.1. Animal Model. Eight-week-old male C57BL/6 mice were purchased from the Biotechnology Corporation of Dashuo (Chengdu, China). All procedures were in accordance with the guidelines of the Institutional Animal Care and Use Committee of the Southwest Medical University.

At 10 weeks of age, all mice were randomly allocated to two groups: a control group (NC group, $n = 8$) and a T2D group ($n = 32$). The NC group received a normal diet until the end of experiments whereas the T2D mouse group was given a HFD (40% kcal fat; Biotechnology Corporation of Dashuo) for 8 weeks, and then diabetes was induced by intraperitoneal injection of single low-dose (40 mg/kg) STZ (Sigma-Aldrich, St Louis, MO, USA) dissolved in 0.05 M sodium citrate buffer (pH 4.5), followed by continued HFD feeding for an additional 12 weeks. Random blood glucose (RBG) levels of ≥16.7 mmol/L (mM) lasting 3 days were confirmed as being "diabetic." At the moment of an STZ intraperitoneal injection, the T2D mice were further randomly classified into four groups ($n = 8$/group) with an equal average initial body weight: (1) acetate (Ac group): T2D mice treated with an intraperitoneal injection (100 mg/(kg·48 h) of sodium acetate (Sigma-Aldrich) for 12 weeks; (2) propionate (Pr group): T2D mice were treated with sodium propionate (Sigma-Aldrich) at a similar dose and frequency to those used for the Ac group; (3) butyrate (But group): T2D mice received the same dose and frequency of sodium butyrate (Sigma); and (4) T2D control group (T2D group): T2D mice treated with an intraperitoneal injection of phosphate-buffered saline (PBS) solution at the same volume and frequency. Meanwhile, the NC groups also received injections of equivalent volumes and frequency of PBS buffer. All mice were weighed, and blood samples from tail veins and urine specimens were collected every two weeks. At the 20th week of experiments, all mice were sacrificed and fasting heart blood collected; kidneys were used for pathology, western blotting, and qRT-PCR.

2.2. Biochemical Measurements. RBG and fasting blood glucose (FBG) levels were measured by Accu-Chek (Roche Diagnostics GmbH, Mannheim, Germany). Random urine albumin-creatinine ratios (ACR) were calculated every two weeks according to manufacturers' procedures as outlined in kits (Afinion™ ACR; Axis-Shield PoC AS, Oslo, Norway). Fasting insulin (FINS) levels were assayed by insulin enzyme-linked immunosorbent assay (ELISA) detection kit (Alpco, Salem, NH, USA), and HOMA-IR values were calculated according to a formula. Blood urea nitrogen (BUN), serum creatinine (SCr), serum cystatin C, total cholesterol (TC), triglyceride (TG), and low-density lipoprotein-cholesterol (LDL-C) levels were measured by an automatic biochemistry analyzer (Hitachi 7150, Hitachi Group, Tokyo, Japan).

2.3. Renal Histology. Kidneys from mice were fixed in 4% paraformaldehyde and embedded in paraffin, and 4 μm sections were cut. Sections were stained with hematoxylin and eosin (H&E), periodic acid-Schiff (PAS), and Masson's trichrome staining. For each mouse, images of six different fields of view were evaluated under ×400 magnification by light microscopy (Leica, Wetzlar, Germany). The numbers of glomeruli with mesangial expansion and vasodilation were counted according to previously established methods [16]. The number of grid points on the mesangial matrix (PAS-

stained glomerulus-positive areas) was divided by the total number of points in each glomerulus to calculate the relative mesangial matrix area as a percentage of the total grid of the glomerulus. Masson's trichrome-stained tissue images and relative fibrotic areas were evaluated by Image-Pro Plus 6.0 software. The histopathologist was blind to the treatment groups when analyses were made.

2.4. Immunohistochemistry Staining. Sections were incubated with the following primary antibodies: anti-GPR43 (rabbit polyclonal antibody; 1 : 100 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-β-arrestin-2 (rabbit polyclonal antibody; 1 : 100 dilution; Santa Cruz Biotechnology), anti-NF-κBp65 (goat polyclonal antibody; 1 : 100 dilution; Santa Cruz Biotechnology), and anti-MCP-1 (rabbit monoclonal antibody; 1 : 100 dilution; CST, Danvers, MA, USA) overnight at 4°C. After sections were washed with PBS, they were incubated with horseradish peroxidase (HRP) or fluorescein isothiocyanate fluorescent dye-conjugated secondary antibodies (1 : 200 dilution; Beijing Biosynthesis Biotechnology, Beijing China) for 2 h at room temperature. For visualizing the signals of immunohistochemistry, sections were treated with peroxidase substrate 3,3-diaminobenzidine and counterstained with hematoxylin. Positive staining areas were evaluated by Image-Pro Plus 6.0 software.

2.5. Cell Culture, Treatment, and Viability Assay. Mouse glomerular mesangial cells (SV-40 MES 13) were obtained from the China Center for Type Culture Collection and cultured in Dulbecco's modified Eagle's medium (Gibco, Waltham, MA, USA) containing 5.6 mM glucose and 10% fetal bovine serum (Gibco) at 37°C and 5% CO_2.

Initially, to determine proper concentrations of each SCFA and GPR43 agonist, cells were randomly divided and the following treatments were applied: (1) Ac at 0.1, 1, 10, and 100 mM concentrations; (2) Pr at 0.1, 1, 10, and 100 mM concentrations; (3) But at 0.05, 0.5, 5, and 50 mM concentrations; and (4) a phenylacetamide compound (Merck Millipore, Burlington, MA, USA) that acted as an allosteric agonist of GPR43 at 0.01 0.1, 1, and 10 μmol/L (μM) concentrations. GMCs were seeded in 96-well plates at concentrations of 1×10^5 cells/mL to 50% confluence in DMEM complete growth medium, followed by treatments respectively supplemented with different concentrations of SCFAs and GPR43 agonist as described above for 24 h in full media. The viability of the cells was determined by a combination method including MTT analysis. MTT experiments were performed in 5 biological replicates. According to MTT results and literature reviews, 10 mM acetate, 10 mM propionate, 5 mM butyrate, and 1 μM GPR43 agonist were used in the *in vitro* study.

2.6. GPR43 Overexpression Vector Construction and Transfection. Open reading frames of the mouse *GPR43* (Gene ID: 233079) gene were amplified by PCR and inserted in a pCD513B-1 plasmid (Public Protein/Plasmid Library) to construct a pCD513B-1-GPR43 expression vector (CMV promoter). PCR and restriction enzyme digestion were used to confirm a successfully constructed recombinant GPR43 overexpression vector. Transfection was done by a Lipofectamine®3000 RNAiMax reagent (Invitrogen, Karlsruhe, Germany) following the manufacturer's instructions.

2.7. Small Interfering RNA Transfection. siRNA targeting *GPR43* (sense: 5′-CCAGCCTGGATCCATTATT-3′, antisense: 5′-AAUAAUGGAUCCAGGCUGG-3′) or control siRNA (sense: 5′-UUCUCCGAACGUGUCACGU-3′; antisense: 5′-ACGUGACACGUUCGGAGAA-3′) was synthesized by Ribo Biotech (Guangzhou, China). Transfections were performed using the Lipofectamine® 3000 RNAiMax reagent (Invitrogen, Karlsruhe, Germany) following the manufacturer's instructions. Experiments were performed with these cells at 24 h posttransfection.

2.8. Detection of ROS, MDA, and SOD. After incubation with different compounds as described above, intracellular production of ROS was measured using an ROS assay kit (Beyotime, Haimen, China); the contents of MDA and total superoxide dismutase (SOD) were determined using a Lipid Peroxidation MDA Assay Kit (Beyotime) and a Total Superoxide Dismutase Assay Kit with WST-8 (Beyotime) according to the manufacturer's instructions. Values were expressed as the mean absorbance normalized to a percentage of the normal control.

2.9. Western Blotting. Total protein was extracted using a protein extraction kit (Kaiji, Shanghai, China), which contains a protease inhibitor and phosphatase inhibitor. Protein concentrations were assessed by using a BCA Protein Assay Kit (Bioworld Technology, USA). The OD value was measured at a wavelength of 562 nm, and the standard curve ($r > 0.99$) calculated the protein sample concentration. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane (Merck Millipore). Immunoblotting was performed using anti-GPR43 (rabbit polyclonal antibody; 1 : 1000 dilution; Santa Cruz Biotechnology), anti-β-arrestin-2 (rabbit polyclonal antibody; 1 : 1000 dilution; Santa Cruz Biotechnology), anti-I-κBα antibody (mouse monoclonal antibody; 1 : 1000; CST, number 4814), anti-p-I-κBα antibody (ser32/36; mouse monoclonal antibody; 1 : 1000; CST; number 9246), anti-NF-κBp65 antibody (rabbit polyclonal antibody; 1 : 1000; Beyotime; number AF0246), anti-p-NF-κBp65 antibody (ser536; mouse monoclonal antibody; 1 : 2000; CST; number 13346), anti-MCP-1 (rabbit monoclonal antibody; 1 : 100 dilution; CST), anti-IL-1β (rabbit polyclonal antibody; 1 : 800 dilution; CST), and anti-GAPDH antibody (mouse; 1 : 800 dilution; Beyotime) overnight at 4°C. The second antibodies of GPR43, β-arrestin-2, NF-κBp65, MCP-1, and IL-1β (1 : 2000, anti-rabbit) and I-κBα, p-I-κBα, p-NF-κBp65, and GAPDH (1 : 2000, anti-mouse) were obtained from the Beyotime Institute of Biotechnology, Shanghai, China. The proteins were detected with HRP chemiluminescence reagent (Millipore, USA), and images were captured with the UVP imaging system (Bio-Rad, USA). Quantity One software was used for the analysis of bands.

2.10. Quantitative Real-Time PCR Analysis. Total RNA was isolated from the kidney and GMCs using an RNA extraction kit (ComWin Biotech, Beijing, China). The isolated RNA was subjected to reverse transcription using a PrimeScript RT

Table 1: Primer sequences for quantitative real-time PCR.

Gene	Forward sequence	Reverse sequence
GPR43	5′-GGTGGAGGCTGTGGTGTT-3′	5′-GCATAGAGGAGGCAGGATT-3′
GPR41	5′-CTCATCACCAGCTACTGCCG-3′	5′-AATTCAGGGTGCTGAGGAGC-3′
β-Arrestin-2	5′-CCATTGTGAAGGAGGGAG-3′	5′-GCATTAGGACGAAGGGTAG-3′
β-Arrestin-1	5′-ACCTTTGAGATCCCGCCAAA-3′	5′-CTTTCTGATGATAAGCCGCACA-3′
β-Actin	5′-ACCTCTATGCCAACACAGTG-3′	5′-GGACTCATCGTACTCCTGCT-3′

Reagent Kit (TaKaRa, Kusatsu, Japan). The synthesized cDNA was used as a template for quantitative PCR analysis. The housekeeping gene, β-actin, was quantified as an internal RNA control. Quantitative RT-PCR was performed on a 7900HT Fast Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA, USA). The primer sequences for all studied genes are listed in Table 1. The thermal cycling program used was as follows: an initial step at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 s, annealing at 60°C for 34 s, and extension at 72°C for 15 s. The melting curve of each PCR product was obtained by continuous fluorescence monitoring at a temperature gradient ramp from 60 to 95°C. Quantitative PCR reactions were performed in triplicate to remove any outliers. The relative changes in gene expression were analyzed by the 2-$\Delta\Delta$CT method.

2.11. ELISA Assay. MCP-1 and IL-1β protein levels in cell culture supernatants were determined using commercially available MCP-1 and IL-1β ELISA kits (Neobioscience, Shanghai, China) according to the manufacturer's protocols. MCP-1 and IL-1β protein levels were determined by comparing the samples with a standard curve generated using the kit.

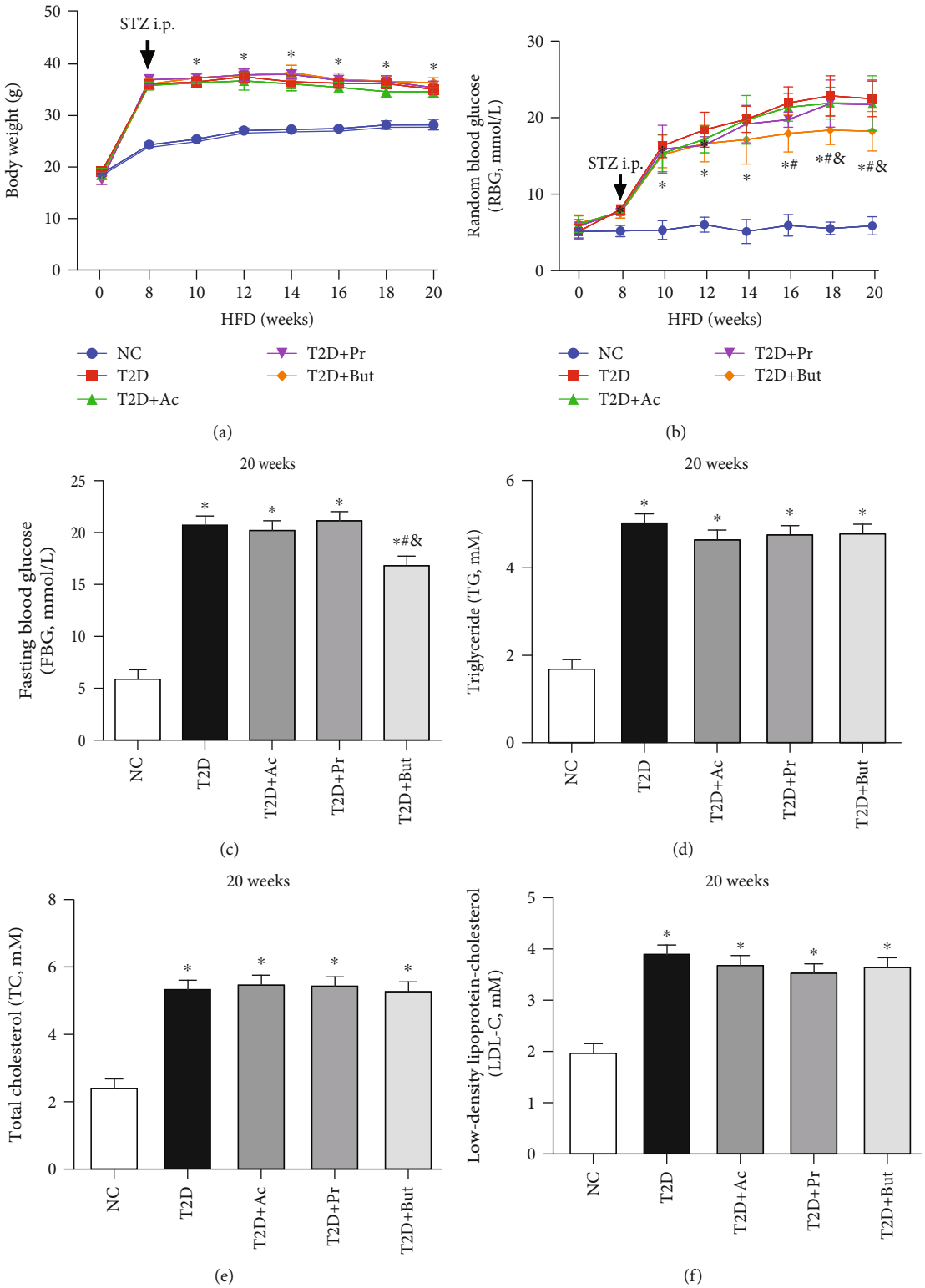
2.12. Statistics. All data were obtained from at least five independent experiments and were expressed as the mean ± standard deviation (SD). Between-group comparisons were analyzed using one-way analysis of variance (ANOVA), followed by post hoc Tukey's correction test for multiple comparisons (SPSS 20.0 software). $p < 0.05$ was considered significant.

3. Results

3.1. SCFAs Ameliorated Hyperglycemia and Insulin Resistance of Experimental T2D. In the current study, we investigated the role of SCFAs in a nongenetic rodent model of T2D, HFD/STZ mice. In this model, overt hyperglycemia results from a combination of insulin resistance induced by HFD feeding and defects in insulin secretion induced by single low-dose (40 mg/kg) STZ treatment. To examine the effects of SCFAs on glycolipid metabolism in the development of obesity and insulin resistance, body weight (BW, Figure 1(a)) and RBG (Figure 1(b)) levels were assessed at baseline and from 8 to 20 weeks. After 8 weeks, compared with the NC group, significant changes in BW and RBG levels in T2D mice were noted. This pattern was also seen for FBG (Figure 1(c)), blood lipid spectrum (TC, TG, and LDL-C; Figures 1(d)–1(f)), and FINS (Figure 1(g)) levels, suggesting that T2D models were successfully achieved. Next, we found that intraperitoneal injections of three main SCFAs for 12 weeks did not have a significant effect on BW, FINS, and the blood lipid spectrum in experimental T2D mice. However, when supplemented with SCFAs, especially butyrate, RBG and FBG levels were partially reversed (Figures 1(a)–1(c)). Finally, SCFA-supplemented mice showed significantly lower homeostatic model assessment of HOMA-IR (Figure 1(h)) than T2D controls and enhanced insulin sensitivity, without affecting the FINS (Figure 1(g)). Collectively, our observations indicated that exogenous SCFAs did not significantly affect obesity, FINS, and lipid metabolism; however, SCFAs, especially butyrate, improved insulin resistance and protected mice from experimentally induced T2D.

3.2. SCFAs Prevented Renal Dysfunction in Experimental T2D. To assess whether SCFAs were nephroprotective *in vivo*, random ACR, an important feature of kidney injury in DN, were measured every two weeks. We found that SCFA treatment, especially butyrate, resulted in lowering the levels of urine ACR (Figure 2(a)) and in lowering serum urea (Figure 2(b)), creatinine (Figure 2(c)), and cystatin C (Figure 2(d)) levels, markers of the severity of renal dysfunction in DN. Histopathological examination of renal tissues by H&E, PAS stains, and Masson's trichrome (Figure 2(e)) revealed that mesangial expansion (Figure 2(f)), the glomerular tuft (Figure 2(g)), and the accumulation of collagen (Figure 2(h)) were substantially elevated in the T2D group when compared to the NC group. Notably, these histomorphometric changes were significantly attenuated by treatment with SCFAs, especially butyrate. These results indicated that T2D-induced renal histomorphometric changes and renal dysfunction were effectively ameliorated by SCFAs.

3.3. SCFAs Inhibited T2D-Induced Renal NF-κB Activation and Regulated GPR43-β-Arrestin-2 Signaling. We searched for potential target genes of SCFAs in the treatment of DN. According to the literature, a possible candidate was NF-κBp65, which forms a key part in the multiple signal transduction of chronic inflammation in the pathogenesis of DN. Consistent with prior reports, western blotting showed that the protective effects of SCFAs, especially butyrate, were associated with the increased expression of I-κBα, which inhibits the phosphorylation and nuclear translocation of NF-κBp65, and inhibited the downstream inflammatory cytokine, MCP-1, and IL-1β expression (Figure 3(a)). In



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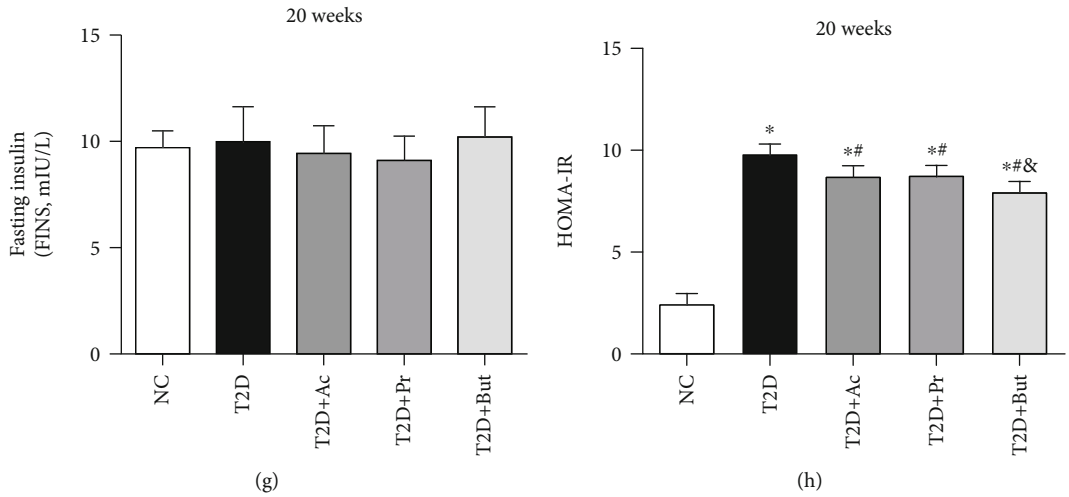
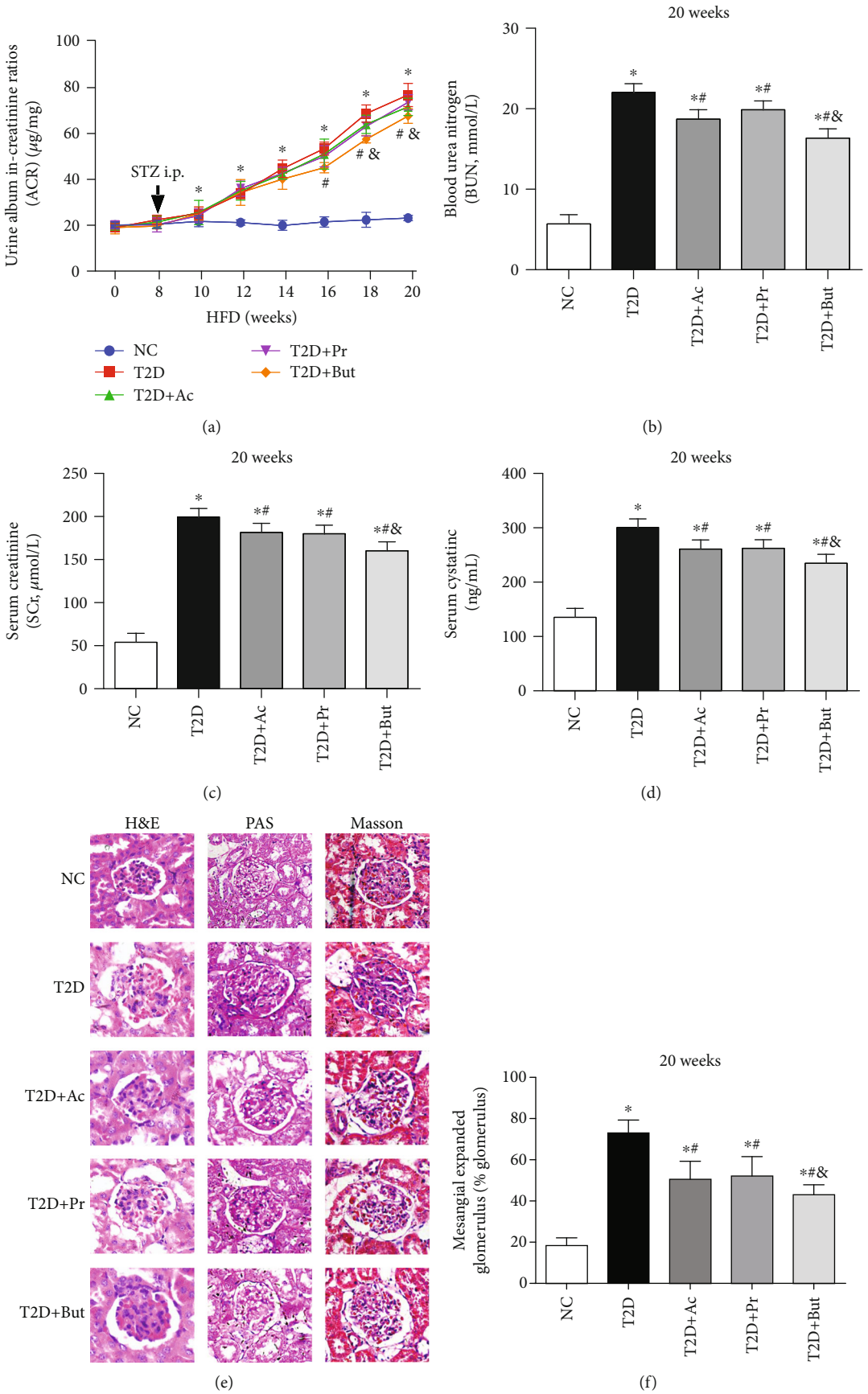


Figure 1: SCFAs ameliorated hyperglycemia and insulin resistance of experimental *T2D*. Mice were subjected to a high-fat diet (HFD) for 8 weeks, intraperitoneally (i.p.) injected with STZ, and then treated with three main SCFAs, acetate (Ac), propionate (Pr), and butyrate (But), for 12 weeks. Body weight (BW) (a) and random blood glucose (RBG) (b) were measured every 2 weeks; fasting blood glucose (FBG) (c), total cholesterol (TC) (d), total glyceride (TG) (e), low-density lipoprotein-cholesterol (LDL-C) (f), fasting insulin (FINS) (g), and homeostatic model assessment of insulin resistance (HOMA-IR) (h) values were measured at the 20th week of the experiment before sacrifice. $^{*}p < 0.05$ compared with the NC group; $^{\#}p < 0.05$ compared with the T2D group; $^{\&}p < 0.05$ compared with the Ac or Pr group.

order to determine if there was a relationship between GPCRs and β-arrestins in established experimental DN mice, we measured mRNA levels of GPR43, GPR41, β-arrestin-1, and β-arrestin-2 by qRT-PCR. Compared with the NC group, mRNA expression of GPR43 was inhibited, but β-arrestin-2 were upregulated in the kidneys of T2D mice. However, no obvious response was found for GPR41 and β-arrestin-1; SCFAs induced the mRNA expression of GPR43 (Figure 3(b)) but inhibited that of β-arrestin-2 (Figure 3(c)) simultaneously. Furthermore, both western blotting (Figure 3(d)) and immunohistochemistry (Figure 3(e)) confirmed that SCFAs reversed the T2D-induced downregulation of GPR43 and upregulation of β-arrestin-2 and inhibited p-NF-κBp65 and MCP-1 expression, suggesting that SCFAs have a protective effect on DN by regulating GPR43-β-arrestin-2 and inhibiting the activation of NF-κB signaling.

