Research Paper

Probucol ameliorates renal injury in diabetic nephropathy by inhibiting the expression of the redox enzyme p66Shc

Shikun Yang, Li Zhao, Yachun Han, Yu Liu, Chao Chen, Ming Zhan, Xiaofen Xiong, Xuejing Zhu, Lin Xiao, Chun Hu, Fuyou Liu, Zhiguang Zhou, Yashpal S. Kanwar, Lin Sun

Aims: Probucol is an anti-hyperlipidemic agent and a potent antioxidant drug that can delay progression of diabetic nephropathy (DN) and reverses renal oxidative stress in diabetic animal models; however, the mechanisms underlying these effects remain unclear. p66Shc is a newly recognized mediator of mitochondrial ROS production in renal cells under high-glucose (HG) ambience. We previously showed that p66Shc can serve as a biomarker for renal oxidative injury in DN patients and that p66Shc up-regulation is correlated with renal damage in vivo and in vitro. Here, we determined whether probucol ameliorates renal injury in DN by inhibiting p66Shc expression.

Results: We found that the expression of SIRT1, Ac-H3 and p66Shc in kidneys of DN patients was altered. Also, probucol reduced the levels of serum creatinine, urine protein and LDL-c and attenuated renal oxidative injury and fibrosis in STZ induced diabetic mice. In addition, probucol reversed p-AMPK, SIRT1, Ac-H3 and p66Shc expression. Correlation analyses showed that p66Shc expression was correlated with p-AMPK and Sirt1 expression and severity of renal injury. In vitro pretreatment of HK-2 cells with p-AMPK and SIRT1 siRNA negated the beneficial effects of probucol. Furthermore, we noted that probucol activates p-AMPK and Sirt1 and inhibits p66Shc mRNA transcription by facilitating the binding of Sirt1 to the p66Shc promoter and modulation of Ac-H3 expression in HK-2 cells under HG ambience.

Innovation and conclusion: Our results suggest for the first time that probucol ameliorates renal damage in DN by epigenetically suppressing p66Shc expression via the AMPK-SIRT1-AcH3 pathway.

1. Introduction

Diabetic nephropathy (DN) is the most common complication of diabetes mellitus leading to end-stage renal disease, and it is associated with a high mortality rate [18]. The initial phases of the DN are characterized by glomerular and tubular epithelial hypertrophy, and thickening of glomerular and tubular basement membranes, which is followed by ensuing hyperfiltration, albuminuria, glomerulosclerosis and tubulo-interstitial fibrosis [12,18]. Recently, various investigators have demonstrated that increased reactive oxygen species (ROS) generation seem to be one of the main pathophysiological change induced by high-glucose (HG) ambience [20]. The increased ROS production results in extracellular matrix (ECM) accumulation and another notable pathological event, i.e., apoptosis. Adaptor protein p66Shc is a newly recognized mediator of mitochondrial ROS production [10,18]. Previously, our laboratory reported that the p66Shc is mainly expressed in the renal tubular epithelium, and to a limited extent in the glomerulus. This mediator can be phosphorylated by PKC-β and PKC-δ under HG conditions [34,35], and that results in oxidative damage secondary to mitochondrial ROS overproduction in DN. Other studies have shown that p66Shc mRNA transcription is regulated by NAD-dependent deacetylase sirtuin-1 (Sirt1) via epigenetic Acetyl-Histone H3 (Ac-H3)
chromatin modification [50]. Furthermore, AMP-activated protein kinase (AMPK) acts as regulator of Sirt1 activity [4], which suggests that the AMPK/Sirt1/Ac-H3 signaling pathway plays a critical role in attenuating renal oxidative damage by modulating p66Shc expression in DN.