3.4. SCFAs Partially Reversed High Glucose-Induced Oxidative Stress and NF-κB Signaling Activation In Vitro. Next, we explored whether oxidative stress and NF-κB signaling could be causally involved in the renal protective effect of SCFAs *in vitro*. Treating GMCs for 24 h with 0.1-10 mM concentration range of acetate or propionate, 0.05-5 mM butyrate, or 0.1-1 μM GPR43 agonist promoted GMC proliferation in a dose-dependent manner; however, higher concentrations of these SCFAs or GPR43 agonist inhibited cell viability (Figure 4(a)). According to the MTT assay and literature review, 10 mM acetate, 10 mM propionate, 5 mM butyrate, and 1 μM GPR43 agonist were used as intervention reagents *in vitro*; 30 mM high glucose was then used as a stimulating factor, which induced abnormal levels of oxidative stress-relevant molecules such as ROS (Figure 5(b)) and MDA (Figure 5(c)), as well as the release of MCP-1 (Figure 5(d)) and IL-1β (Figure 5(e)). We found that these high glucose-induced abnormalities were significantly abolished by SCFAs or the GPR43 agonist, and these intervention reagents have no significant effect on oxidative stress and inflammation. In line with *in vivo* results, western blotting showed that the high glucose-induced phosphorylation of NF-κBp65 and MCP-1 protein level was significantly decreased by these SCFAs or a GPR43 agonist (Figure 5(f)), suggesting that either SCFAs or a GPR43 agonist at a certain concentration range inhibited high glucose-induced oxidative stress and NF-κB activation in GMCs.

3.5. SCFA-Mediated Antioxidant and Anti-Inflammatory Effects Were Partially Reversed by siRNA-GPR43. To determine whether GPR43 and GPR41 are regulated by high glucose *in vitro*, we firstly detected GPR43 and GPR41 mRNA in GMCs by qRT-PCR. Compared with the NC group, the relative GPR43 mRNA expression gradually decreased after treatment with 30 mM glucose from 6 h to 24 h; however, a change in the GPR41 level was not found with high glucose treatment (Figure 5(a)), in line with *in vivo* results. Western blotting (Figure 5(b)) confirmed that GPR43 expression in GMCs was gradually decreased by 30 mM glucose in a time-dependent manner, accompanied by the high expression of MCP-1, revealing an intrinsic relationship between GPR43 and chronic inflammation in GMCs. However, the high glucose-inhibited GPR43 as well as high glucose-induced MCP-1 were abolished by SCFAs or the GPR43 agonist (Figure 5(c)). To find out whether GPR43 mediate the role of SCFAs, siRNAs were constructed to silence the GPR43 gene in GMCs. The results showed that the inhibition of ROS (Figure 5(d)) and MDA (Figure 5(e)) by SCFAs or the GPR43 agonist was significantly reversed by siRNA-GPR43. Furthermore, SCFAs or the GPR43 agonist-inhibited NF-κB activation, MCP-1 expression (Figure 5(f)), and MCP-1 release (Figure 5(g)) were also abolished by siRNA-GPR43,



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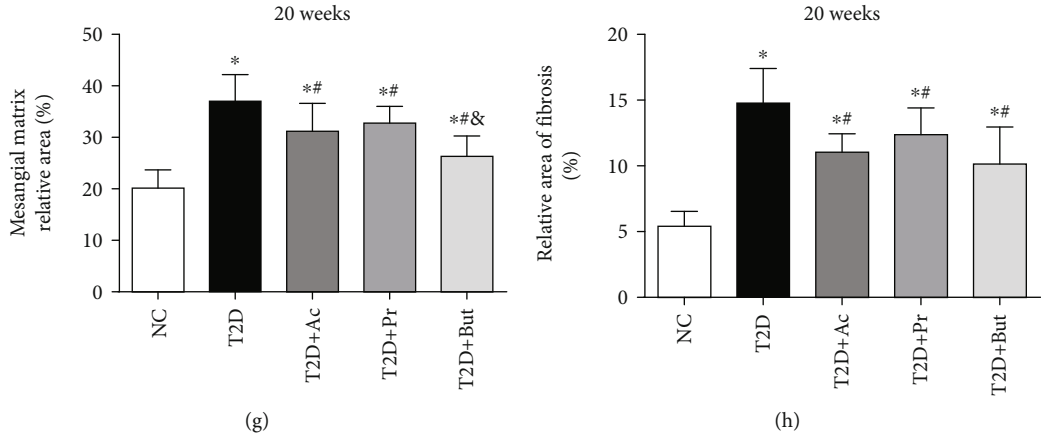


Figure 2: SCFAs prevented the renal dysfunction and kidney injury. Urine ACR (a) of T2D mice were measured every 2 weeks, and blood urea nitrogen (BUN) (b), serum creatinine (SCr) (c), and serum cystatin C (d) were assayed at the 20th week of the experiment. Histopathological examination of renal tissues was by H&E, PAS, and Masson's trichrome staining (400x) (e). Mesangial expansion (f), glomerular tuft (g), and the accumulation of collagen (h) were measured. Ac: acetate; Pr: propionate; But: butyrate; $^{*}p < 0.05$ compared with the NC group; $^{\#}p < 0.05$ compared with the T2D group; $^{\&}p < 0.05$ compared with the Ac or Pr group.

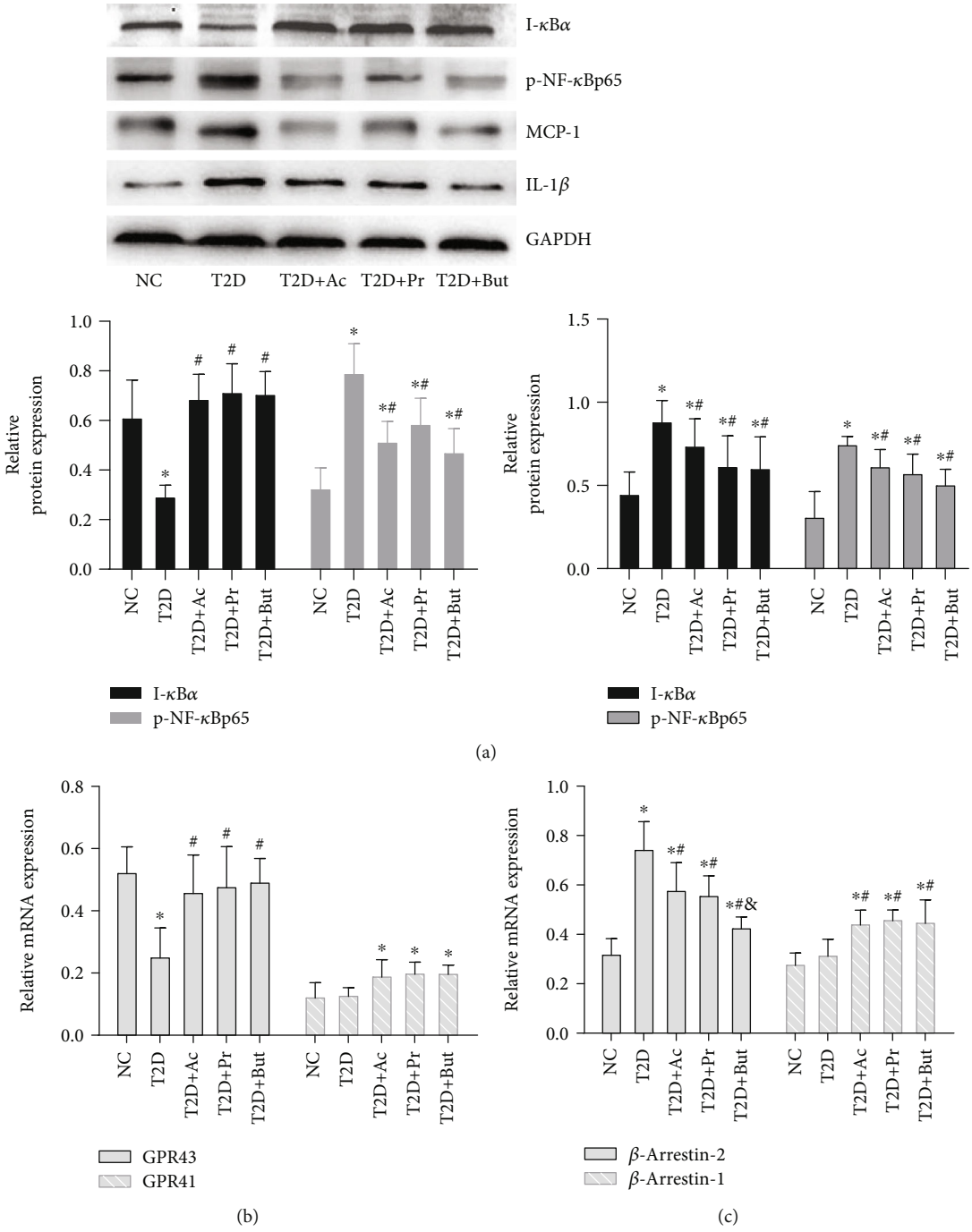
suggesting that GPR43 was partially involved in SCFA-mediated antioxidant and anti-inflammatory effects.

3.6. SCFA-Mediated Beneficial Effects Were Significantly Facilitated by GPR43 Overexpression. To assess the involvement of GPR43 in the inhibition of NF-κB signaling, plasmids overexpressing mouse GPR43 with pCD513B-1 (a GFP tag) or the pCD513B-1 control plasmid was constructed to determine the effects of overexpressed GPR43 on SCFA-mediated benefits. GMCs showed green fluorescence (Figure 6(a)), suggesting plasmid transfection was successful and the pCD513B-1-GPR43 fusion protein was expressed. Western blotting showed that the GPR43 were overexpressed in GMCs after transfection (Figure 6(b)). Since the most dramatic changes were observed in the butyrate intervention group, we only measured butyrate-mediated benefits in the next study. The results showed that butyrate-inhibited phosphorylation of NF-κBp65 and MCP-1 expression (Figure 6(c)) was enhanced by overexpressed GPR43. Furthermore, the butyrate-inhibited ROS (Figure 6(d)) and NF-κB components (Figure 6(e)) and MCP-1 and IL-1β release (Figure 6(f)) were also reversed by siRNA-GPR43 but were facilitated by GPR43 overexpression. Taken together, these data indicated that the inhibition of oxidative stress and NF-κB signaling by SCFAs was partially dependent on GPR43.

3.7. The Interaction of β-Arrestin-2 and I-κBα Was Induced by SCFAs via GPR43. To investigate the underlying mechanism by which GPR43-β-arrestin-2 signaling was involved in SCFA-inhibited NF-κB signaling, we first performed experiments to determine whether β-arrestins respond to high glucose and SCFAs. qRT-PCR showed that compared with β-arrestin-1, 30 mM high glucose induced the mRNA expression of β-arrestin-2 in a time-dependent manner (Figure 7(a)). SCFAs, especially butyrate, or the GPR43 agonist, inhibited high glucose-induced β-arrestin-2 expression (Figure 7(b)). Consistent with mRNA expression, western blotting confirmed that the β-arrestin-2 expression was induced by high glucose in a time-dependent manner (Figure 7(c)), but this trend was reversed by SCFAs or the GPR43 agonist (Figure 7(d)). Next, cell extracts were prepared and subjected to immunoprecipitation using anti-β-arrestin-2 antibody, and I-κBα coimmunoprecipitation was detected by immunoblotting with anti-I-κBα antibodies. As shown in Figure 7(e), β-arrestin-2 antibodies immunoprecipitated a polypeptide of about 100 kDa that was recognized by the I-κBα-specific antibody (Figure 7(e)), indicating that β-arrestin-2 and I-κBα were able to form a complex on endogenously expressed proteins in GMCs. Interestingly, the results also revealed that the interaction between β-arrestin-2 and I-κBα was decreased by 30 mM high glucose, but this effect was reversed by 5 mM butyrate. To investigate the underlying mechanism, GPR43 overexpression plasmids and siRNA-GPR43 were transfected into GMCs; the coimmunoprecipitation showed that butyrate-induced interaction between β-arrestin-2 and I-κBα was significantly reversed by siRNA-GPR43 but was facilitated by GPR43 overexpression (Figure 7(f)), suggesting that butyrate inhibited NF-κB signaling by GPR43-mediated interaction between β-arrestin-2 and I-κBα.

4. Discussion

SCFAs were identified as a link between gut microbiota and T2D: a number of studies in the last decade have shown that increased intake of dietary fiber and dietary supplementation with butyrate prevented or treated diet-induced weight gain, insulin resistance, and its related metabolic comorbidities [17, 18]. Recently, the effect of oral butyrate administration on blood HbA1c, inflammatory cytokines, and lipopolysaccharide (LPS) was demonstrated in db/db mice by restoring the composition of gut microbiota and preserving the integrity of the gut epithelial barrier [19]. SCFAs have been shown to modulate intestinal hormones (such as glucagon-like



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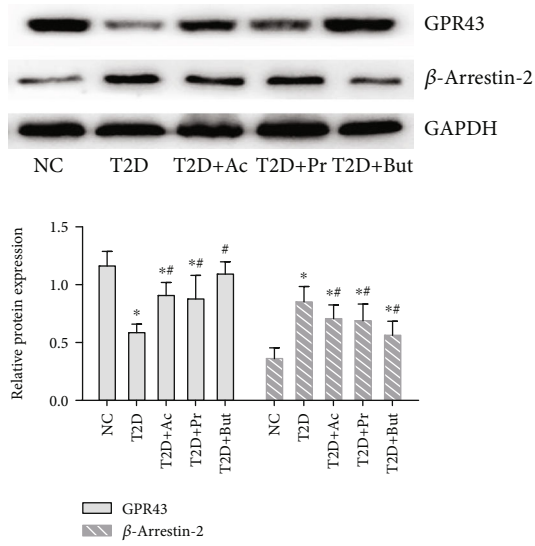
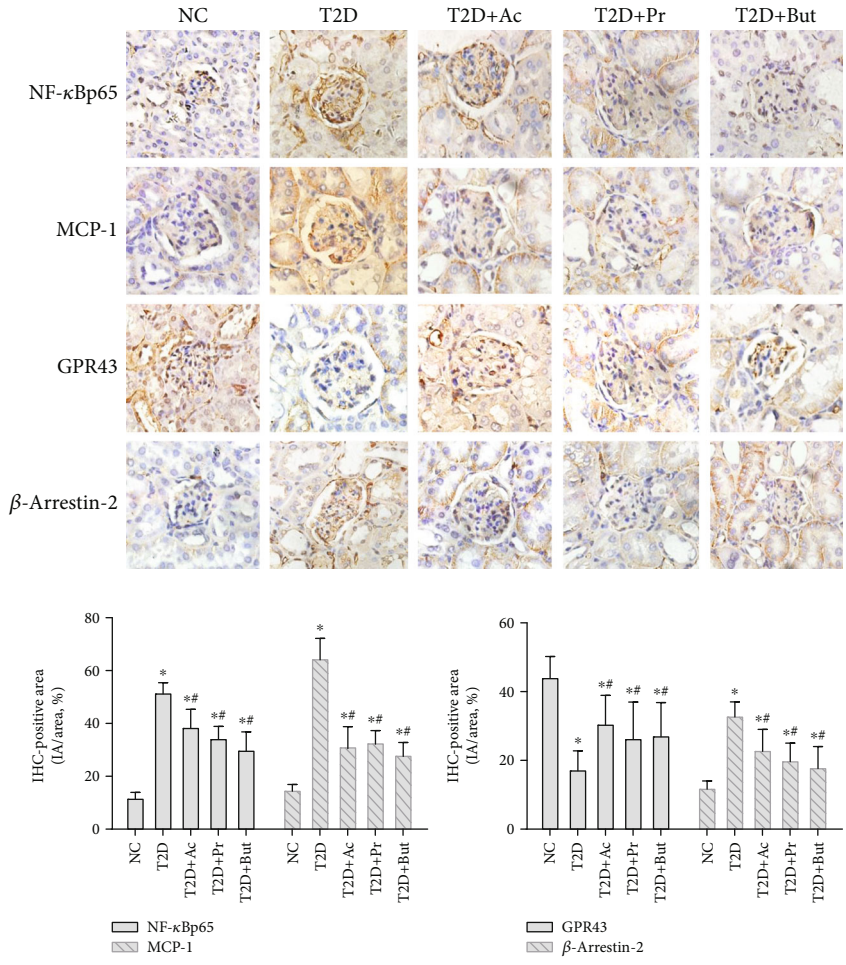
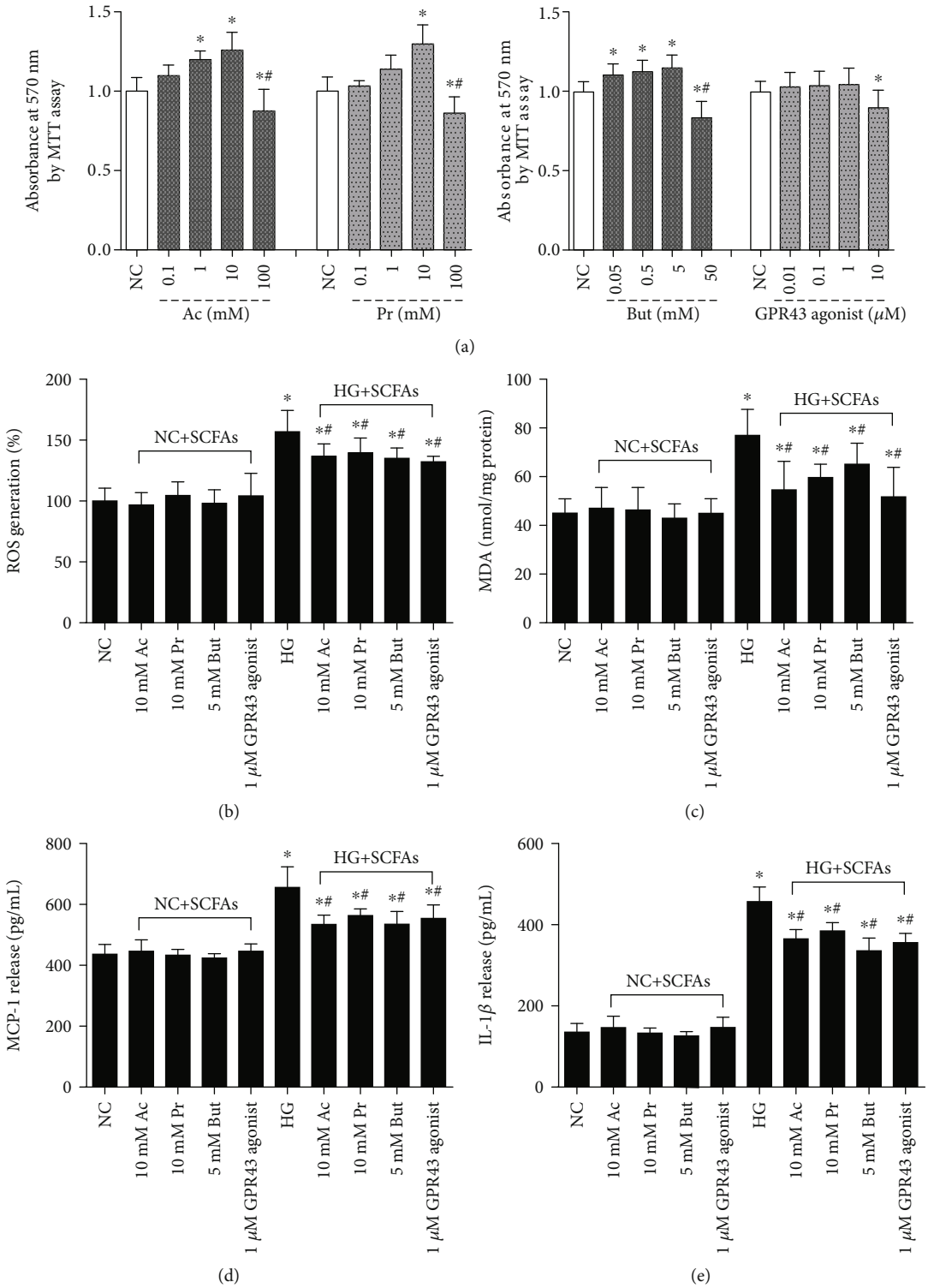


(d)



(e)

Figure 3: SCFAs inhibited T2D-induced NF-κB activation and regulate GPR43-β-arrestin-2 signaling. (a) Western blotting revealed the expression of I-κBα, p-NF-κBp65, MCP-1, and IL-1β in T2D kidney tissue after SCFA treatment. (b, c) qRT-PCR of GPR43, GPR41, β-arrestin-2, and β-arrestin-1 in kidney tissue after SCFA treatment. (d) Western blotting-based assays for the expression of GPR43 and β-arrestin-2 in kidney tissue after SCFA treatment. (e) Immunohistochemistry- (400x) based assays for the expression of p-NF-κBp65, MCP-1, GPR43, and β-arrestin-2 after SCFA treatment. Ac: acetate; Pr: propionate; But: butyrate; $^{*}p < 0.05$ compared with the NC group; $^{\#}p < 0.05$ compared with the T2D group; $^{\&}p < 0.05$ compared with the Ac or Pr group.