Probucol is a compound with two characteristic phenolic rings, and it has been used for years as an anti-hyperlipidemic agent in patients with DN [32]. Over the last several years many clinical studies have shown that probucol can reduce urinary protein excretion and prevent DN progression [8]. In addition, there are certain reports in the literature which indicate that probucol acts as a potent oxygen radical scavenger, and thus can effectively prevent oxidative stress-induced tissue damage [16,26]. Along these lines it has been demonstrated that probucol suppresses oxidative stress and attenuates oxidative injury in the renal podocytes of db/db mice by inhibiting NADPH oxidase 2 (Nox2) expression [49]. Conceivably, by virtue of probucol being an antioxidant it has been proposed to inhibit renal tubular epithelial-mesenchymal transition (EMT) in mouse models of DN [6,31]. However, the molecular mechanism(s) underlying this phenomena are not precisely delineated. Interestingly, recent studies have suggested that succinobucol, a metabolically stable derivative of probucol, is a more efficient modulator of mitochondrial homeostasis and mitochondrial ROS production than its parent compound [5]. Additionally, probucol has been shown to suppress human glioma cell proliferation in vitro by inhibiting ROS production and activating AMPK phosphorylation [17].

Given the above findings, it is plausible that the AMPK/Sirt1/p66Shc signaling pathway plays a critical role in DN development. In this study, we noted altered expression of Sirt1, Ac-H3 and p66Shc in renal biopsy tissue samples of DN patients. We analyzed the relationship between the expression of these signaling proteins and patient clinical characteristics. In addition, we assessed the efficacy and novel pharmacological mechanism of probucol in the alleviation of renal injury in STZ induced diabetic mice and demonstrated that probucol attenuates renal injury by inhibiting p66Shc expression via the AMPK/Sirt1 signaling pathway.

2. Results

2.1. Renal p66Shc, Sirt1 and Ac-H3 expression in DN patients

The clinical characteristics of the DN patients and non-diabetic renal disease (NDRD) patients, who served as controls in this study, are shown in Table 1. DN patients exhibited significantly increased blood glucose and hemoglobin A1c (HbA1C) levels, as well as increased 24 h urine protein excretion, compared with control subjects. There were no significant differences between DN patients and control subjects with respect to age, sex. Changes in glomerular and tubulo-interstitial morphology, namely, glomerulosclerosis and focal tubular atrophy and interstitial fibrosis, were illustrated by H & E, PAS, PASM and Masson's trichrome staining of biopsy tissues of DN patients. Mesangial region expansion and tubular atrophy were noted in H & E and PAS staining. PASM and Masson's trichrome staining demonstrated tubular atrophy and interstitial fibrosis in DN patients compared with control subjects (Fig. 1A). Immunohistochemical (IHC) staining demonstrated significantly increased p66Shc and acetylated histone3 (Ac-H3) expression in the glomeruli and renal tubules of DN patients compared with NDRD patients, as well as decreased Sirt1 expression in DN patients compared with NDRD patients (Fig. 1B). Quantitatively, the tubular interstitial damage noted in DN patients was more severe than that noted in NDRD patients (Fig. 1C). P66Shc staining intensity was increased by 50% in DN patients (Fig. 1D), while Sirt1 staining intensity was decreased by ~ 30%. In contrast, Ac-H3 staining was increased by ~ 32% in DN patients (Fig. 1E). Scatter plot analysis demonstrated a positive correlation between p66Shc expression and tubulo-interstitial damage severity and AcH3 expression (Fig. 1F and H) and a negative correlation between p66Shc expression and SIRT1 expression (Fig. 1G).

2.2. Effects of probucol on biochemical parameters, p66Shc mRNA expression and glomerular injury in diabetic mice

To determine the long-term effects of probucol in a diabetic mice model, specific functional characteristics and biochemical indicators were analyzed. As shown in Table 2, probucol administration for 20 weeks had no effect on body weight in STZ-induced diabetic mice compared with DN mice (Table 2 and Fig. 2A). No changes in blood glucose levels were observed (Fig. 2B), while a ~ 7.5-fold increase in proteinuria was observed in STZ mice compared with control mice. This increase was modestly reversed by probucol treatment (1.06 ± 0.21 mg/24 h vs 1.48 ± 0.68 mg/24 h, P < 0.05, Fig. 2C). Likewise, changes in serum creatinine, blood urea nitrogen (BUN), total triglyceride (TG) and total cholesterol (TC) levels were noted to be increased in STZ mice compared with control mice (Table 2). In addition, STZ mice exhibited significantly increased levels of urinary H2O2 and TBARS. These increases were reversed following probucol treatment. No changes were observed in DMSO-treated mice (Fig. 2D and E). Furthermore, renal malondialdehyde (MDA) and LDL-c levels were significantly increased in STZ-induced diabetic mice. These increases were reversed by probucol treatment (Fig. 2F and G). Significantly enhanced p66Shc mRNA expression was observed in the kidneys of STZ-induced mice. This was reversed by probucol treatment (Fig. 2H). Correlation analysis revealed a significantly positive correlation between p66Shc mRNA expression and urinary H2O2 (R = 0.743, p < 0.01) (Fig. 2I) and LDL-c levels (R = 0.733, p < 0.01) (Fig. 2J). Further investigation demonstrated that probucol treatment significantly attenuated these changes in the kidneys of diabetic mice, particularly in the mesangial matrix and glomerulosclerosis, compared to untreated STZ-induced diabetic mice. No significant changes were observed in DMSO-treated mice compared to STZ-induced diabetic mice (Fig. 2K). In addition, probucol treatment significantly decreased the kidney weight/body weight ratio compared to untreated STZ-induced diabetic mice. No changes were observed in DMSO-treated mice (Fig. 2L). Similar findings were noted with respect to glomerulosclerosis, as indicated by glomerular damage scores (Fig. 2M) and glomerular tuft areas (Fig. 2N). Glomerular damage scores were positively correlated with p66Shc mRNA expression (R = 0.6, p < 0.05) (Fig. 2O).

2.3. Effects of probucol on tubulo-interstitial damage and renal oxidative injury in diabetic mice

To determine the effects of probucol on renal tubulo-interstitial...
Fig. 1. p66Shc, Sirt1 and Ac-H3 expression in patients with DN. Panel A: HE, PAS, PASM and Masson’s trichrome staining showed that compared to NDRD patients, which served as controls (Aa–Ad), DN patients exhibited notable glomerular damage, glomerulosclerosis, tubular injury and interstitial fibrosis on kidney biopsy (Ae–Ah). Panel B: Immunohistochemistry (IHC) studies revealed that p66Shc was mainly localized in “hypertrophic renal tubules” and the glomerulus. p66shc expression was increased in DN patients (left upper panel), compared to control subjects (left lower panel). DN patients exhibited very low Sirt1 expression levels compared to control subjects (middle panels). Ac-H3 expression was mainly localized in renal tubular cell nuclei and glomeruli and was significantly increased in DN patients (right upper panel) compared to NDRD patients (right lower panel). The bar graphs are representative of tubulo-interstitial damage scores (Panel C). Panels D and E reflect the average relative staining intensity following p66Shc, Sirt1 and Ac-H3 staining in the kidney biopsy specimens of DN versus NDRD patients. Panels F–H: Scatter plots show the relationship between p66Shc expression and tubulo-interstitial damage (F), SIRT1 expression (G) and Ac-H3 expression (H) in the kidneys of DN patients. *P < 0.01. Values are the mean ± SE (magnification ×400).
Further analysis showed that p66Shc expression was positively correlated with Sirt1 expression (Fig. 4H). To confirm the above results, IHC staining was performed on kidney tissues sections (Fig. 4K). Sirt1 (upper panel) and AcH3 (middle panel) were mainly expressed in the glomeruli and tubular nuclei, and renal Sirt1 expression was notably reduced in STZ-induced diabetic mice compared with control mice (Fig. 4K, b vs a), while Ac-H3 expression was significantly increased in these mice compared to control mice (Fig. 4Kf vs e). p66Shc was mainly expressed in the renal tubules, as well as in the glomerulus although to a limited extent. Renal p66hc expression was also increased in STZ-induced diabetic mice (Fig. 4K lower panels). However, these changes were reversed by probucol treatment.