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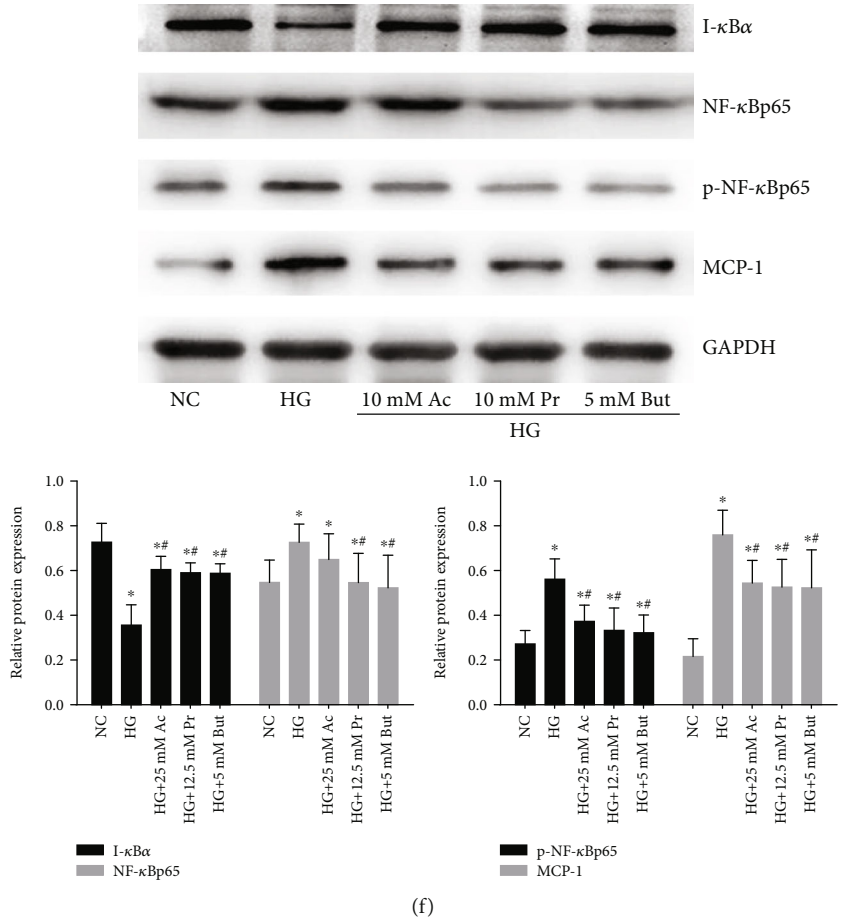
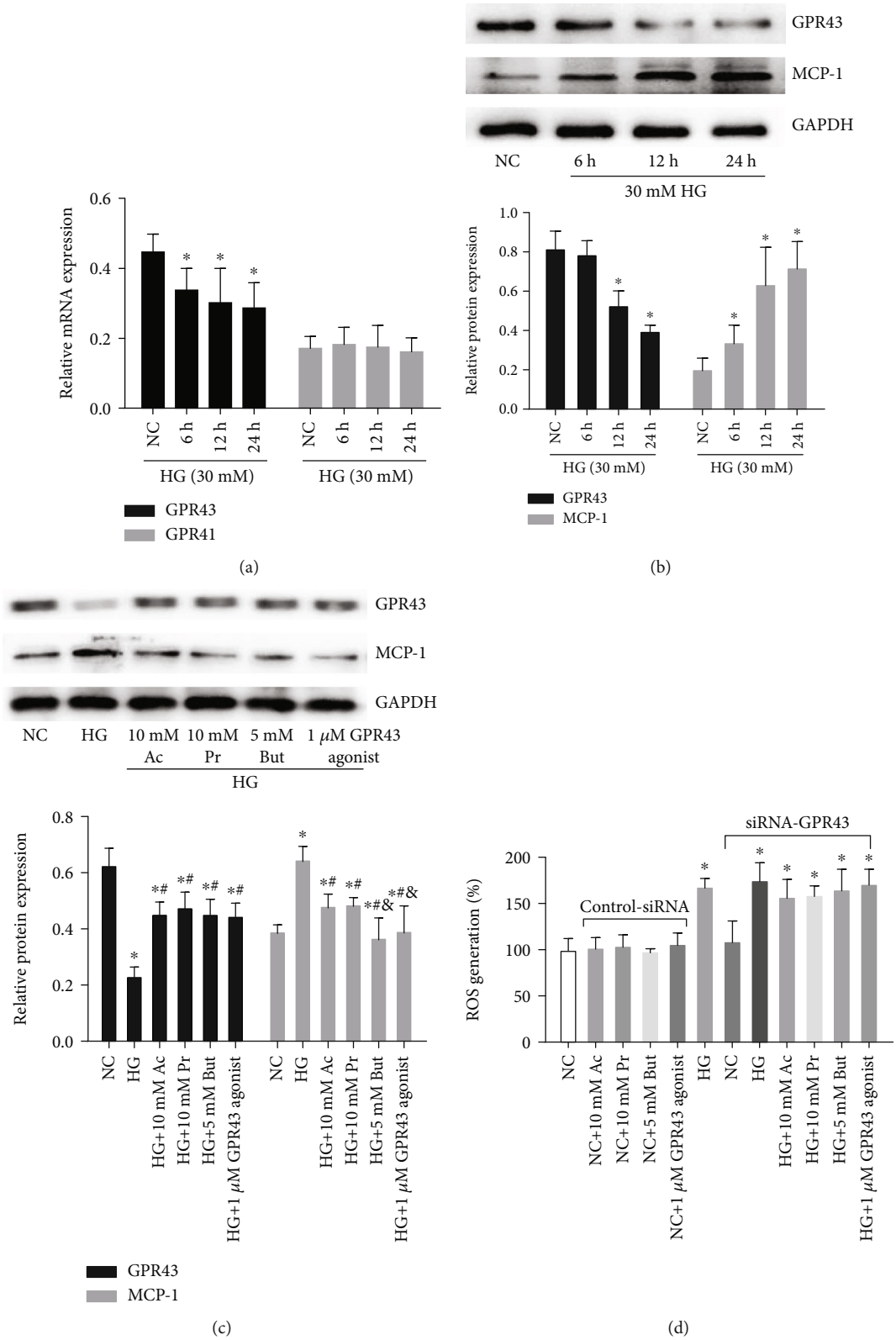


Figure 4: SCFA treatment partially inhibited oxidative stress and NF-κB activation in high glucose-induced GMCs. (a) The effects of a concentration range of SCFAs or GPR43 agonist on GMC proliferation were analyzed by MTT assay. GMCs were stimulated with 30 mM high glucose in the presence of the indicated concentration of SCFAs or GPR43 agonist for 24 h. ROS (b), MDA (c), MCP-1 (d), and IL-1β (e) in the cell culture supernatant were evaluated by kit. The protein expression of I-κBα, NF-κBp65, p-NF-κBp65, and MCP-1 was assayed by western blotting (f). Ac: acetate group; Pr: propionate group; But: butyrate group; $^{*}p < 0.05$ compared with the NC group; $^{\#}p < 0.05$ compared with the HG group.

peptide 1 and peptide tyrosine tyrosine) and to affect intestinal permeability, satiety, gastric emptying, and food intake [20, 21]. Recent studies have revealed that SCFAs regulate the inflammatory response and metabolic homeostasis [22–24]. However, a study of external SCFA administration on glucose and lipid metabolism in HFD and STZ-induced T2D mice is lacking. In the present study, we found that the intraperitoneal injection of three main SCFAs had no significant effect on BW, FINS, and the blood lipid spectrum of T2D mice; however, SCFA treatment, especially butyrate, resulted in decreased RBG, FBG, and HOMA-IR, suggesting a positive effect of SCFAs on insulin sensitivity and glucose homeostasis by a nongastrointestinal interventional manner. We speculate that SCFAs protect T2D mice by improving hepatic and peripheral insulin resistance as reported in the literatures above.

Inadequate levels of SCFAs are associated with kidney and cardiovascular disease [25–27]. Importantly, the latest studies found that an intraperitoneal injection of sodium butyrate or its oral administration can improve renal dysfunction and inhibit renal oxidative stress, inflammation, and fibrosis in the STZ-induced T1D mouse [28, 29]. SCFAs exert their anti-inflammatory effects partly via preventing the proteasomal degradation of the NF-κB inhibitor, I-κBα, both in intestinal and in extraintestinal environments [16, 30]. Here, we found that elevated ACR, serum urea, creatinine, and cystatin C, markers of the severity of renal dysfunction in DN, were decreased by SCFAs. In addition, mesangial expansion, the glomerular tuft, the accumulation of collagen, and the activation of NF-κB in diabetic renal tissues were significantly attenuated by SCFAs, especially butyrate. In *in vitro* studies, the inhibitory effects of SCFAs on oxidative stress and inflammation have been reported in porcine kidney cells and human renal cortical and tubular epithelial cells [31, 32]. GMCs are vulnerable to external stimulation, such as high glucose environment, which induces proliferation, hypertrophy, and extracellular matrix accumulation, as well as consequent renal fibrosis. These pathophysiological changes have been recognized as major events in the progression of DN; therefore, in this study, GMCs were used as a



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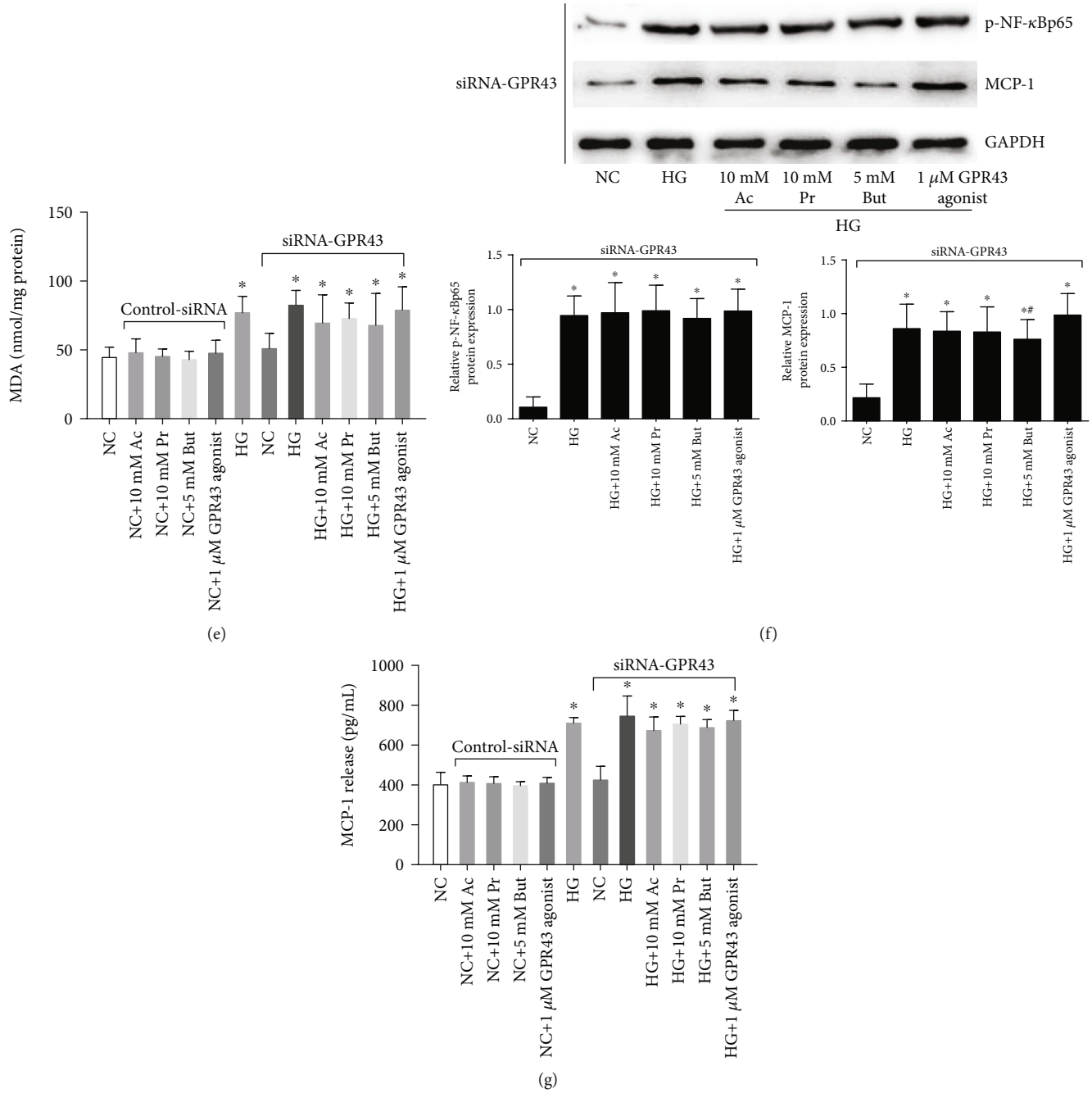
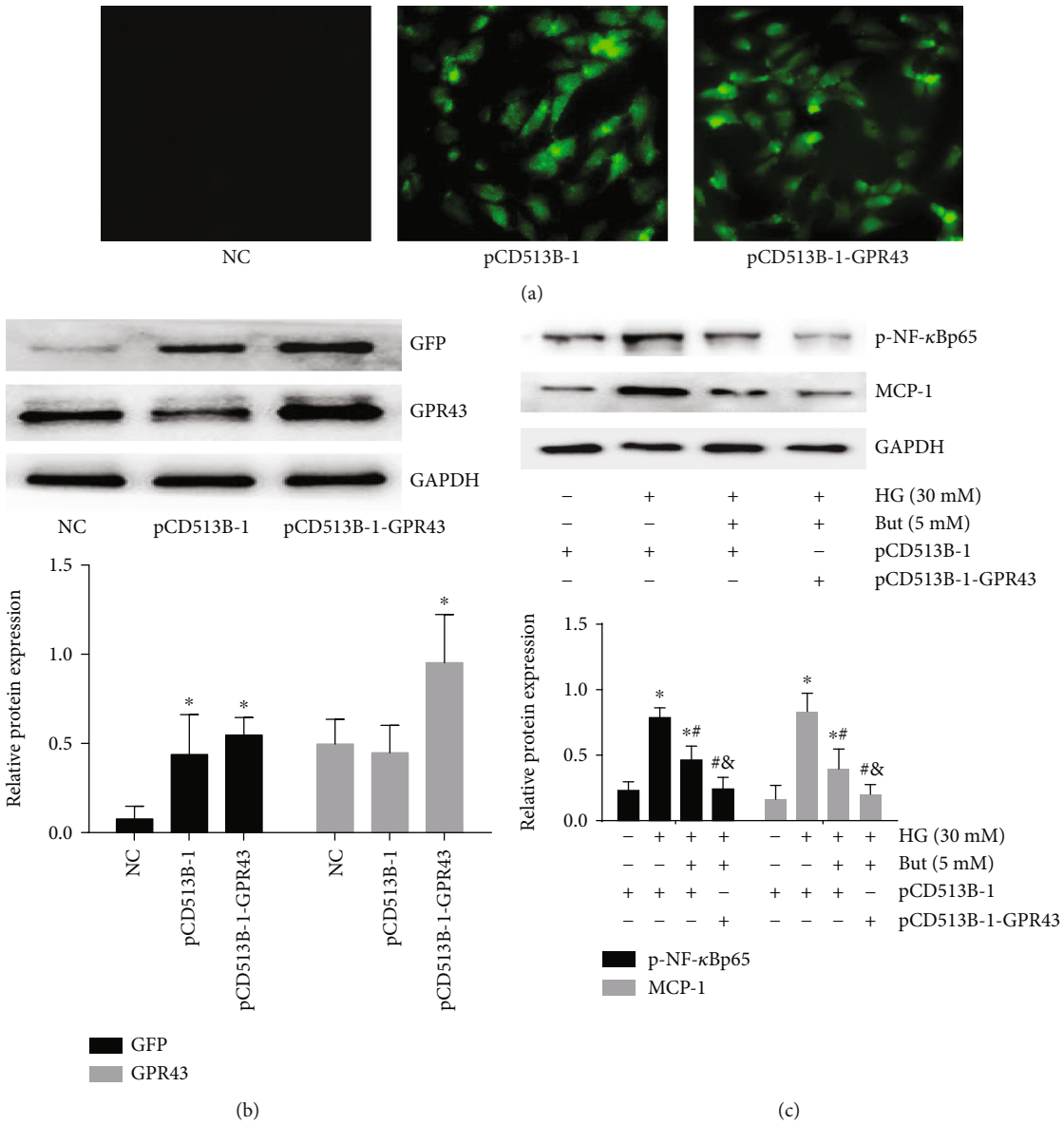


Figure 5: SCFA-mediated antioxidant and anti-inflammatory effects were partly reversed by siRNA-GPR43. qRT-PCR was performed to detect GPR43 and GPR41 mRNA levels. (b) Western blotting-based assay for the expression of GPR43 and MCP-1 after a 30 mM high glucose challenge for 6, 12, and 24 h. (c) The effects of indicated concentrations of SCFAs or a GPR43 agonist on GPR43 and MCP-1 expression were analyzed by western blotting. Following 30 mM high glucose for 24 h, SCFAs or a GPR43 agonist-ameliorated ROS (d), MDA (e), NF-κB signal (g), and MCP-1 (f) were significantly reversed by siRNA-GPR43. Ac: acetate; Pr: propionate; But: butyrate; $^{*}p < 0.05$ compared with the NC group; $^{\#}p < 0.05$ compared with the HG group. $^{\&}p < 0.05$ compared with the Ac or Pr group.

representative cell type [33, 34]. Although limited by cell type, our study found that the protective effects of SCFAs were associated with the inhibition of ROS and MDA and suppressed the degradation of I-κBα and the phosphorylation of NF-κBp65 in high glucose-induced GMCs. Taking into account all of these recent studies and the present results, SCFAs, especially butyrate, have shown positive effects on diabetic kidney injury, inhibition of oxidative stress, and NF-κB signaling and may be potential therapeutic agents in the prevention and treatment of DN.

However, unlike the positive effects of SCFAs on AKI models that have been observed, the influence of SCFAs on chronic kidney disease (CKD) seems to be more controversial: either protective or causative effects exist. Park et al. [35] showed that oral administration of SCFAs induced chronically increasing doses of SCFAs to higher than