2.5. Probucol regulates high-glucose-induced p-AMPK, Sirt1, Ac-H3 and p66Shc expression in HK-2 cells

In vitro studies were performed with HK-2 cells, human proximal tubular cells. Significantly decreased time-dependent p-AMPK expression was observed, as determined by Western blotting, in HK-2 cells exposed to 30 mM γ-glucose (HG) for 1–6 h (Fig. 5A upper panel). No changes were observed in total AMPK expression (Fig. 5A middle panel). Pretreatment with probucol (40 µM) reversed p-AMPK down-regulation (Fig. 5B line 3 vs line 2). No differences were noted between cells treated with HG and HG plus DMSO (Fig. 5B line 4 vs line 2). We subsequently investigated the effects of probucol on Sirt1, Ac-H3 and p66Shc protein expression. Western blot analysis showed that probucol treatment reversed the changes in Sirt1, Ac-H3 and p66Shc expression induced by HG treatment in a dose-dependent manner (Fig. 5F–C). Immunofluorescence studies showed that HG decreased Sirt1 expression in HK-2 cells (Fig. 5G upper panels) but significantly increased p66Shc expression. These effects were reversed by probucol treatment. In addition, we also measured p-AMPK concentrations in HK-2 cells treated with probucol. As shown in Fig. 5H, decreased p-AMPK concentrations were observed in HK-2 cells exposed to HG conditions compared to HK-2 cells exposed to LG conditions. These changes were partially reversed by probucol treatment. Similar changes in Sirt1 expression were observed in HK-2 cells subjected to HG and probucol treatment (Fig. 5I).

2.6. Probucol inhibits p66Shc expression through the AMPK/Sirt1 pathway

To determine whether the AMPK-Sirt1 pathway modulates p66Shc expression regulation by probucol, studies with various inhibitors or activators were performed. HK-2 cells were exposed to different concentrations of γ-glucose. They were also subjected to individual concomitant treatments of probucol and Dorsomorphin (selective AMPK inhibitor), 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR, AMPK activator) or EX-527 (selective SIRT1 inhibitor). Treatment with probucol significantly reduced p66Shc mRNA expression in HK-2 cells exposed to HG conditions (Fig. 6A, line 3 vs line 2). This effect was partially blocked by separate Dorsomorphin and Ex527 treatments (line 4, 6 vs line 3). AICAR treatment enhanced the effects of probucol (line 5 vs line 3), but not in the presence of Ex527 (Fig. 6A, line 7 vs line 3). These findings indicate that probucol reduces p66Shc

### Table 2

| Group    | n  | BW (g) | KW (mg) | KW/BW (mg/g) | BS (mmol/l) | Scr (µmol/l) | BUN (µmol/l) | TC (µmol/l) | TG (µmol/l) | Proteinuria (mg/24 h) |
|----------|----|--------|---------|-------------|-------------|-------------|-------------|------------|------------|----------------------|
| Control  | 15 | 47.67 ± 3.72  | 203.73 ± 11.59  | 4.28 ± 0.21   | 5.63 ± 0.98 | 12.68 ± 1.66 | 6.57 ± 0.76 | 5.89 ± 0.79 | 1.92 ± 0.37 | 0.21 ± 0.07         |
| STZ      | 15 | 41.53 ± 3.11*  | 219.20 ± 7.32*  | 5.29 ± 0.27** | 21.33 ± 6.44 | 17.01 ± 2.28* | 9.67 ± 1.74* | 6.13 ± 0.68 | 2.19 ± 0.43 | 1.48 ± 0.68*         |
| STZ + DMSO | 15 | 42.80 ± 2.89*  | 215.07 ± 8.02*  | 5.03 ± 0.23** | 22.53 ± 5.90 | 17.93 ± 1.39* | 9.13 ± 1.19 | 6.17 ± 0.53 | 2.06 ± 0.27 | 1.49 ± 0.39         |
| Proprobol | 15 | 41.13 ± 2.85*  | 204.20 ± 8.14** | 4.98 ± 0.24** | 22.06 ± 7.30 | 14.25 ± 1.81** | 7.47 ± 1.17** | 5.29 ± 0.72* | 1.61 ± 0.19** | 1.06 ± 0.21**        |