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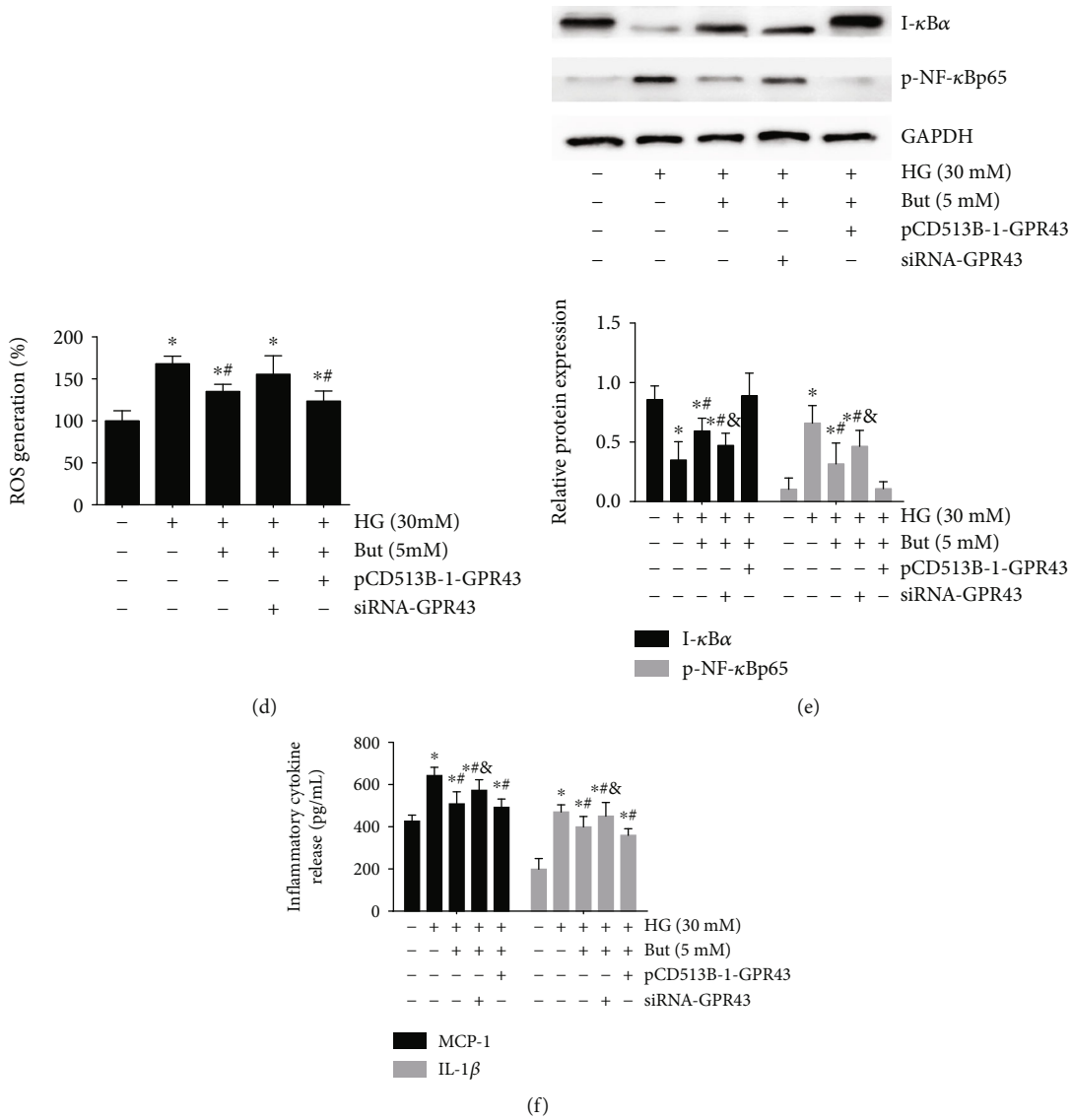
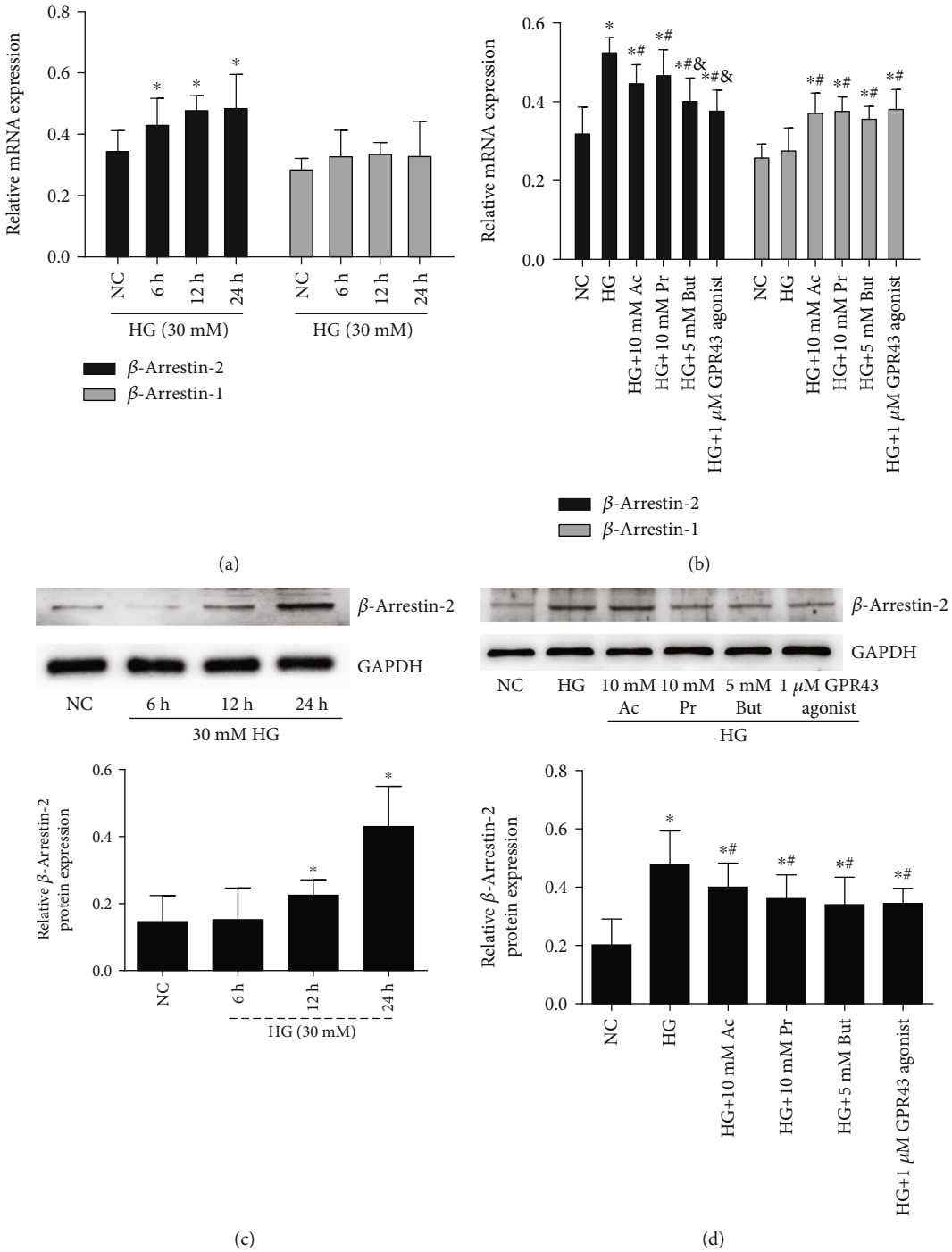


Figure 6: SCFA-mediated antioxidant and anti-inflammatory effects were significantly facilitated by GPR43 overexpression. Plasmids expressing GPR43 with pCD513B-1 (an N-terminal GFP tag) and pCD513B-1 control plasmid that expresses GFP but cannot overexpress GPR43 were constructed to determine the effect of overexpressed GPR43 and GFP protein in GMCs according to fluorescence images (200x) (a) and western blotting (b). Following 30 mM high glucose for 24 h, 5 mM But-mediated inhibition of p-NF-κBp65 and MCP-1 protein expression (c) was significantly facilitated by GPR43 overexpression. The But-mediated inhibition of ROS (d) and p-NF-κBp65 (e) and MCP-1 and IL-1β release (f) were reversed by siRNA-GPR43 but facilitated by GPR43 overexpression. But butyrate: $^{*}p < 0.05$ compared with the NC group, $^{\#}p < 0.05$ compared with the HG group, and $^{\&}p < 0.05$ compared with the But group.

physiological levels in mice and led to kidney hydronephrosis. Therefore, SCFAs were confirmed to play a dual role in the inflammatory system depending on the stimulus concentration [36]. Furthermore, although SCFAs are weak acids, too high concentrations or too frequent injections may stimulate the peritoneum to induce aseptic inflammation and ascites formation. Based on literature reports on *in vivo* experiments [13–15], our study indicated that a 100 mg/kg/48 h dose of SCFAs had positive and tolerable effects in T2D model mice. Previous studies *in vitro* have revealed that 25 mM acetate, 12 mM propionate, and 3.2 mM butyrate improved hypoxia-induced MitoSOX in renal tubular epithelial cells (HK-2 cells) [13]. In addition, 0.5-10 mM butyrate uppressed high glucose-stimulated TGF-β1 synthesis in HK-2 cells in a dose-dependent manner [37]. In line with the literature above, we showed that 10 mM acetate, 10 mM propionate, or 5 mM butyrate has protective effects on high glucose-induced oxidative stress and inflammation. It would seem contradictory that the SCFA pharmacological concentrations used herein or in the literature should be at least at the mM level to inhibit the expression of cytokines; however, the concentration of SCFAs in the peripheral circulation is very low (19-160 μM), especially that of propionate and butyrate [38]. Therefore, low



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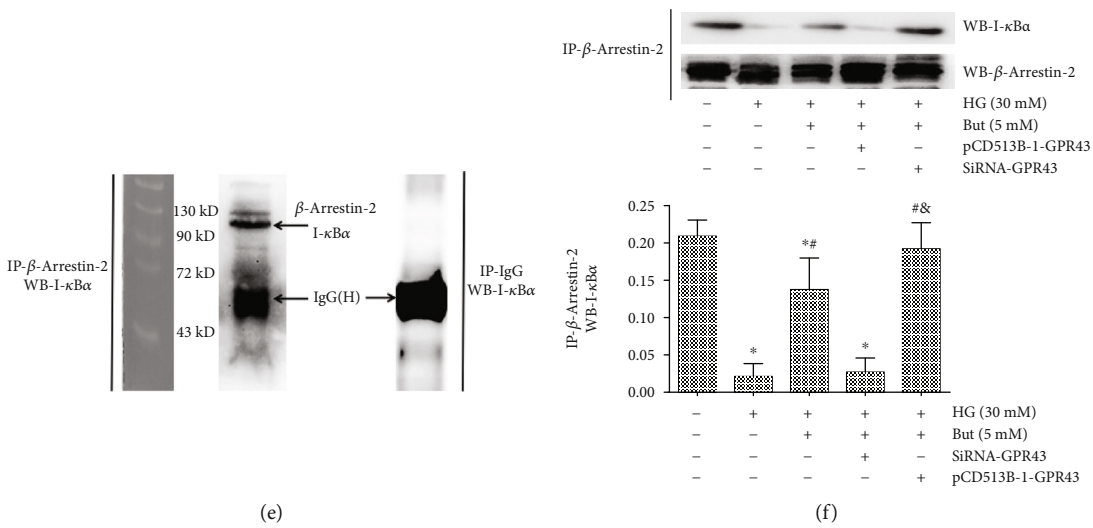

Figure 7: Interaction between β-arrestin-2 and I-κBα was induced by SCFAs via GPR43. (a) GMCs were treated with 30 mM high glucose for 6, 12, and 24 h. RT-PCR was performed to detect β-arrestin-2 and β-arrestin-1 mRNA levels. (b) The effects of indicated concentrations of SCFAs or an GPR43 agonist on β-arrestin-2 and β-arrestin-1 expression were analyzed by RT-PCR. (c) Western blot assay for the expression of β-arrestin-2 after 30 mM high glucose challenge for 6, 12, and 24 h. (d) High glucose-induced β-arrestin-2 expression was significantly reversed by SCFAs or GPR43 agonist. (e) The interaction between β-arrestin-2 and I-κBα under physiological conditions was detected by immunoprecipitation (IP) with anti-β-arrestin-2 antibody or normal mouse IgG antibody (negative control), followed by western blotting with an anti-I-κBα antibody. β-Arrestin-2 was conjugated with I-κBα in *vitro*. (f) The interaction between β-arrestin-2 and I-κBα was decreased by 30 mM high glucose but was reversed by 5 mM butyrate. And these butyrate-mediated effects were significantly reversed by siRNA-GPR43 but were facilitated by overexpressed GPR43. IgG-H marks the IgG heavy chain. But butyrate: $^{*}p < 0.05$ compared with the NC group, $^{\#}p < 0.05$ compared with the HG group, and $^{\&}p < 0.05$ compared with the HG+But group. Ac: acetate; Pr: propionate.

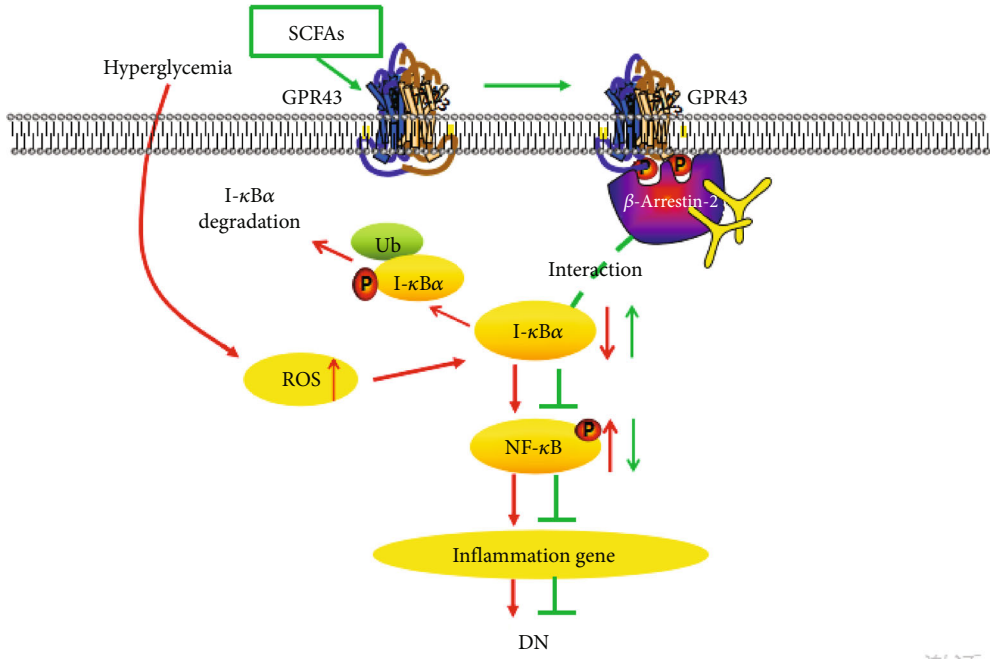

Figure 8: Overview on the effects of SCFAs on oxidative stress and NF-κB activation in DN. High glucose induces the production of ROS and the polyubiquitination of phosphorylated I-κBα, followed by NF-κB activation and the expression of various inflammatory cytokines that are important factors in the development of DN (red arrows). However, SCFAs inhibit the oxidative stress and NF-κB inflammatory signaling possibly via activating GPR43 and increasing the interaction between β-arrestin-2 and I-κBα (green arrows), suggesting that SCFA-mediated GPR43-β-arrestin-2 signaling may be a novel and promising target for DN.

concentration of SCFAs would not be able to induce antioxidant and anti-inflammatory effects; however, if the concentration of SCFAs is elevated by exogenous supplementation, some tissues and cells may not be able to tolerate it [39]. These studies and our results suggest the importance of determining appropriate concentrations when testing the benefits of SCFAs in kidney disease and encourage further studies to identify the most appropriate interventional manner and related pharmacological concentration.

Finally, we intended to explore the molecular mechanism (s) by which SCFAs mediate antioxidant and anti-inflammatory effects. Compared with the GPR41 receptor, GPR43 is mostly involved in the regulation of immune function and inflammation [40–42]. Our previous study revealed that SCFAs or a GPR43 agonist markedly upregulated the expression of GPR43 inhibited by high glucose but diminished the expression of MCP-1 and IL-1β [43]. In the present study, we showed that compared with GPR43, GPR41 was rarely expressed in kidney tissues and in GMCs and did not show an obvious response to diabetes-relative stimulation; SCFAs reversed the downregulation of GPR43, along with inhibition of oxidative stress and NF-κB signaling, indicating that GPR43 was critically involved in SCFA-mediated beneficial effects. Recently, several studies found that β-arrestin-2, which regulates desensitization, internalization, intracellular signaling, and recycling of GPCRs, directly binds to and blocks phosphorylation and degradation of I-κBα and finally leads to the inhibition of NF-κB activity [44, 45]. The most important finding presented here is that high glucose induced the expression of β-arrestin-2, but not β-arrestin-1, in a time-dependent manner, suggesting that β-arrestin-2 may represent a new target for an anti-inflammatory therapy, in response to SCFAs. Our study also revealed that I-κBα coimmunoprecipitated with β-arrestin-2 under physiological conditions, but the interaction was attenuated by high glucose; furthermore, upon butyrate treatment, an increased amount of I-κBα was associated with β-arrestin-2, illustrating that butyrate blocks phosphorylation and degradation of I-κBα by inducing the interaction between β-arrestin-2 and I-κBα, finally leading to the inhibition of NF-κB signaling. Last but not least, the butyrate-induced positive effects described above were significantly inhibited by siRNA-GPR43 or facilitated by overexpressed GPR43. These results collectively suggest that the interaction between β-arrestin-2 and I-κBα is induced by SCFAs via GPR43; GPR43-β-arrestin-2 signaling may be a new and promising target for DN.

It must be pointed out that some of the beneficial effects of butyrate administration were statistically significant in *in vivo* and *in vitro* experiments; however, SCFA treatment did not totally reverse T2D-induced renal dysfunction, and high glucose-induced oxidative stress and NF-κB activation were not totally inhibited by SCFAs or a GPR43 agonist, suggesting the presence of other mechanisms, such as the inhibition of HDAC, which are involved in the crosstalk of SCFAs and the kidney in the prevention and treatment of DN [8, 28, 29]. Therefore, highly selective agonists and antagonists for receptors that sense SCFAs as well as tissue-specific GPR43 and/or β-arrestin-2 knockout or overexpressing mice are needed in future studies to elucidate the molecular mechanisms involved in SCFA-mediated benefits [46]. Finally, compared with other SCFAs, butyrate has shown a better therapeutic effect, but in relatively small absolute numbers; how to select the best efficient SCFA subtype, control the concentration, and avoid potential side effects are important challenges for future research.

5. Conclusion

As summarized in Figure 8, our study provides compelling evidence that exogenous SCFAs, especially butyrate, ameliorate hyperglycemia and insulin resistance, improve renal function, ameliorate histopathological changes, and restore high glucose-induced inflammatory damage, which is attributable to the role GPR43-β-arrestin-2 signaling plays in buffering oxidative stress and blocking NF-κB activation. These results from the current study establish a novel role of SCFAs in improvement of glucose metabolism and renal protection through GPR43-β-arrestin-2 machinery and may have implications for DN therapy.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that they have no conflict of interest.

Authors' Contributions

WH conceived and designed the study, performed the experiments, analyzed the data, and wrote the manuscript. YX and YHX supervised the study and edited the manuscript. CLG, HWX, and YL operated the animal experiments. LPZ and JLG operated the molecular experiments. QW and LC helped analyze the data and conduct animal experiments. All authors reviewed and approved the manuscript for submission. Wei Huang, Yi Man and Chenlin Gao contributed equally to this work.

Acknowledgments

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Research Article

Ameliorative Effects of Bredemolic Acid on Markers Associated with Renal Dysfunction in a Diet-Induced Prediabetic Rat Model

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Recently, studies have shown that renal dysfunction is associated not only with overt diabetes but also with the preceding stage known as prediabetes. Diet and pharmacological interventions are the therapeutic approaches to managing prediabetes, but the compliance in combining the two interventions is low. Hence, the efficacy of pharmacological intervention is reduced without diet modification. In our previous study, we established that bredemolic acid (BA) ameliorated glucose homeostasis via increased GLUT 4 expression in the skeletal muscle of prediabetic rats in the absence of diet intervention. However, the effects of bredemolic acid on renal function in prediabetic condition are unknown. Therefore, this study was aimed at investigating the ameliorative effects of bredemolic acid on renal dysfunction in a diet-induced prediabetic rat model. Thirty-six Sprague-Dawley male rats (150–180 g) were divided into two groups: the nonprediabetic ($n = 6$) and prediabetic ($n = 30$) groups which were fed normal diet (ND) and high-fat high-carbohydrate (HFHC) diet, respectively, for 20 weeks. After the 20th week, the prediabetic groups were subdivided into prediabetic control (PD) and 4 other prediabetic groups which were treated with either BA (80 mg/kg) or metformin (MET, 500 mg/kg) for further 12 weeks (21st to 32nd). Plasma, urine, and kidney samples were collected for biochemical analysis. The untreated prediabetic (PD) rats presented increased fluid intake and urine output; increased creatinine, urea, and uric acid plasma concentrations; albuminuria; proteinuria; sodium retention; potassium loss; increased aldosterone and kidney injury molecule (KIM-1) concentration; and increased urinary podocin mRNA expression. However, BA administration attenuated the renal markers and oxidative stress and decreased the urinary podocin mRNA expression. In conclusion, BA administration, regardless of diet modification, attenuates renal dysfunction in an experimentally induced prediabetic state.

1. Introduction

More than 25% of type 1 and type 2 diabetes mellitus patients have been reported to develop renal dysfunction [1, 2]. However, the renal dysfunction does occur not only in overt diabetes but also in the early stages of impaired glucose metabolism [3, 4]. Renal dysfunction is defined by the appearance of abnormal kidney functional changes such as a reduced glomerular filtration rate (GFR), increased serum creatinine and urea, albuminuria, increased excretion of kidney injury molecule (KIM-1), and glomerular podocyte injury with urinary loss of podocin. Podocin is an exclusive integral membrane protein in the podocytes that directly interact with nephrin and CD2-associated protein [5]. Hence, urinary loss of podocin is an apparent indication of podocyte injury and renal dysfunction [6–8].

Moreover, literatures have shown that impaired glucose metabolism promotes renal dysfunction via activation of

oxidative stress and renin-angiotensin-aldosterone system (RAAS) [9–11]. The activation of RAAS triggers the release of aldosterone which stimulates serum/glucocorticoid-regulated kinase 1 (SGK1) that regulate epithelial sodium channel (ENaC) and consequently lead to sodium retention and potassium loss in diabetic conditions [12–14]. Of note, literature evidence showed that about one-third of individuals with newly diagnosed diabetes mellitus have varying degrees of renal dysfunction [15]. This can only be attributed to the abnormal changes that occur during prediabetes. The prediabetic stage often precedes the onset of type 2 diabetes mellitus and is said to be caused by chronic consumption of high-caloric diets coupled with a sedentary lifestyle [15, 16]. Cross-sectional clinical studies have confirmed that prediabetes is associated with the onset of chronic kidney disease (CKD) [4, 17]. Therefore, screening of markers of renal function during the prediabetic state offers an early window of opportunity of preventing and managing CKD [15]. More importantly, diet modification and pharmacological intervention have been reported as the therapeutic approaches to managing prediabetes [18–20]. However, the compliance of combining the two interventions is low as patients adhere to pharmacological intervention without diet modification, and consequently, the efficacy of the pharmacological intervention is reduced [21, 22]. Hence, antidiabetic agents that can possibly ameliorate CKD regardless of diet intervention are considered necessary.

Studies in our laboratory have demonstrated that pentacyclic triterpenes, such as oleanolic acid, ursolic acid, and maslinic acid, are antidiabetic agents which attenuate renal dysfunction in streptozotocin-induced diabetes mellitus [23, 24]. Similarly, we have previously demonstrated that a maslinic acid isomer, bredemolic acid, is an antihyperglycaemic agent that regulated blood glucose concentration via increased expression of GLUT 4 in the gastrocnemius muscle of the prediabetic rat model without diet intervention [25]. However, the biological effects of bredemolic acid on renal dysfunction in the prediabetic state are unknown. Therefore, this study sought to investigate the effects of bredemolic acid on selected markers of renal function in a diet-induced prediabetic rat model, and we also treated the prediabetic rats with metformin, a common first-line drug in the therapy of type 2 diabetes and obesity [26].

2. Materials and Methods

2.1. Animals. Thirty-six (36) male Sprague-Dawley rats with body weight 150–180 g were used for this study as described in previous research [25]. The rats were obtained from the Biomedical Research Unit (BRU), University of KwaZulu-Natal (UKZN). The animals were kept and maintained in a standard animal facility under controlled environmental conditions at room temperature ($22 \pm 2^{\circ}$C), humidity ($55 \pm 5\%$), and 12 h day : 12 h night cycle. The animals consumed standard rat chow (Meadow Feeds, South Africa) and water *ad libitum* for 2 weeks to acclimatize before being exposed to the experimental diet (high-fat high-carbohydrate). The components of the high-fat high-carbohydrate (HFHC) diet are carbohydrate (55%%kcal/g), fats (30% kcal/g), and proteins (15% kcal/g) as described in the previous research [22]. All experimental procedures in this study were carried out in absolute compliance with the animal care guidelines and approved with ethical number (AREC/024/018D) by the Animal Research Ethics Committee (AREC) of the UKZN, Durban, South Africa.

2.2. Experimental Design. After the acclimatization, the animals were divided into 2 main groups: the nonprediabetic control group ($n = 6$) and the prediabetic group ($n = 30$). The nonprediabetic (NPD) control animals (negative control) were given standard rat chow (ND) and water *ad libitum* for 20 weeks while the prediabetic animals were given HFHC diet and drinking water supplemented with fructose (15%) for 20 weeks to induce prediabetes. At the 20th week, prediabetes was confirmed via assessment of fasting blood glucose and oral glucose tolerance test (OGTT) as described by the American Diabetes Association and our previous study [25, 27]. Of notes, this study is a continuation of the previous study, and the data on body weight, food intake, fasting blood glucose, oral glucose tolerance test, fasting insulin concentration, and insulin resistance in the previous study are relevant for this present study.

2.3. Treatment of Animals. The treatment period lasted for 12 weeks (21st–32nd). The nonprediabetic control group (Group 1) fed on standard rat chow (ND) without treatment for 12 weeks while the prediabetic animals ($n = 30$) were further divided into the 5 groups (Group 2–Group 6, $n = 6$) and fed on HFHC or ND for 12 weeks as well. Group 2 served as the prediabetes control group (PD) and continuously fed on the HFHC diet without treatment for 12 weeks. The other 4 groups of the prediabetic animals continuously fed on HFHC diet or switched to ND and were treated with either oral administration of BA (80 mg/kg) or metformin (MET, 500 mg/kg) every third day for 12 weeks due to the three-day pharmacokinetic activity of pentacyclic triterpenes as previously described [28, 29]. The switch of diet from HFHC to ND is the dietary intervention while the continuous feeding on HFHC diet is the absence of dietary intervention. The ND+MET (Group 3) rats changed diet from HFHC to ND and received MET orally whereas the HFHC+MET (Group 4) rats were continuously fed on the HFHC diet and received MET orally. The ND+BA (Group 5) rats changed diet from HFHC to ND and received BA orally while HFHC +BA (Group 6) rats continuously fed on the HFHC diet and were treated with BA. After the 12 weeks of treatment, the animals were sacrificed; blood samples and the kidneys were collected from all the animals for biochemical analysis. The fluid intake and urine volumes were assessed in all the animals at the 20th week and every 4 weeks (24th, 28th, and 32nd week). The renal function parameters and other biochemical parameters were measured at the end of the experiment.

2.4. Determination of Fluid Intake and Urine Output. At the 20th week and every 4 weeks thereafter, all the animals in each group were placed in different metabolic cages for 24

hours to measure fluid intake and urine output. The urine samples were measured and centrifuged at 13000 rpm for 5 minutes at 4°C, and the supernatants were stored at -80°C in a Bio Ultra freezer (Snijders Scientific, Tilburg, Holland) until ready for kidney function parameter analysis.

2.5. Blood Collection and Tissue Harvesting. All the animals were placed in a gas anaesthetic chamber (Biomedical Research Unit, UKZN, Durban, South Africa) and anaesthetised with 100 mg/kg of Isofor (Safeline Pharmaceuticals (Pty) Ltd., Roodeport, South Africa). In an unconscious state, blood samples were collected from all the animals via a cardiac puncture into different precooled EDTA containers. The blood samples were centrifuged (Eppendorf centrifuge 5403, Germany) 503 g for 15 minutes at 4°C to obtain plasma. Thereafter, the plasma samples were aspirated into plain sample bottles and stored in a Bio Ultra freezer (Snijders Scientific, Tilburg, Holland) at -80°C until ready for biochemical analysis. Also, the kidneys were removed, rinsed with cold normal saline solution, weighed on the weighing balance, snapped frozen in liquid nitrogen, and stored at -80°C in a Bio Ultra freezer for biochemical analysis of selected parameters.

2.6. Biochemical Analysis. The biochemical analysis of kidney function parameters (such as creatinine, urea, uric acid, albumin, and total protein) and electrolytes (Na^+ and K^+) was determined at the 32nd week in the plasma and urine samples by using their respective assay kits (Elabscience Biotechnology Co., Ltd., Houston, TX, USA) as instructed by the manufacturer. However, the kidney injury molecule (KIM-1) and aldosterone plasma concentrations were determined from their specific ELISA kits as instructed by the manufacturer (Elabscience Biotechnology Co., Ltd., Houston, TX, USA) via the microplate reader, SPECTROstar Nano spectrophotometer (BMG LABTECH, Ortenburg, LGBW, Germany).

2.7. Determination of GFR. The GFR of all the animals were determined at the 32nd week of the experiment from the estimation of creatinine in the plasma and urine (creatinine clearance) as follows:

$$\mathrm{GFR}[\mathrm{mL/min}] = \frac{\text{Urine creatinine (mg/dL)} \times \text{24hrs urine volume (mL)}}{\text{Plasma creatinine (mg/dL)} \times 60\ \text{min} \times \text{24hrs}}. \quad (1)$$

2.8. Lipid Peroxidation and Antioxidant Status. The lipid peroxidation was assessed by determination of the concentration of malondialdehyde (MDA) in the kidney homogenized tissue according to the previously established protocol [24]. However, the antioxidant status of the kidney homogenate was assessed by determination of the concentration of superoxide dismutase (SOD), glutathione peroxidase (GPx), and total antioxidant capacity (TOAC) by using their specific ELISA kits according to the instruction of the manufacturer (Elabscience Biotechnology Co., Ltd., Houston, TX, USA).

2.9. Urine RNA Isolation. RNA was isolated from urine (4 mL) by using a ZR Urine RNA Isolation Kit™ (Zymo Research Corp., Irvine, USA) according to the manufacturer's protocol. The purity of the RNA was confirmed by the relative absorbance of ratio 260/280 nm via a Nanodrop 1000 spectrophotometer (Thermo Scientific, USA). Urine RNA (100 ng) was reverse transcribed to complementary DNA (cDNA) by using the iScript™ cDNA Synthesis Kit (Bio-Rad, California, USA) through incubation in a thermal cycler (SimpliAmp Thermal Cycler, Applied Biosystems, Life Technologies).

2.10. Urine Complementary DNA (cDNA) Synthesis. For cDNA synthesis, urine RNA (2 μL) was mixed with 5x iScript reaction (4 μL), iScript reverse transcriptase enzyme (1 μL) (Bio-Rad, USA), and nuclease-free water to a final volume of 20 μL. The mixture was incubated in the thermal cycler (SimpliAmp Thermal Cycler, Applied Biosystems, Life Technologies) at 25°C for 5 minutes, 42°C for 30 minutes, and finally at 85°C for 5 minutes. Thereafter, the synthesized cDNA was stored at -80°C until use for real-time PCR (polymerase chain reaction).

2.11. Real-Time PCR. The urinary mRNA level of podocin was quantified by real-time PCR LightCycler (Roche LightCycler 96, USA). cDNA template (2 μL), SYBR Green PCR master mix (5 μL) (Bio-Rad, USA), podocin forward primer (1 μL), podocin reverse primer (1 μL), and nuclease-free water were mixed to a final volume of 10 μL. Thereafter, the sample mixtures were cycled 40 times at 95°C for 10 seconds, 60°C for 20 seconds, and 72°C for 20 seconds in the LightCycler (Roche LightCycler 96, USA). All the samples were run in duplicate, and β-actin mRNA levels were used as a housekeeping gene to normalize the podocin mRNA level. The sequences of the used oligonucleotide primers (Metabion International AG, Planegg, Germany) were as follows: *podocin forward 5′-TGG AAG CTG AGG CAC AAA GA-3′* and *podocin reverse 5′-AGA ATC TCA GCC GCC ATC CT-3′*.

2.12. Statistical Analysis. The data were normally distributed and presented as the mean ± SEM. Multiple intergroup differences were analysed by one-way ANOVA with the Bonferroni test as a post hoc test through GraphPad Prism 7 software. The results were considered statistically significant at $p < 0.05$.

3. Results and Discussion

3.1. Effects of BA Administration with or without Diet Intervention on Plasma and Urinary Albumin and Total Protein. High-fat or high-fructose diet has been associated with impaired glucose metabolism and insulin resistance which in turn leads to metabolic disturbances with complications that result in renal dysfunction such as decreased plasma concentration of albumin and total protein, albuminuria, proteinuria, and diffuse thickening of the glomerular capillary basement membrane [30–33]. In this study, a significant decrease in plasma concentrations of albumin (Figure 1(a)) and total protein (Figure 1(b)) was observed in the prediabetic control rats when compared to nonprediabetic control rats. In addition, albuminuria (Figure 1(c)) and proteinuria (Figure 1(d)) which are apparent indicators of

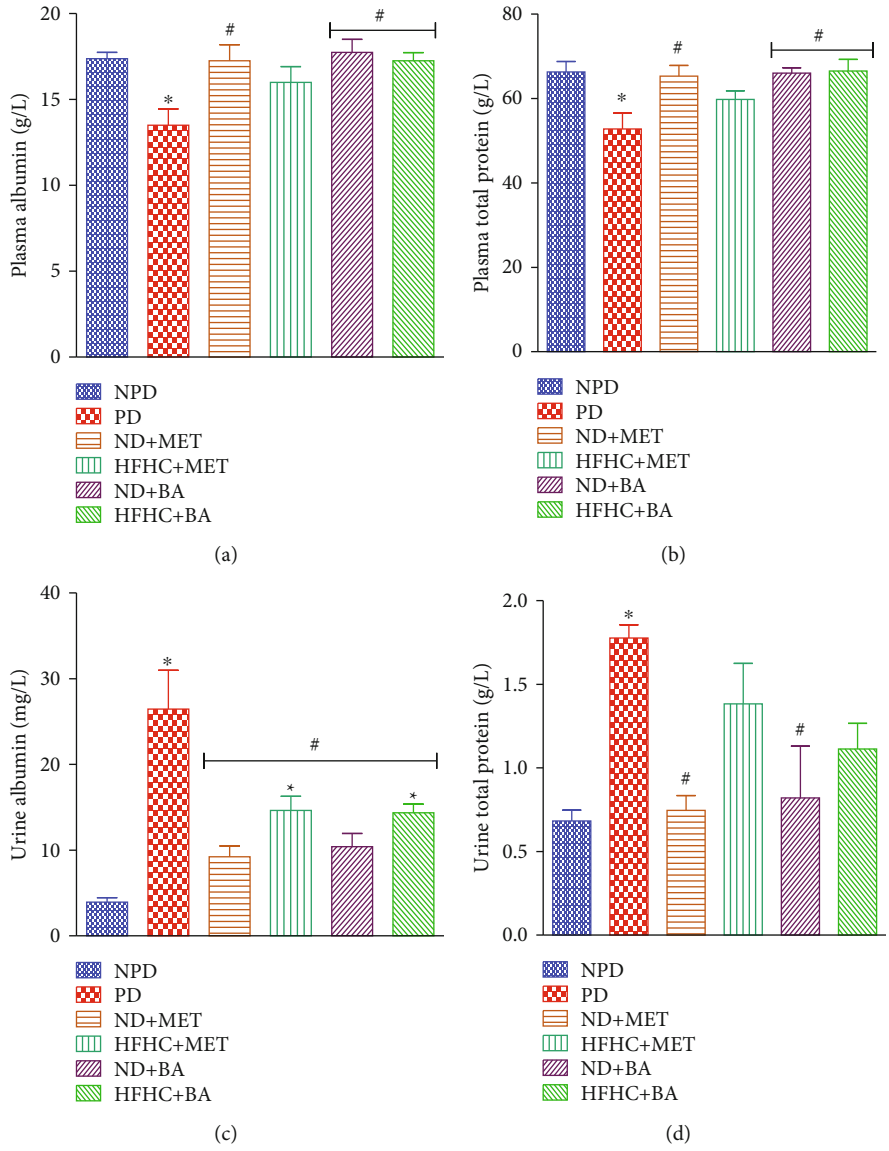

Figure 1: Effects of BA on plasma albumin (a), plasma total protein (b), urine albumin (c), and urine total protein (d) in prediabetic rats in the presence or absence of dietary intervention. $^{*}p < 0.001$ (vs. NPD), $^{\#}p < 0.05$ (vs. PD).

renal damage were observed in the prediabetic control rats compared to nonprediabetic rats. These observations may be attributed to the impaired filtration barrier which has been reported in prediabetic condition in other studies [30, 34]. Therefore, we suggest that the abnormal glucose homeostasis and insulin resistance that are associated with prediabetes due to chronic consumption of high-caloric diet might have resulted into the impaired filtration barrier with consequent loss of plasma albumin and protein, thus resulting into significant albuminuria and proteinuria [3, 35]. However, the administration of BA in the presence or absence of diet intervention as well as metformin administration with diet intervention attenuated albuminuria and proteinuria in the BA- and metformin-treated prediabetic rats, and this in turn contributed to the improved plasma concentrations of albumin (Figure 1(a)) and total protein (Figure 1(b)). We therefore suggest that BA attenuated these renal dysfunction markers by its antihyperglycaemic property and the improved insulin sensitivity which we have earlier reported in our study [25].

3.2. Effects of BA Administration with or without Diet Intervention on Plasma KIM-1. Apart from albuminuria or proteinuria, another indicator of renal damage is the KIM-1, which is an expressed biomarker on the apical membrane of proximal tubular cells [36]. The observed significant increase in the plasma concentration of KIM-1 in the prediabetic control rats compared to nonprediabetic control rats in this study (Figure 2) was also an indication of decline in renal function, and this observation on KIM-1 correlated with other studies in insulin-resistant states [37, 38]. However, the KIM-1 plasma concentration of BA-treated prediabetic rats with or without dietary intervention as well as metformin-treated prediabetic rats with diet intervention

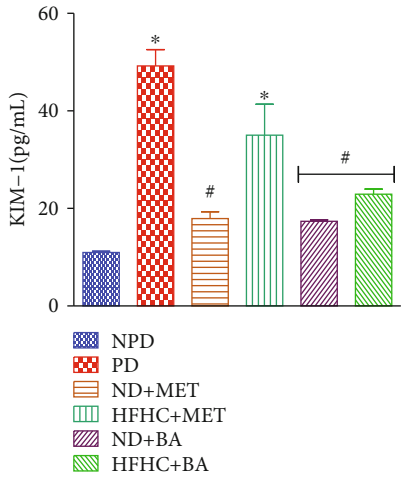

Figure 2: Effects of BA on plasma kidney injury molecule (KIM-1) concentrations in prediabetic rats in the presence or absence of dietary intervention.

was significantly decreased in comparison to the prediabetic control rats.

3.3. Effects of BA Administration with or without Diet Intervention on Plasma and Urinary Uric Acid and Urea. In this study, the plasma concentrations of urea and uric acid (Figure 3(a) and Figure 3(b), respectively) significantly increased in the prediabetic control rats when compared to the nonprediabetic control rats. The alterations in the plasma or urinary concentrations of urea may be suggested to be due to impaired excretory or regulatory function of the kidney in maintaining constant homeostasis in the prediabetic or diabetic state [39]. Moreover, decreased urinary concentration of urea (Figure 3(c)) in the prediabetic control rats in comparison to the nonprediabetic control rats was observed in this study. This observation was in accordance with the results of previous studies [24, 40]. Administration of BA in the absence or presence of dietary intervention as well as metformin in the presence of dietary intervention significantly decreased the plasma and increased the urinary concentrations of urea.

Of note, high fructose diet has been reported to result in ATP depletion due to utilization of two molecules of ATP for each fructose molecule metabolized [41, 42]. Therefore, the resultant ADP is further degraded to AMP. In the insulin-resistant state (prediabetes), xanthine dehydrogenase enzyme is activated and triggered the conversion of the AMP to uric acid, hence resulting into the observed hyperuricaemia and elevated uric acid excretion in this study [43, 44]. Therefore, we suggest that the significant increase in uric acid levels in the plasma may be due to the chronic consumption of fructose diet which triggered insulin resistance and further leads to the observed hyperuricaemia and significant urinary excretion of uric acid in prediabetic control rats (Figure 3(d)). However, we hypothesized that the administration of BA and metformin in the presence of dietary intervention significantly ameliorated the hyperuricaemia probably due to the improved insulin sensitivity in the BA- and metformin-treated prediabetic rats.

3.4. Effects of BA Administration with or without Diet Intervention on Lipid Peroxidation and Antioxidant Status in the Kidney. The observed increase in the lipid peroxidation (MDA) and decrease in the concentration of antioxidant enzymes (SOD, GPx, and TOAC) in the prediabetic control rats in comparison to the nonprediabetic control rats are apparent indicators of oxidative stress (Table 1). Increased glucose influx into the cells (due to consumption of high-caloric diet) which results into increased glucose catabolism through the Krebs cycle and production of electron donors (NADH and FADH2) at quantities that overwhelm the capacity of oxidative phosphorylation electron transport chain triggers oxidative stress under hyperglycaemic conditions [45]. This process occurs in microvascular endothelial cells such as the glomerular endothelial cells which are unable to decrease glucose influx during a hyperglycaemic state [46]. The glomerular endothelium plays a significant role in the pathogenesis of diabetic nephropathy directly and through its interaction with podocytes [45]. Therefore, we suggest that another mechanism for the antioxidant effect of BA may probably be due to the decreased postmeal glucose in BA-treated prediabetic animals.

3.5. Effects of BA Administration with or without Diet Intervention on Plasma, Urine Creatinine, and GFR. The plasma concentrations of creatinine significantly increased (Figure 4(a)) while the urinary concentration of the same parameter (Figure 4(b)) in the prediabetic control rats was significantly decreased in comparison to the nonprediabetic control rats. These observations were correlated with the results of other studies [24, 40]. The impaired creatinine clearance altered the plasma and urine creatinine concentrations and further contributed to the decreased GFR in the prediabetic control rats (Figure 4(c)) [2]. Studies have shown that insulin resistance triggers oxidative stress in renal tissues [47, 48]. Therefore, we suggest that the impaired creatinine clearance which resulted into the decreased GFR may be due to insulin resistance which further triggered oxidative stress as reported in other studies [49, 50]. However, the administration of BA in the absence or presence of diet intervention and metformin administration in the presence of diet intervention significantly increased the urine creatinine by comparison to the prediabetic control rats. Also, the GFR of BA and metformin-treated prediabetic rats with diet intervention significantly increased by comparison to the PD control rats (Figure 4(c)). Therefore, we suggest that the improved creatinine clearance in BA-treated prediabetic rats is due to the antioxidant activity of the pentacyclic triterpene.

3.6. Effects of BA Administration with or without Diet Intervention on Plasma Aldosterone. A high-fat diet has been reported to activate the renin-angiotensin-aldosterone system (RAAS) in insulin-resistant states [14, 20, 30]. Also, literatures have shown that due to hyperinsulinaemia in insulin-resistant states, aldosterone production increases, and this in turn activates the aldosterone-induced SGK1 signaling

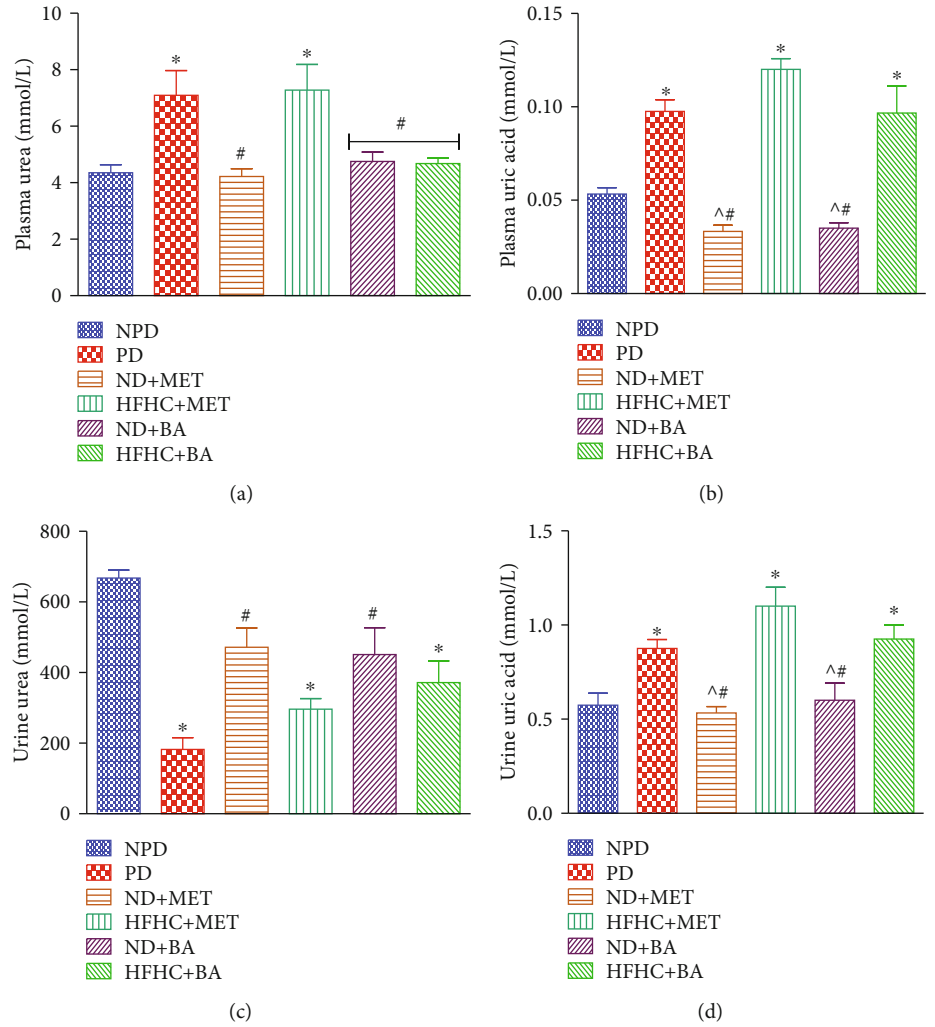

Figure 3: Effects of BA on plasma urea (a), plasma uric acid (b), urine urea (c) and urine uric acid (d) in prediabetic rats in the presence or absence of dietary intervention. $^{*}p < 0.001$ (vs. NPD), $^{\#}p < 0.001$ (vs. PD), and $^{\wedge}p < 0.001$ (vs. HFHC+MET).

Table 1: The effects of BA on lipid peroxidation and antioxidant status in prediabetic rats in the presence or absence of dietary intervention. Values are presented as the mean ± SEM ($n = 6$).