Note: BS, blood glucose; BW, body weight; KW, kidney weight of right kidney; Scr, serum creatinine; BUN, blood urea nitrogen; TC, total cholesterol; TG, triglyceride.

*P < 0.01, vs control group; **P < 0.05, vs control group.

**P < 0.01, vs STZ group; ***P < 0.05, vs STZ group.
Fig. 2. Effects of probucol on blood glucose, urine protein, urinary H$_2$O$_2$, and TBARS levels and p66Shc mRNA expression, as well as glomerulosclerosis, in the kidneys of STZ-induced diabetic mice. A: Body weight (g), B: serum glucose levels and C: proteinuria/24 h in mice from 0 to 20 weeks following STZ-mediated diabetes induction. D: Urinary H$_2$O$_2$ levels, E: urinary thiobarbituric acid reactive substances (TBARS), and F: malondialdehyde (MDA) concentrations in the kidney tissues of various groups. G: LDL-c levels. H: Bar graph representation of p66Shc mRNA expression in the kidneys of STZ-induced mice. I-J: Correlation analysis of p66Shc mRNA expression and urinary H$_2$O$_2$ levels (I) and LDL-c levels (R = 0.733, p < 0.01) (J). K: Mouse kidney sections were stained with H & E (upper panels, magnification ×100), and glomerular morphology was evaluated by PAS staining (lower panels, magnification ×400). L: Kidney weight/body weight ratio, M: glomerular damage score, and N: glomerular tuft area. O: Analysis of the association between p66Shc mRNA expression and glomerulosclerosis (R = 0.6, p < 0.05). Data are presented as the mean ± SE, R: correlation coefficient, *P < 0.01 vs. control, #P < 0.01 vs. STZ groups.
A) Masson staining showing collagen fibers in control (a), STZ (b), STZ+DMSO (c), and STZ+Probucol (d) groups. Fibronectin immunostaining in control (e), STZ (f), STZ+DMSO (g), and STZ+Probucol (h) groups.

B) Tubular interstitial damage score and AOD of FN expression were compared across control, STZ, STZ+DMSO, and STZ+Probucol groups.

C) Graph showing p65 mRNA level and Tubular interstitial damage score with a correlation coefficient R=0.749.

D) Graph showing p65 mRNA level and Tubular interstitial damage score with a correlation coefficient R=0.887.

E) DHE staining and TUNEL assay showing relative ROS score across control, STZ, STZ+DMSO, and STZ+Probucol groups.

F) Graph showing p65 mRNA level and TUNEL positive cells with a correlation coefficient R=0.136.
expression through the AMPK/Sirt1 pathway. Similar results were noted regarding cellular lipid peroxidation injury, as determined by malondialdehyde (MDA) measurements (Fig. 6B). Immunofluorescence showed that probucol treatment significantly ameliorated the decreases in p-AMPK and Sirt1 expression elicited by HG conditions (Fig. 6C, upper and middle panels). However, these changes were almost restored to baseline in cells treated with Dorosomorphin or Ex5-527. AICAR treatment enhanced the effects of probucol, but not in the presence of Ex5-527. The results regarding Sirt1 contrasted with those regarding p66Shc expression (Fig. 6C middle panels), which were similar to those regarding p66Shc mRNA expression. In addition, probucol treatment reduced HG-stimulated ROS generation, and this effect was partially blocked partially by treatment with Dorosomorphin or Ex5-527, as shown by DHE staining (Fig. 6C, lower panels). These findings were also confirmed by Western blot analyses (Fig. 6D–F). These data suggest that probucol inhibits p66Shc expression and ROS generation via the AMPK-Sirt1 pathway in HK-2 cells exposed to HG conditions.