Groups Parameters	NPD	PD	ND+MET	HFHC+MET	ND+BA	HFHC+BA
MDA (nmol/g protein)	5.10 ± 0.13	7.72 ± 0.41***	5.69 ± 0.19$^{\#\#}$	6.75 ± 0.40**	5.07 ± 0.08$^{\#\#\#}$	5.63 ± 0.25$^{\#\#\#}$
SOD (ng/mL)	8.66 ± 0.27	3.14 ± 0.38***	9.92 ± 0.52$^{\#\#\#}$	6.62 ± 0.12$^{\#\#\#}$	11.45 ± 0.63$^{*\#\#\#}$	8.08 ± 0.81$^{\#\#\#}$
GPx (pg/mL)	1793.00 ± 42.38	849.27 ± 24.69***	1820.11 ± 25.88$^{\#\#\#}$	1274.50 ± 36.14***	1914.21 ± 37.18$^{\#\#\#}$	1698.61 ± 33.17$^{\#\#\#}$
TOAC (U/mL)	44.40 ± 2.57	14.80 ± 1.03***	31.45 ± 1.02$^{*\#\#\#}$	22.14 ± 3.03***	41.31 ± 1.65$^{\#\#\#}$	24.17 ± 3.10$^{***\#}$

$^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ (vs. NPD); $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, and $^{\#\#\#}p < 0.001$ (vs. PD).

pathway [13, 51]. In correlation with other studies [40, 52], significantly elevated plasma concentration of aldosterone was also observed in the prediabetic control rats when compared to the nonprediabetic control rats (Figure 5). Therefore, we suggest that the consumption of the high-fat diet contributed to the elevated aldosterone concentration through the activation of RAAS in the prediabetic control rats. In this study, the administration of BA and metformin in the absence or presence of diet intervention significantly decreased the plasma aldosterone concentration in the BA- and metformin-treated prediabetic rats. Therefore, we suggest that the administration of BA probably improved insulin sensitivity which in turn reduced the activation of RAAS and consequently leads to the significantly decreased plasma

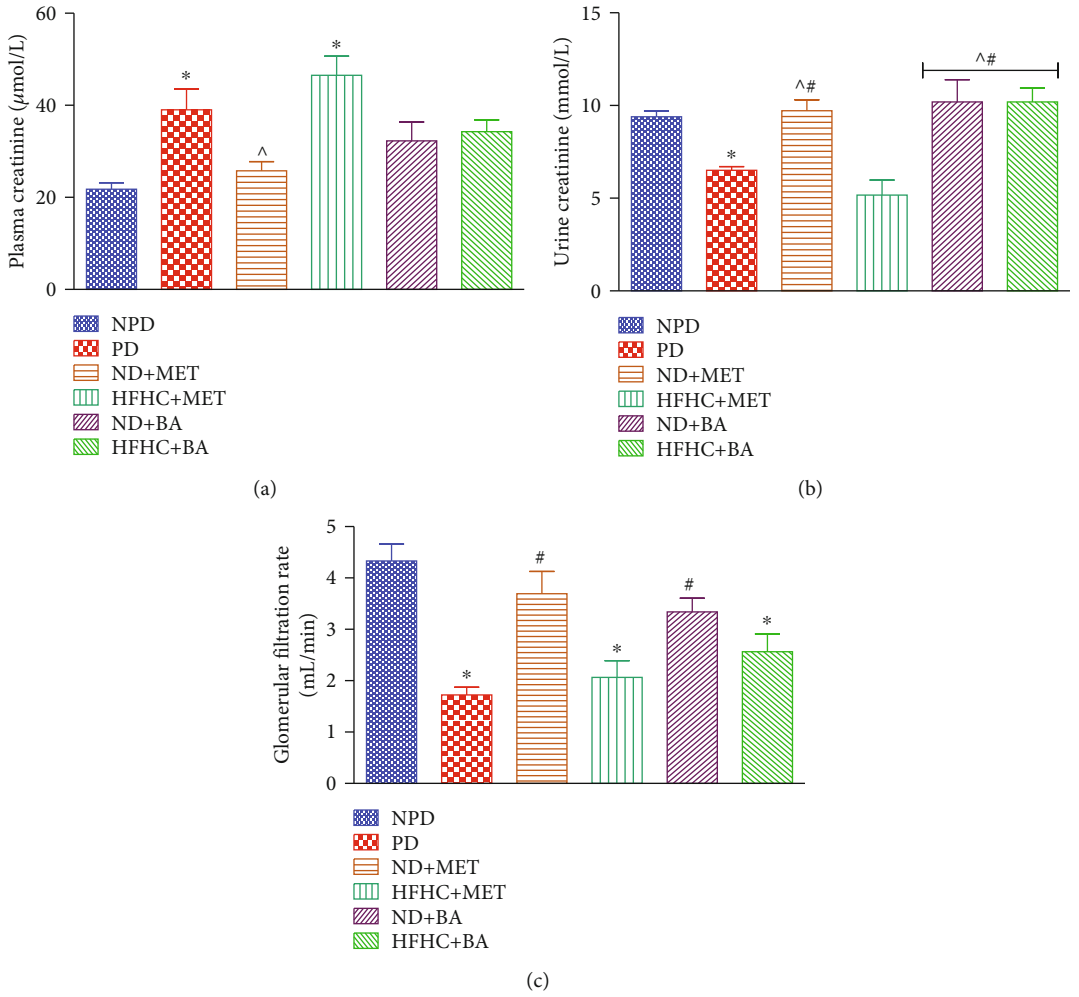

Figure 4: Effects of BA on plasma creatinine (a), urine creatinine (b), and GFR (c) in prediabetic rats in the presence or absence of dietary intervention. $^{*}p < 0.001$ (vs. NPD), $^{\#}p < 0.001$ (vs. PD), and $^{\wedge}p < 0.01$ (vs. HFHC+MET).

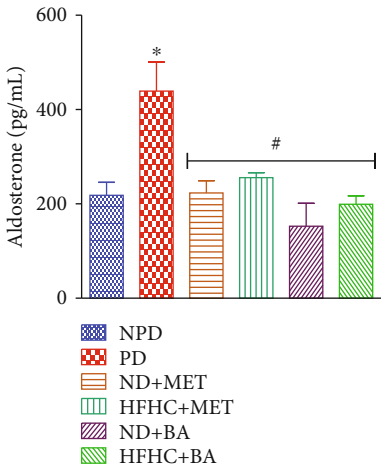

Figure 5: Effects of BA on plasma aldosterone concentrations in prediabetic rats in the presence or absence of dietary intervention. $^{*}p < 0.001$ (vs. NPD), $^{\#}p < 0.001$ (vs. PD).

aldosterone concentration in BA-treated prediabetic rats even in the absence of diet intervention.

3.7. Effect of BA Administration with or without Diet Intervention on Plasma and Urinary Sodium and Potassium, Fluid Intake, and Urine Output. Due to the aforementioned RAAS activation and elevated plasma concentration of aldosterone in insulin-resistant states, the fluid intake, urine output, sodium reabsorption, and potassium loss significantly increased in the prediabetic control rats in this study. Literature has shown that the activation of RAAS subsequently activates the serum/glucocorticoid-regulated kinase 1 (SGK1) which further triggers the stimulation of the epithelial sodium channel (ENaC) to cause sodium retention, hypokalemia, and increased fluid intake [13, 51]. In this study, the fluid intake and urine output of the prediabetic control rats were significantly increased in comparison to the nonprediabetic control rats throughout the treatment period (Table 2). However, in the presence or absence of dietary intervention with BA administration as well as metformin administration with diet intervention, the fluid intake and urine output significantly decreased when compared to

Table 2: Effects of BA on fluid intake and urine output in prediabetic rats in the presence or absence of dietary intervention. Values are presented as the mean ± SEM ($n = 6$).

Parameters	Groups					
	NPD	PD	ND+MET	HFHC+MET	ND+BA	HFHC+BA
Fluid intake (mL)						
0 week	21.50 ± 0.96	54.50 ± 4.54*	59.00 ± 3.63*	51.00 ± 4.73*	66.17 ± 6.43*	52.83 ± 5.10*
4 weeks	23.17 ± 1.76	34.50 ± 4.07*	22.67 ± 2.16#^	39.83 ± 7.10*	25.00 ± 3.37^	30.33 ± 3.33
8 weeks	20.83 ± 2.39	35.00 ± 2.89*	23.33 ± 3.33	32.5 ± 2.81	25.50 ± 2.93	33.33 ± 2.47
12 weeks	19.50 ± 1.38	34.17 ± 2.01*	20.83 ± 1.54#	30.00 ± 2.24	22.50 ± 2.14#	22.17 ± 2.32#
Urine output (mL)						
0 week	8.67 ± 0.67	31.33 ± 3.82*	33.17 ± 3.21*	30.33 ± 2.60*	39.00 ± 3.00*	34.33 ± 3.77*
4 weeks	9.00 ± 0.86	26.67 ± 3.41*	17.00 ± 2.46#^	29.67 ± 2.89*	17.67 ± 2.39#^	18.33 ± 2.45*^
8 weeks	11.00 ± 0.45	23.33 ± 4.28*	15.67 ± 1.75	20.83 ± 2.34*	14.00 ± 2.00#	18.00 ± 2.00
12 weeks	11.00 ± 1.44	26.33 ± 2.03*	16.67 ± 1.12#	22.17 ± 2.23*	16.50 ± 1.67#	16.00 ± 2.19#

*$p < 0.001$ (vs. NPD), #$p < 0.05$ (vs. PD), and ^$p < 0.05$ (vs. HFHC+MET).

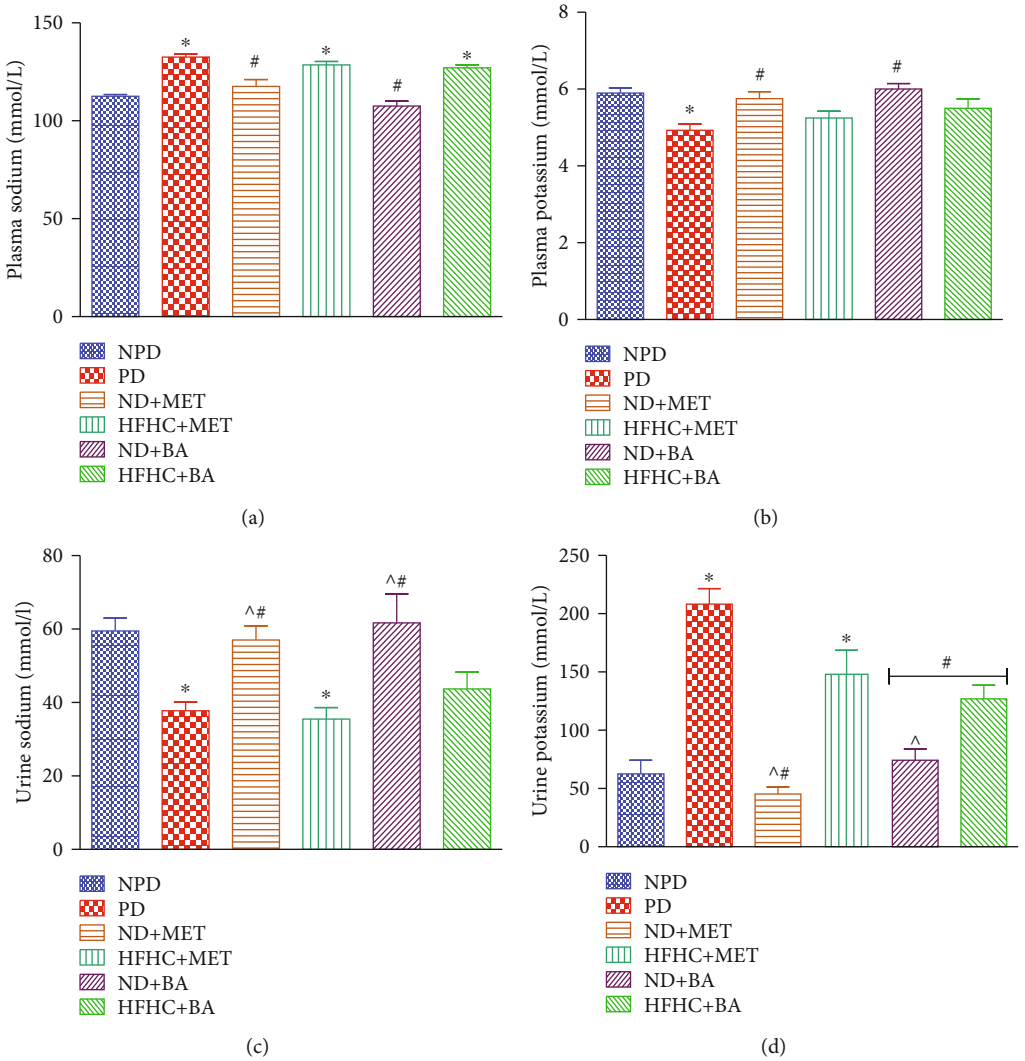

Figure 6: Effects of BA on plasma sodium (a), plasma potassium (b), urine sodium (c), and urine potassium (d) in prediabetic rats in the presence or absence of dietary intervention. *$p < 0.001$ (vs. NPD), #$p < 0.05$ (vs. PD), and ^$p < 0.001$ (vs. HFHC+MET).

the prediabetic control rats, especially at the 12th week period of treatment ($p < 0.05$).

Moreover, the administration of BA or metformin with diet intervention significantly decreased the plasma sodium concentration (Figure 6(a)) and increased the plasma potassium concentration (Figure 6(b)) when compared to the prediabetic control rats ($p < 0.05$). On the other hand, the BA- or metformin-treated prediabetic rats with diet intervention had significantly increased urinary sodium (Figure 6(c)) and decreased urinary potassium (Figure 6(d)) by comparison to the prediabetic control rats. Apart from RAAS, other mechanisms that can possibly be associated with the increased fluid intake, urine output, and electrolyte imbalance in the prediabetic control rats are hyperglycaemia and glycosuria. Therefore, we suggest that the amelioration of fluid intake, urine output, and the electrolytes by administration of BA may be attributed to the improved hyperglycaemia and glycosuria in the BA-treated prediabetic rats as reported in the previous study [25].

3.8. Effect of BA Administration with or without Diet Intervention on Urinary Podocin mRNA Expression. Literature evidences revealed that elevated aldosterone concentration induced proteinuria and glomerular podocyte injury with decreased gene expression of podocin in the kidney tissues and increased gene expression of podocin mRNA in the urine [53, 54]. Also, it has been established that podocytes express mineralocorticoid receptors (MR); hence, podocytes are targeted cells for aldosterone hormone [53, 55]. Therefore, when aldosterone concentration is increased, oxidative stress is induced in the podocytes, and this subsequently promotes podocyte injury by increased reactive oxygen species (ROS) production in the mitochondria [56]. In addition, it has been demonstrated that podocytes are insulin-responsive cells that similarly respond to insulin in the same manner as the skeletal muscle [57]. This showed that podocyte survival is modulated by insulin signaling [57]. Similarly, in this study, the aforementioned increase in urinary podocin mRNA expression was observed in prediabetic control rats, and this correlated with other similar studies [8, 58]. The podocin mRNA expression in the urine of prediabetic control rats was significantly increased by 12.04-fold when compared to the nonprediabetic control rats (Figure 7). The podocin mRNA expressions in the urine of BA and metformin-treated prediabetic rats in the presence or absence of diet intervention were significantly decreased when compared to the prediabetic control rats.

However, we suggest that the administration of BA probably improved insulin sensitivity and ameliorated the insulin signaling in podocytes, and this further contributed to the observed decreased gene expression of urinary podocin mRNA in BA-treated prediabetic rats in this study. Moreover, pentacyclic triterpenes have been reported to selectively inhibit 11β-hydroxysteroid dehydrogenase type I enzyme, an enzyme that converts inactive cortisone into active cortisol, thus preventing activation of mineralocorticoid receptors in aldosterone tissue such as the kidney [59, 60]. Therefore, we hypothesized that the same enzymatic inhibition may probably prevent aldosterone biological actions on podocyte mineralocorticoid receptors and this subsequently led to reduced podocyte injury which in turn contributed to the decreased urinary gene expression of podocin mRNA in the BA-treated prediabetic rats with or without diet modification.

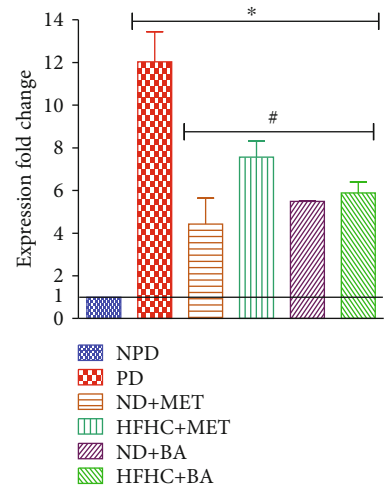

Figure 7: Effects of BA on urinary podocin mRNA expression in prediabetic rats in the presence or absence of dietary intervention. $^{*}p < 0.001$ (vs. NPD), $^{\#}p < 0.001$ (vs. PD).

4. Conclusion

Administration of BA with or without diet modification has been shown in this study to attenuate renal dysfunction markers and urinary expression of podocin mRNA in the prediabetic state. These biological actions of BA may be due to the earlier reported combination of the improved insulin sensitivity, antihyperglycaemic and antioxidant properties of the pentacyclic triterpene (BA) [61, 62]. Pentacyclic triterpenes have been reported as nontoxic antioxidants that have low pharmacokinetic activity of three days without side effects [28, 29]. Therefore, we suggest that the ameliorative effects of BA on renal function markers compared to metformin in this study may be attributed to the low pharmacokinetic feature of BA even in the absence of dietary intervention. However, this is a preliminary study, more structural and molecular findings are still needed to clarify the mechanisms by which BA ameliorates renal function.

Data Availability

The data used to support our findings in this study are available upon request from the corresponding author. However, the data on body weight, food intake, fasting blood glucose, and oral glucose tolerance test have been reported in our previous study.

Conflicts of Interest

The authors declare no conflicts of interest.

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Research Article

Renoprotective Effects of a New Free Radical Scavenger, XH-003, against Cisplatin-Induced Nephrotoxicity

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Acute renal injury has an incidence of 25%–30% in patients with tumors who are treated with cisplatin and in patients for whom no specific drugs are available for treatment. Amifostine is the only FDA-approved chemoprotective drug; however, its clinical application is limited because of side effects. The small-molecule antioxidant XH-003, an acute radiation syndrome- (ARS-) protective drug independently developed in our laboratory, with 100% intellectual property rights, overcomes the side effects of amifostine but retains its high efficacy. In this study, XH-003 showed a chemoprotective effect similar to that of amifostine. A mechanistic study showed that XH-003 could significantly reduce cisplatin-induced increases in serum creatinine and urea nitrogen, increase the activity of antioxidant enzymes (SOD, CAT, and GSH-Px), reduce oxidative stress and tissue inflammation, and alleviate renal tissue damage by blocking the activity of the mitochondrial apoptosis pathway. Most importantly, XH-003 could reduce the accumulation of cisplatin in renal tissue by regulating the expression of proteins involved in cisplatin uptake and excretion, such as organic cation transporter 2 and MRP2. Moreover, in an *in vivo* xenotransplantation model, XH-003 did not interfere with the antitumor effect of cisplatin. These data provide strong evidence that the ARS-protective agent has a great potential for protecting against chemotherapy-induced toxicity. Thus, XH-003 can be considered in antitumor therapy.

1. Introduction

Cisplatin (DDP), a potent chemotherapeutic agent, is widely used to treat various types of solid tumors, such as bladder, cervical, head and neck, esophageal, triple-negative breast, and small-cell lung cancers [1–3]; however, it has severe side effects including ototoxicity, neurotoxicity, and nephrotoxicity. Mounting clinical evidence has shown that acute kidney injury (AKI) is developed in approximately 25%–30% of patients treated with DDP [4]. AKI is associated with preferential accumulation of DDP in renal tubules, resulting in renal dysfunction [5]. However, the detailed mechanism of DDP-induced AKI remains elusive.

The proposed pathophysiological mechanisms of DDP-induced nephrotoxicity primarily involve DNA damage, the mitochondrial apoptosis pathway, inflammation, and oxidative stress [6–8]. The uptake of DDP by renal tubular epithelial cells involves organic cation transporter 2 (OCT2) and copper transporter 1 (CTR1). After entering cells, the chlorine atom of DDP is replaced by water by hydration. Therefore, electrophilic compounds produced by DDP can interact with nuclear DNA and activate the p53 protein. DDP can also interact with mitochondrial DNA, reduce the expression of electron transport chain proteins, damage respiration, and increase the production of reactive oxygen species (ROS). ROS, in turn, can induce oxidative stress and activate p53, which ultimately activates the apoptotic pathway. The increase in ROS can also induce proinflammatory factors, resulting in inflammation. In general, DDP-enhanced ROS production is the key contributor to renal dysfunction. Therefore, inhibition of ROS by antioxidants is a potential approach to the treatment of DDP-induced nephrotoxicity [9].

Amifostine [10] (Ethyol®), a highly efficient ROS scavenger, has been developed by the Walter Reed Army Institute in 1959 as an acute radiation syndrome- (ARS-) protective

agent for soldiers in the Cold War and has been approved by FDA for the reduction of cumulative renal toxicity associated with repeated administration of DDP in patients with advanced ovarian cancer in 1995. However, owing to its short half-life, injection-only administration, strong side effects (nausea, vomiting, hypotension, etc.), and poor patient compliance, the clinical application of amifostine is limited [10]. At present, hydration and diuresis are primarily used to protect against DDP-induced nephrotoxicity [11] by reducing the concentration of DDP in renal tubules and by reducing renal damage. However, this method requires consumption of large volumes of water, resulting in frequent urination, which is inconvenient for patients. Meanwhile, the guidance on DDP hydration requires improvements. More importantly, hydration and diuresis do not protect from renal dysfunction in a percentage of treated patients. In addition, in the primary stage, researches have reported that natural antioxidants, such as capsaicin [12, 13], curcumin [14–16], ellagic acid [17–19], epigallocatechin-3-*O*-gallate [20–22], α-lipoic acid [23, 24], lycopene [25, 26], quercetin [27, 28], resveratrol [29, 30], sulforaphane [31, 32], tannic acid [33, 34], and vitamins [35–39], can alleviate the DDP-induced increase in serum creatinine and urea nitrogen levels, inhibit the activation of p53, reduce the level of ROS in renal tissue, block the activation of the mitochondrial and endoplasmic reticulum apoptosis pathways, and reduce the inflammatory response; however, these natural antioxidants have a weak antioxidant capacity and should only be used as dietary supplements. These compounds have been proved to not have renal protective properties. Furthermore, sodium thiosulfate can prevent DDP-induced nephrotoxicity and inhibits the antitumor effect of DDP, limiting its application. Thus, there remains a need for a specific drug for protection against DDP-induced nephrotoxicity.

Compound XH-003, an ARS-protective drug, was independently developed by our laboratory with 100% intellectual property rights. XH-003 is currently in a preclinical study, and the results show that XH-003 not only retains the strong antioxidant ability of amifostine but also overcomes the drawbacks of the drug. XH-003 can be administered orally, has a bioavailability of up to 42%, and reduces the toxic effects of amifostine. The properties of XH-003 as an ARS-protective agent have been shown to far exceed those of amifostine. Therefore, the aim of this study is to explore the potential of XH-003 against DDP-induced nephrotoxicity and to not only broaden the indications of XH-003 but also benefit more patients with cancer and improve their quality of life.

2. Concise Methods

2.1. Animals. Female SD rats (200–300 g) were used in all acute nephrotoxicity experiments. The animals were purchased from SPF Biotechnology Co. Ltd. (Beijing) and were bred in a certified animal facility at the Institute of Radiation Medicine (IRM) of the Chinese Academy of Medical Sciences (CAMS).

2.2. Ethics Approval and Consent to Participate. All experimental procedures were carried out in accordance with the NIH Guidelines for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of the IRM, CAMS (permit number: 2017053). The animals were cared for in accordance with the guidelines of the National Animal Welfare Law of China.

2.3. DDP Dose Screening. A total of 12 female SD rats (230–260 g) were divided into four groups ($n = 3$ each), including a control group and three DDP treatment groups (5, 7.5, and 10 mg/kg, respectively). DDP was administered as a single intraperitoneal (i.p.) injection. The rats were monitored twice daily and weighted once a day, with their survival and behavior recorded. The experiment was terminated after 3 days, and all the rats were anesthetized and sacrificed. Blood samples were obtained, and sera were separated. Serum creatinine and urea were calculated using the SMT-100V portable automatic animal biochemical analyzer.

2.4. Determination of XH-003 Administration Time. A total of 15 female SD rats (230–260 g) were divided into five groups ($n = 3$ each), including a control group, DDP (5 mg/kg) group, XH-003 (800 mg/kg) group, and DDP (5 mg/kg)+XH-003 (800 mg/kg) groups A and B. Group A received a single PO dose of XH-003 (800 mg/kg) 30 min before DDP administration, and group B received a single PO dose of XH-003 (800 mg/kg) 4 h before DDP administration. On day 3, all the animals were anesthetized and sacrificed; blood samples were obtained, and sera were separated. Serum creatinine and urea were measured according to the abovementioned method.

2.5. Comparison of Chemoprotective Effects of XH-003 and Amifostine in Chemotherapy. A total of 18 female SD rats (230–260 g) were divided into six groups ($n = 3$ each), including a control group, DDP (5 mg/kg) group, XH-003 (800 mg/kg) group, amifostine (200 mg/kg) group, DDP (5 mg/kg)+XH-003 (800 mg/kg) group, and DDP (5 mg/kg)+amifostine (200 mg/kg) group. The DDP+XH-003 group received a single PO dose of XH-003 (800 mg/kg) 30 min before DDP administration, and the DDP+amifostine group received a single 200 mg/kg i.p. dose of amifostine 30 min before DDP administration. The rats were monitored twice daily and weighted once a day. The experiment was terminated after 3 days, and all the rats were anesthetized and sacrificed. Blood samples were obtained. The right kidney was fixed in 4% neutral formaldehyde for histological examination, and the left kidney was frozen in liquid nitrogen and stored at −80°C until analysis.

2.6. Kidney Function Tests. Serum samples were collected by centrifugation at 4,000 rpm for 10 min. Serum creatinine and urea nitrogen levels were measured according to the abovementioned method.

2.7. Hematological Evaluation. Blood was obtained from the rats via the orbital sinus and was collected in a micropipette coated with K_3EDTA. The blood parameters measured included white blood cells (WBCs), red blood cells, platelets

(PLTs), hemoglobin, monocytes (MOs), and basophils (BAs). Cells were counted using a Celltac E hemocytometer (Nihon Kohden, Japan).

2.8. Malondialdehyde (MDA) Assay. The MDA content was measured in kidney tissue using an MDA detection kit (No. A003-1; Nanjing Jiancheng Bioengineering Institute, Nanjing, China), according to the manufacturer's instructions.

2.9. T-SOD, GSH-Px, and CAT Assays. The activities of T-SOD, GSH-Px, and CAT in rat kidney tissues were tested using commercial kits (catalog numbers: A-001-1, A005-1, and A007-2-1, respectively), as specified by the manufacturer (Nanjing Jiancheng Bioengineering Institute).

2.10. Hematoxylin and Eosin (HE) Staining. The samples of renal tissue fixed in 4% paraformaldehyde were embedded in paraffin and cut into 5 μm sections using a paraffin section machine. Subsequently, the sections were dewaxed with xylene and absolute ethanol to water, then stained with HE (71014460; Shanghai Jingke Chemical Technology Co., Ltd.) and analyzed under a microscope.

2.11. Terminal Deoxynucleotidyl Transferase-Mediated dUTP Nick-End Labeling (TUNEL) Staining. The 5 μM tissue sections were treated according to the protocol for a TUNEL kit (11684817910; Roche). Analysis was performed under a microscope.

2.12. Western Blotting. Proteins were extracted from renal tissue using an ice-cold lysis buffer, and the protein concentrations were quantified using a BCA assay kit (PC0020; Solarbio). SDS-PAGE was used to separate equal amounts of protein. The blocked membrane was incubated using anti-Bax (1 : 500; WL01637, WanleiBio), anti-P-p53 (1 : 2,000; ab1431, Abcam), anti-PUMA (1 : 3,000; ab9643, Abcam), anti-caspase-3 (1 : 2,000; 9661, CST), anti-Nrf2 (1 : 500; ab89443, Abcam), anti-HO-1 (1 : 500; WL01637, WanleiBio), anti-OCT2 (1 : 500; ab243153, Abcam), anti-MRP2 (1 : 500; ab203397, Abcam), and anti-β-actin (1 : 1,000; WL01372, WanleiBio) antibodies overnight at 4°C. Then, the membranes were incubated with a suitable horseradish peroxidase-conjugated secondary antibody for 60 min at room temperature. Finally, an ECL western blotting substrate was used to visualize the immunoblots.

2.13. Platinum Uptake Assay. To evaluate the effect of XH-003 on the uptake of DDP by renal tissue, we examined the platinum (Pt) content in renal tissue using inductively coupled plasma mass spectrometry. In brief, tissue samples were lyophilized using a freeze dryer system. The lyophilized samples were digested with 0.2 mL of high-purity nitric acid at room temperature for 4 h, then diluted with 2 mL of deionized water and centrifuged at 6,000 rpm for 15 min. Finally, a 0.2 mL aliquot was transferred to an inductively coupled plasma mass spectrometry autosampler tube to determine the Pt content (μg/g tissue) at each time point.

2.14. H358 In Vivo Tumor Model. H385 cells were cultured in Dulbecco's modified Eagle medium containing 10% fetal bovine serum. After reaching the exponential growth stage, cells were resuspended in phosphate-buffered saline and mixed (1 : 1) with Matrigel for subcutaneous inoculation into BALB/c nude mice. Fifty female mice were inoculated with 5×10^6 H358 cells. When the average tumor volume reached approximately 200 mm^3, the mice were randomly divided into the following four groups ($n = 6$ each): control group, XH-003 (500 mg/kg) single-dose group, DDP (10 mg/kg) single-dose group, and 500 mg/kg XH-003+10 mg/kg DDP group. XH-003 was administered orally once a week for 2 weeks, the first administration of XH-003 was 30 min before DDP administration, and the second administration of XH-003 was 8 h after the first administration. DDP was administered by tail vein injection once a week for 2 weeks. The therapeutic effect was evaluated based on the relative tumor inhibition rate. The results of tumor volume were expressed by mean ± standard error of mean. All data were analyzed with Prism 6.0. $p < 0.05$ was considered statistically significant.

3. Results

3.1. Optimal Dose of DDP for Building a Renal Injury Model. A single high dose of DDP, ranging from 5 to 15 mg/kg, is widely used to build renal injury models in rats [40]. However, differences in animal species, sources, animal room environments, etc., result in different optimal DDP doses for modeling and in different effects of renoprotective agents. To establish a rat renal injury model suitable for our study, three doses of DDP were tested, and the results are shown in Figure 1 and Supplementary Table S1. All rats in the treatment groups lost weight, whereas those in the control group gained weight (Figure 1(a)). Meanwhile, the levels of creatinine and urea nitrogen (Figures 1(b) and 1(c)) were significantly higher in rats treated with the different doses of DDP than in the control group. The mean serum creatinine and urea nitrogen levels were 21.67 ± 5.56 μmol/L and 5.86 ± 0.53 mmol/L in the control group and increased to 173.53 ± 49.53 μmol/L and 37.45 ± 8.85 mmol/L in the 5 mg/kg DDP group ($p < 0.05$), 328 ± 104.5 μmol/L and 41.8 ± 1.91 mmol/L in the 7.5 mg/kg DDP group ($p < 0.05$), and 370.8 ± 69.3 μmol/L and 46.69 ± 2.69 mmol/L in the 15 mg/kg DDP group ($p < 0.05$), respectively. Notably, one rat died in each 7.5 and 15 mg/kg DDP group, which was attributed to DDP intolerance. Considering these factors, the optimal single dose of DDP of 5 mg/kg was applied to study the protective effect of XH-003 against DDP-induced nephrotoxicity.

3.2. Optimal Time of XH-003 Administration prior to DDP Administration. The most optimum administration time for XH-003 was 4 h before irradiation, and its half-life was 6.65 h. However, it remains unknown whether XH-003 would show a chemoprotective effect at this time of administration. Therefore, two times of administration were tested, 30 min and 4 h prior to DDP administration. The results are shown in Figure 2 and Supplementary Table S2. Compared with those in the control group, the levels of creatinine and urea nitrogen in the DDP group were significantly higher: 7.8 (159.3 ± 21.64 vs. 20.43 ± 3.82 μmol/L; $p < 0.01$) and 4.7 (28.87 ± 6.36 vs. 6.09 ± 0.49

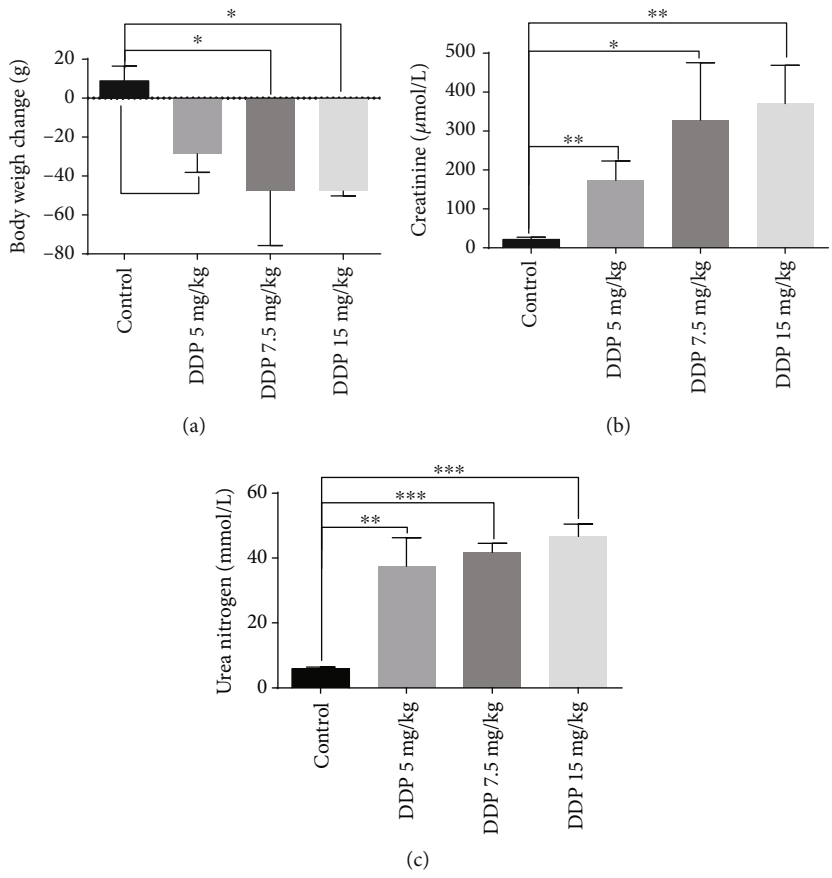

Figure 1: Changes in body weight (a), creatinine (b), and urea nitrogen (c) in rats after treatment with DDP (5–15 mg/kg). $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

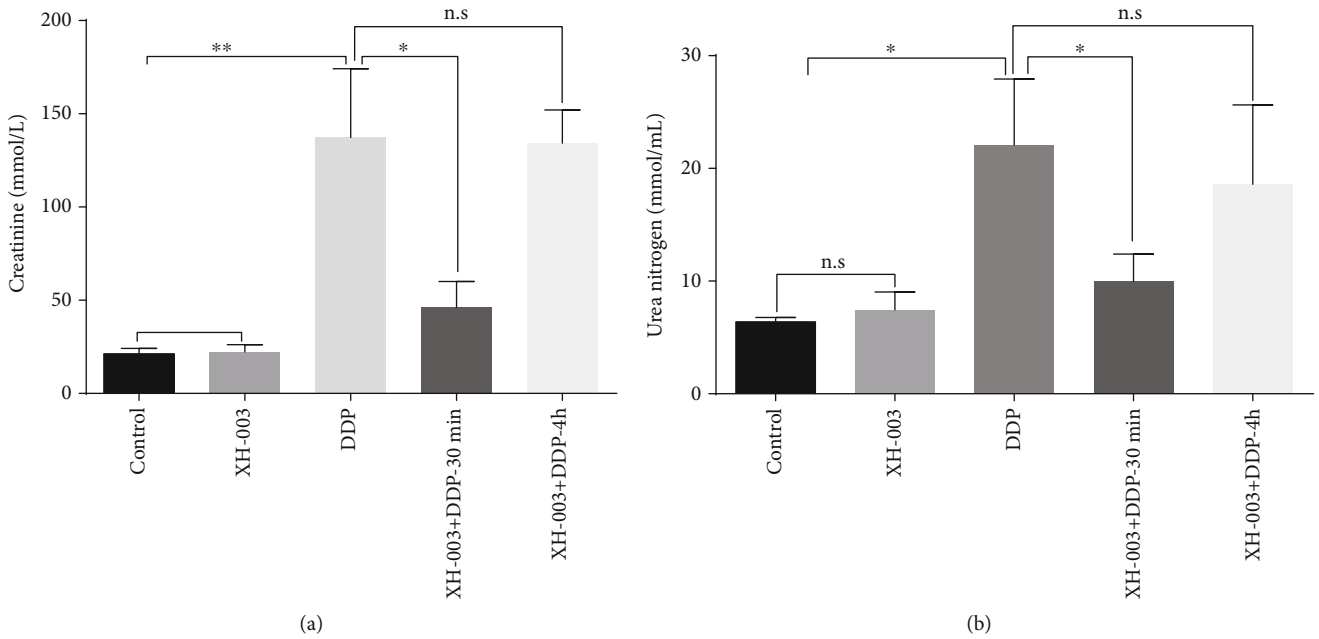

Figure 2: Effects of the time of administration of XH-003 on DDP-induced increases in serum creatinine (a) and urea nitrogen (b) levels. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$. n.s: no difference.

mmol/L; $p < 0.05$) times higher, respectively. Interestingly, compared with those in the DDP group, the levels of creatinine ($87.17 \pm 14.10\,\mu$mol/L; $p > 0.05$) and urea nitrogen (15.51 ± 5.36 mmol/L; $p > 0.05$) slightly improved upon administration of XH-003 4 h prior to DDP administration, with no statistically significant differences. However, the administration of XH-003 30 min prior to DDP administration significantly decreased the levels of creatinine ($46.17 \pm 13.85\,\mu$mol/L; $p < 0.05$) and urea nitrogen (10.91 ± 4.04 mmol/L; $p < 0.05$). In addition, compared with the control group, the XH-003-alone treatment group did not show any differences in the levels of creatinine ($19.77 \pm 1.90\,\mu$mol/L; $p > 0.05$) and urea nitrogen (6.41 ± 0.7 mmol/L; $p > 0.05$). Therefore, administration of XH-003 30 min prior to DDP administration could significantly improve DDP-induced nephrotoxicity and play a chemoprotective role.

3.3. Renal Protection Effects of XH-003 Are Similar to Those of Amifostine. The primary purpose of designing XH-003 was to retain the efficacy of amifostine and overcome its drawbacks, such as nonoral administration, a short half-life, and strong side effects. As an ARS-protective agent, XH-003 has achieved this goal. However, it remains unknown whether the efficacy of XH-003, as a chemoprotective agent, is similar to that of amifostine. Therefore, we compared the protective effects of XH-003 and amifostine under the same experimental conditions. The results are shown in Figure 3 and Supplementary Table S3. The significant increases in serum creatinine (143.15 ± 8.25 vs. $61.39 \pm 13.80\,\mu$mol/L; $p < 0.001$) and urea nitrogen (22.33 ± 4.33 vs. 10.07 ± 1.17 mmol/L; $p < 0.05$), caused by DDP, were prevented in the XH-003+DDP group. Although amifostine significantly decreased creatinine (143.15 ± 8.25 vs. $72.14 \pm 14.17\,\mu$mol/L; $p < 0.01$) and urea nitrogen (22.33 ± 4.33 vs. 17.50 ± 5.16 mmol/L; $p > 0.05$), the decrease in the latter was not statistically significant. In addition, DDP-induced severe weight loss was alleviated by XH-003 and amifostine (Figure 3). It should be noted that there were no significant differences in the protective effects (61.39 ± 13.80 vs. $72.14 \pm 14.17\,\mu$mol/L for creatinine, $p > 0.05$; 10.07 ± 1.17 vs. 17.50 ± 5.16 mmol/L for urea nitrogen, $p > 0.05$; data not shown) of XH-003 and amifostine against DDP-induced nephrotoxicity. Thus, XH-003 retained the advantages of amifostine in protection against nephrotoxicity.

3.4. XH-003 Alleviates DDP-Induced Abnormalities in Peripheral Blood. The evaluation of peripheral blood (Figure 4 and Supplementary Table S4) showed that in the DDP group, MO% (25.23 ± 4.03 vs. 7.85 ± 1.59; $p < 0.01$), BA% (0.29 ± 0.04 vs. 0.02 ± 0.04; $p < 0.01$), PLTs (416.33 ± 74.14 vs. $195.67 \pm 19.66 \times 10^9$/L; $p < 0.01$), and WBCs (10.62 ± 1.02 vs. $4.08 \pm 1.49 \times 10^9$/L; $p < 0.01$) significantly increased compared with their levels in the control group, suggesting that DDP induced inflammatory responses. However, XH-003 significantly improved DDP-induced levels of MO% (9.76 ± 2.88 vs. 25.23 ± 4.03; $p < 0.01$), BA% (0.13 ± 0.04 vs. 0.29 ± 0.04; $p < 0.01$), PLTs (257.33 ± 35.11 vs. $416.33 \pm 74.14 \times 10^9$/L; $p < 0.05$), and WBCs (4.93 ± 0.21 vs. $10.62 \pm 1.02 \times 10^9$/L; $p < 0.001$). There were no changes in other peripheral blood indexes (data not shown).

3.5. XH-003 Improves DDP-Induced Renal Inflammatory Infiltration. Given the results of the peripheral blood evaluation, HE staining was used to observe whether DDP caused the inflammatory response in renal tissue and whether XH-003 could alleviate this effect (Figure 5). In the DDP group, the glomerular structure was clear, with no obvious lobular atrophy of the glomerulus. However, vascular congestion was observed in the stroma, and inflammatory cell infiltration was observed in the tissue. Meanwhile, XH-003 alleviated this inflammatory reaction, showing significant protection to the kidneys of the rats challenged with DDP.

3.6. XH-003 Relieves Oxidative Stress in the Kidneys of DDP-Treated Rats. Oxidative stress influences DDP-induced renal injury. The increase of MDA level and the decrease of antioxidant enzyme level are observed in DDP-induced renal injury. Next, we examined whether XH-003 could attenuate oxidative stress in the kidneys of DDP-treated rats. The results are shown in Figure 6 and Supplementary Table S5. DDP induced a significant increase in the MDA level (254.98 ± 44.82 vs. 47.71 ± 7.27 nmol/mL; $p < 0.01$) and decreased the levels of the antioxidant enzymes SOD (140.43 ± 9.45 vs. 186.80 ± 13.97 U/mg; $p < 0.01$), GSH-Px (274.69 ± 70.82 vs. 578.41 ± 66.91 U/mg; $p < 0.01$), and CAT (219.70 ± 14.32 vs. 349.14 ± 48.92 U/g; $p < 0.05$) in renal tissue compared with those in the control, respectively. Meanwhile, compared with DDP group, XH-003 significantly reduced the MDA level (46.43 ± 12.63 vs. 254.98 ± 44.82 U/mg; $p < 0.01$) and increased the antioxidant enzymes SOD (201.02 ± 4.03 vs. 140.43 ± 9.45 U/mg; $p < 0.001$), GSH-Px (666.91 ± 76.82 vs. 274.69 ± 70.82 U/mg; $p < 0.01$), and CAT (299.72 ± 37.52 vs. 219.70 ± 14.32 U/g; $p < 0.05$). These results indicated that XH-003 effectively alleviated the DDP-induced oxidative stress.

3.7. XH-003 Inhibits DDP-Induced Renal Apoptosis. We used TUNEL staining to evaluate apoptosis *in vivo* (Figure 7). Compared with the control and XH-003-only groups, which showed no apoptosis, DDP induced apoptosis in renal tissue, which was alleviated by XH-003, showing a significant protective effect against DDP-induced renal injury.

3.8. XH-003 Prevents the DDP-Induced Activation of the Mitochondrial Apoptosis Pathway. To examine whether XH-003 prevents the activation of the DDP-induced apoptosis pathway, the expression levels of apoptosis pathway-related proteins were analyzed by western blotting (Figure 8). We found that XH-003 significantly reduced the expression levels of DDP-induced mitochondrial apoptosis pathway-related proteins such as cleaved caspase-3, PUMA, Bax, and phospho-p53. XH-003 also alleviated the DDP-induced decreases in the expression of the antioxidation-related proteins Nrf2 and HO-1.

3.9. XH-003 Reduces the Content of DDP in Renal Tissue. To examine whether the renal protection effects of XH-003 are

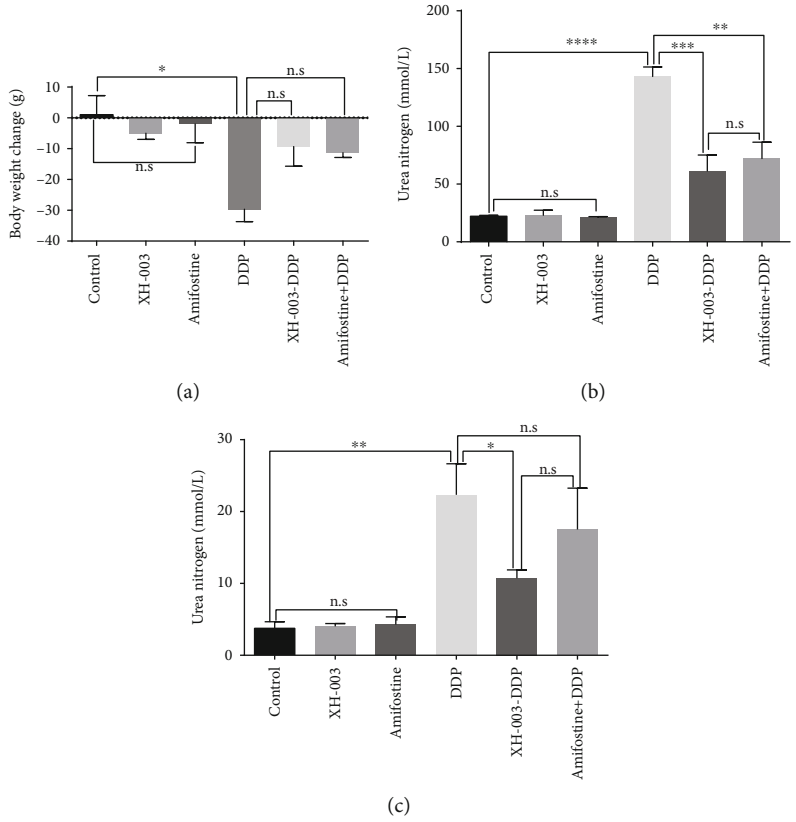

Figure 3: Comparison of renal protective effects between XH-003 and amifostine. (a) Changes in body weight in each group of rats. (b) Changes of serum creatinine level. (c) Changes of urea nitrogen in the serum. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$. n.s: no difference.

because of a decrease of DDP in kidney tissue, Pt levels were determined 3 days after DDP administration. The results are shown in Figure 9 and Supplementary Table S6. In the XH-003+DDP group, the level of Pt ($42.58 \pm 2.13\,\mu$g/g) was half of that in the DDP group ($83.46 \pm 1.57\,\mu$g/g; $p < 0.001$), indicating that XH-003 significantly reduced the level of Pt in renal tissue.

3.10. XH-003 Affects the Uptake and Excretion of DDP from Renal Tissue. We speculated that XH-003 might affect the uptake or excretion of DDP from renal tissue. OCT2 and MRP2 influence DDP uptake and excretion. Western blotting analysis was used to test whether XH-003 affected the expression of the OCT2 and MRP2 proteins (Figure 10). Compared with those in the DDP group, the expression level of OCT2 was decreased and that of MRP2 was increased by XH-003 treatment, indicating that XH-003 reduced the DDP uptake and increased its excretion from renal tissue. XH-003 alone could also increase the expression of MRP2.

3.11. XH-003 Does Not Affect the Antitumor Effect of DDP. In addition to its ability to reduce DDP-induced nephrotoxicity, it was very important to determine whether XH-003 attenuates the antitumor effect of DDP. The results of the H358 xenotransplantation experiment (Figure 11) showed that compared with those in the control ($510.6 \pm 103.8\,\text{mm}^3$) and XH-003 ($460.4 \pm 80.2\,\text{mm}^3$) groups, tumor volumes significantly declined in the DDP ($247.3 \pm 129.1\,\text{mm}^3$) and DDP+XH-003 ($184.4 \pm 106.5\,\text{mm}^3$) groups. Interestingly, the antitumor effect was significantly better ($p < 0.001$) in the XH-003+DDP group than in the DDP group, suggesting that XH-003 did not affect the antitumor activity of DDP but the combination of the two drugs might have had a synergistic effect.

4. Discussion

DDP, used as an antitumor agent since 50 years, has significantly contributed to the enormous advances in the field of oncology; however, there is no effective solution to the problem of DDP-induced nephrotoxicity. Oxidative stress and DNA damage play key roles in DDP-induced nephrotoxicity. Therefore, the inhibition of ROS by antioxidants is a potential approach to the treatment of DDP-induced nephrotoxicity.

In this study, we found that XH-003 also showed a significant protective effect against DDP-induced nephrotoxicity. XH-003 significantly reduced DDP-increased levels of creatinine and urea nitrogen in the plasma, which are well-known biomarkers of kidney function. Furthermore, XH-003 alleviated the DDP-induced abnormal changes in BA%, MO%, PLTs, and WBCs in the peripheral blood, suggesting that XH-003 could effectively improve DDP-induced inflammation. HE staining confirmed that XH-003 relieved DDP-induced renal tissue inflammation. Together with the results of TUNEL staining for apoptosis, the data confirmed that

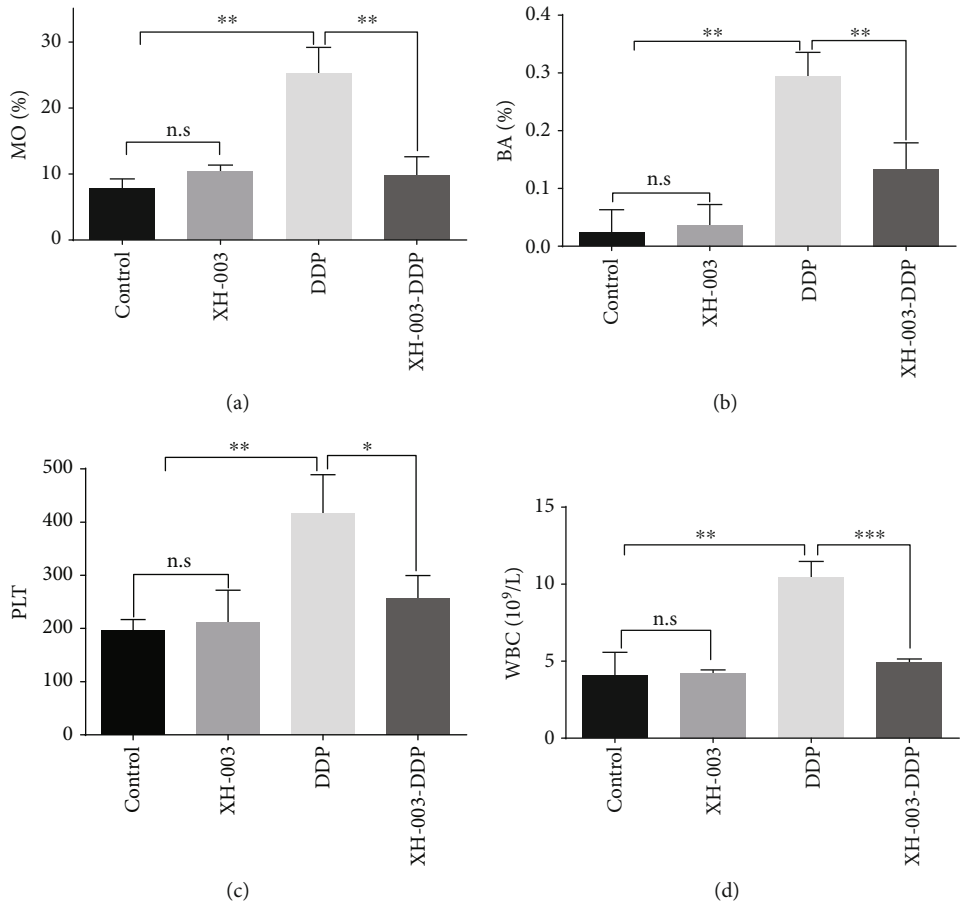

Figure 4: Effects of XH-003 on DDP-induced peripheral blood abnormalities, including (a) MO%, (b) BA%, (c) PLT counts, and (d) WBC counts. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$. n.s: no difference.

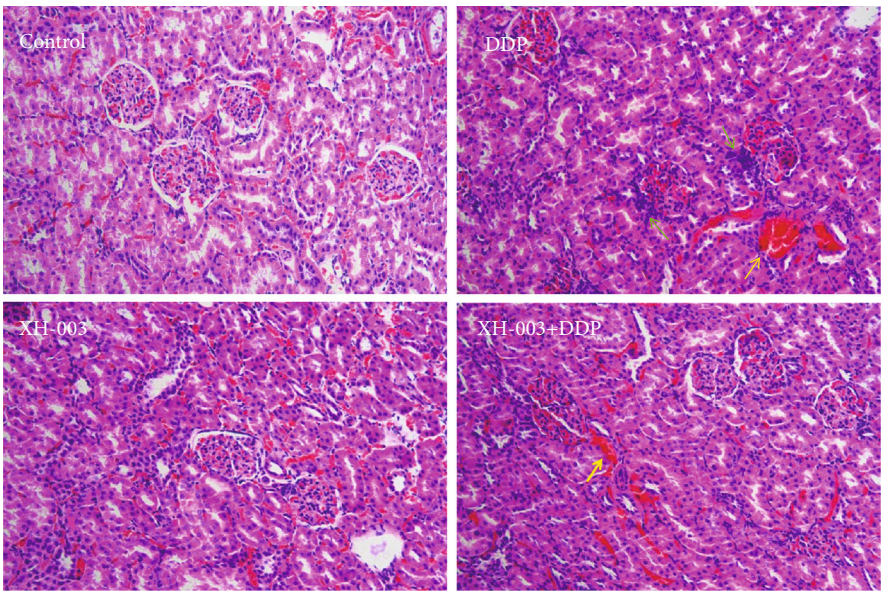

Figure 5: Effects of XH-003 on DDP-induced renal inflammation. Green arrows indicate inflammation, and yellow arrows indicate interstitial hyperemia.

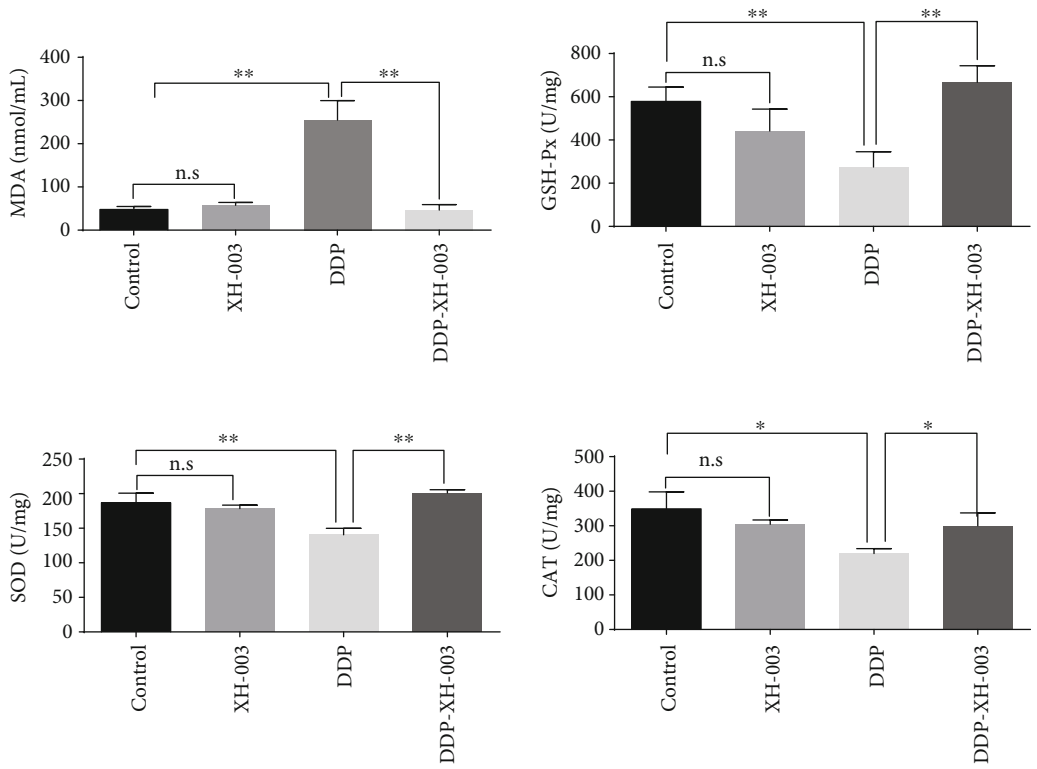

Figure 6: Effects of XH-003 on DDP-induced oxidative stress, based on the levels of MDA, GSH-Px, SOD, and CAT. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$. n.s: no difference.

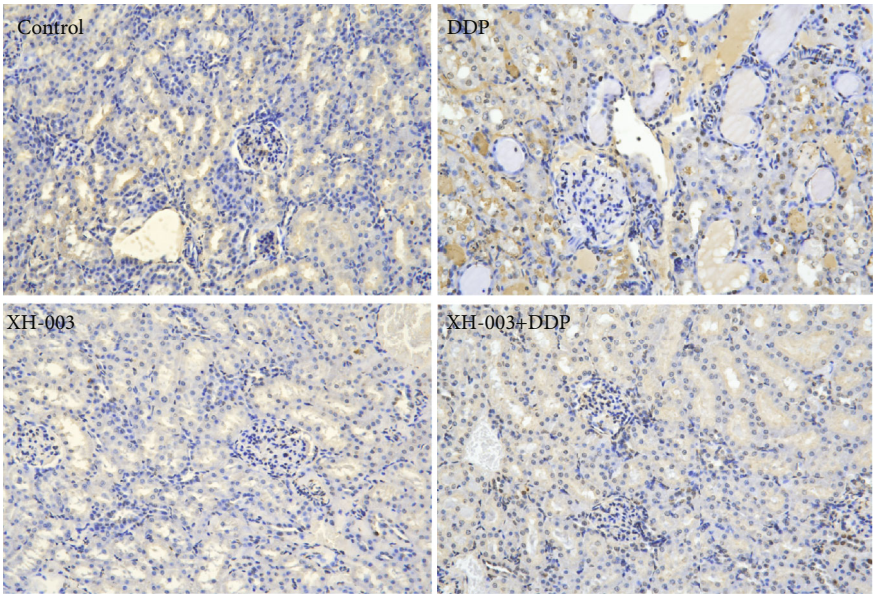

Figure 7: Effect of XH-003 on DDP-induced renal apoptosis, which is indicated by brown TUNEL staining.

XH-003 had a good protective effect against DDP-induced nephrotoxicity.

Evidence obtained in recent years has indicated that DDP not only directly damages DNA through chelation but also induces renal damage by producing large amounts of ROS and indirectly damaging DNA. When DNA is damaged, it immediately activates the ataxia telangiectasia mutant protein, which can cause phosphorylation of p53. The role of p53, which is a well-recognized tumor suppressor protein, was elucidated in renal injury by Cummings and Schnellmann. Activated p53 can promote the expression of PUMA; disturb the interaction between the BCl-XL and Bax proteins;

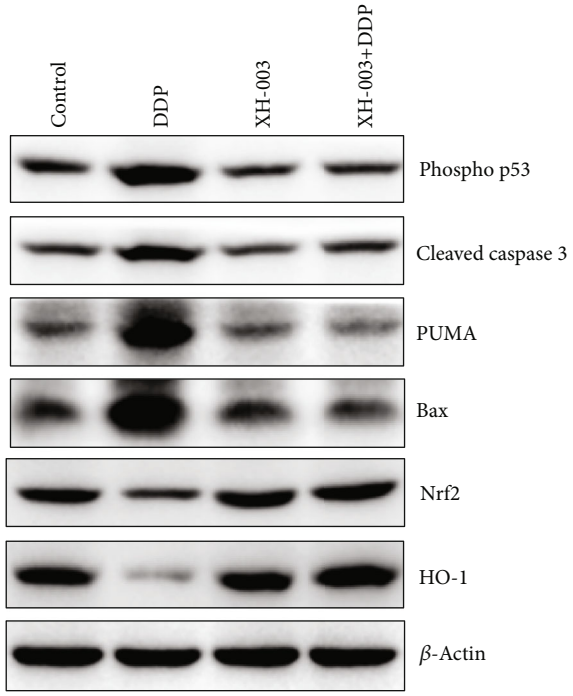

Figure 8: Effects of XH-003 on DDP-induced changes in the expression of mitochondrial apoptosis pathway- and antioxidation-related proteins.

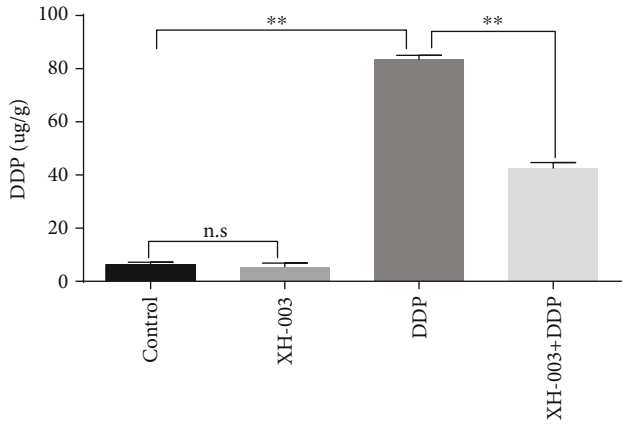

Figure 9: Effect of XH-003 on DDP accumulation in renal tissue.

lead to the formation of the mitochondrial pore and the release of apoptotic proteins, such as cytochrome c; activate the caspase cascade; and induce apoptosis. Moreover, the increase in ROS can activate the endoplasmic reticulum pathway, followed by activation of the caspase-9/3 cascade and induction of apoptosis [41–43]. Our mechanistic study showed that XH-003 could effectively reduce the phosphorylation level of p53 and the expression of apoptosis-related proteins in the mitochondrial pathway, including PUMA, Bax, and cleaved caspase-3.

Oxidative stress in a physiological state can promote the dissociation of Nrf2 and keap-1 and Nrf2 translocation into

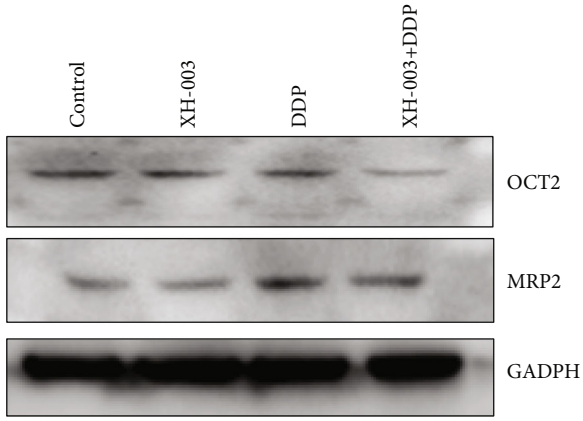

Figure 10: Effects of XH-003 on the expression of proteins related to the uptake and excretion of DDP.

the nucleus. Nrf2 is combined with ARE through MAF and activates the expression of downstream antioxidant genes, promoting the expression of antioxidant proteins, such as HO-1, SOD, and CAT [44, 45]. When DDP enters renal tissue, it induces excessive oxidative stress, which leads to excessive consumption of Nrf2, blocked expression of antioxidant proteins, and reduced antioxidant capacity. In this study, we found that XH-003 prevented DDP-induced inhibition of Nrf2 and HO-1 protein expression. Meanwhile, XH-003 significantly reduced the increase in MDA and alleviated DDP-induced decreases in antioxidant enzyme activities, indicating that XH-003 can effectively improve the DDP-induced oxidative stress in renal tissue.

DDP is easily accumulated, excreted, and metabolized in the kidney. Therefore, reducing the concentration of DDP in the kidney is an important strategy for kidney protection. Interestingly, in this study, XH-003 significantly reduced the DDP concentration in renal tissue, resulting in decreased nephrotoxicity. A previous study has shown that the uptake of DDP by renal tubular cells involves OCT2 and CTR1 and the efflux involves the MRP2 and MRP4 transporters [46]. Our results showed that XH-003 reduced the concentration of DDP in renal tissue by affecting the expression of the DDP transport- and excretion-related proteins OCT2 and MRP2, thereby playing a chemoprotective role.

XH-003 reduced the concentration of DDP in renal tissue, downregulated the expression of apoptosis-related proteins in the mitochondrial pathway, and protected normal renal tissue; however, it is unknown whether it also protected tumor tissue. The XH-003 dose used in this study was not effective in significantly reducing the antitumor effect of DDP and may have had a synergistic effect with DDP. Some antitumor drugs, such as metal drugs and DNA-damaging drugs, whose primary mechanism involves the activation of the apoptosis pathway and induction of cell death through excessive ROS production, can kill tumor cells by increasing ROS levels. However, there are some drugs that cause antitumor effects by removing ROS from tumor cells; these include vitamins C and E, whose primary mechanism targets the

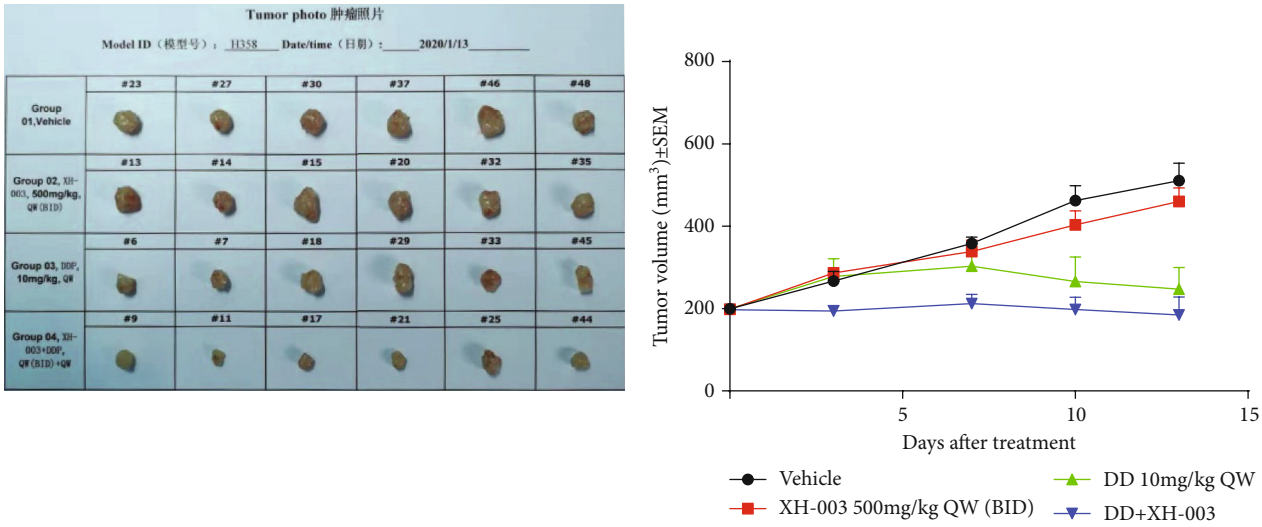

Figure 11: Effect of XH-003 on the antitumor efficacy of DDP.

ability of ROS to promote tumor development via the oxidation of specific chemical groups in cells. This oxidation reaction can induce gene mutations and activate related biochemical pathways, thus promoting cell proliferation and tumorigenic transformation. Therefore, as a powerful antioxidant, XH-003 may also have potential application in the field of antitumor therapy.

Although current studies have shown that XH-003 has a protective effect on DDP-induced nephrotoxicity, there are some limitations. For example, in the experimental condition section, the selection of animal species and feeding conditions in each laboratory have an influence on the dosage of cisplatin, and there is no standardized modeling method, which can lead to variations in results. Although the experiment has been repeated many times, the number of rats in each group is small, which may affect the accuracy. In addition, the renal function index of this experiment evaluated creatinine and urea nitrogen, but did not evaluate other important indexes such as glomerular filtration rate and serum uric acid. Most importantly, the existing animal models can not completely simulate the situation of real clinical patients, so we need to explore new animal models to meet the clinical needs. Therefore, in the follow-up research, it is necessary to improve the experimental conditions and animal model, so that the preclinical research results can better reflect the situation of clinical patients.

5. Conclusion

XH-003 significantly reduced the accumulation of DDP in renal tissue, downregulated the expression of mitochondrial apoptosis pathway-related proteins, and alleviated oxidative stress and inflammation, thus providing renal protection without affecting the antitumor activity of DDP. Based on these results, chemoprotection can be considered an extended indication of XH-003.

Data Availability

The data used to support the findings of this study are included within the article.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Hong-Qi Tian and Ya-Hong Liu conceived and designed the experiments; Ya-Hong Liu and Kui Li carried out the experiments; Ya-Hong Liu analyzed and interpreted the results and prepared the manuscript.

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Supplementary Materials

Table S1: the analysis of creatinine and urea nitrogen in rats (mean ± standard deviation). Table S2: the analysis of creatinine and urea nitrogen in rats treated with different time of XH-003 (mean ± standard deviation). Table S3: comparison of renal protective effects between XH-003 and amifostine (mean ± standard deviation). Table S4: the effect of XH-003 on DDP induced peripheral blood abnormality (mean ± standard deviation). Table S5: effect of XH-003 on biological

indexes of oxidative stress (mean ± standard deviation). Table S6: evaluation the effect of XH-003 on DDP accumulation in renal tissue. *(Supplementary Materials)*

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