2.7. Probuloc negatively regulates p66Shc expression via epigenetic chromatin modification

It has been reported that Sirt1 activation by p-AMPK is dependent upon its binding to the p66Shc promoter region, which decreases Ac-H3 levels and ultimately results in p66Shc gene transcription down-regulation. We examined the effects of probucol on Sirt1 and Ac-H3 expression levels in HK-2 cells that were exposed to HG and treated with probucol or AMPK siRNA. Significantly decreased Sirt1 expression was observed in HK-2 cells exposed to HG conditions or transfected with AMPK siRNA, as determined by Western blotting (Fig. 7A). Sirt1 expression was partially restored by probucol treatment. Contrasting results were observed regarding Ac-H3 protein expression following various treatments (Fig. 7B). We considered the possibility that probucol-mediated p66Shc transcription regulation may occur at the chromatin level because histone deacetylation can inhibit p66Shc gene transcription. To confirm this possibility, we performed a ChIP assay in HK-2 cells under different α-glucose conditions and focused on the 1641-bp region of the p66Shc promoter, which is upstream of the transcription start site. Within this fragment, only one region (−508 bp to −250 bp) from the material immunoprecipitated with the anti-Sirt1 antibody was amplified by real-time PCR (Fig. 7C). We then assessed the interactions among Sirt1, Ac-H3 and the −508-bp to −250-bp region of the p66Shc gene promoter in HK-2 cells. As shown in Fig. 7D, the antibody directed against Sirt1 immunoprecipitated the DNA fragments from HK-2 cells containing potential binding sites for the p66Shc promoter. The binding of Sirt1 to the promoter region was dramatically decreased in HK-2 cells under HG conditions and following AMPK siRNA transfection compared to LG conditions. Binding was partially restored following probucol or AICAR treatment (Fig. 7D). Contrasting results were observed regarding the binding of Ac-H3 to the p66Shc promoter region (Fig. 7E), suggesting that probucol-mediated p66Shc transcription inhibition may involve epigenetic chromatin modification modulated by a Sirt1-AcH3 complex.

3. Discussion

The data of this study indicate that probucol ameliorates renal injury in STZ-induced diabetic mice, and it seems to exert these effects by quelling oxidative stress and apoptosis in diabetic kidneys by repressing p66Shc, an adaptor protein linked to longevity. In addition, the findings suggest that probucol inhibits p66Shc expression via an AMPK-SIRT1-AcH3-dependent pathway. Renal biopsy tissue staining results indicate that renal injury and fibrosis are significantly increased in DN patients (Fig. 1A). Unfortunately, the mechanisms underlying these changes have not been clearly delineated, and no suitable drug that can reverse these changes has been identified. The studies carried out over the last 2 decades indicate that DN is characterized by increased oxidative stress caused by excessive generation of reactive oxygen species (ROS) that ultimately progresses to renal failure [18,20]. Thus, therapies targeting the ROS would conceivably play a key role in attenuating renal injury and delaying the progression of DN.

p66Shc is a member of the adaptor protein family and it has three isoforms encoded by ShcA with relative molecular weights of 46, 52 and 66 kDa. Among them, the p66Shc protein is attracting more attention because it has an additional N-terminal region named CH2, which is responsible for its redox properties, lifespan regulation and apoptosis [11]. Previous studies from our laboratory and of others have shown that p66Shc plays a critical role in renal oxidative injury in DN [10,34,35]. Incidentally, HG and angiotensin II (Ang II) could activate PKC-ζ and PKC-δ, then phosphorylate p66Shc which translocates into mitochondria and increase ROS generation. So P66Shc could serve as a mediator of mitochondrial oxidative stress and apoptosis in the proximal tubule [35]. In this regard, the increased p66Shc mRNA expression has been linked to renal oxidative damage in DN patients [42]. Another molecule that can modulate the biology of p66Shc is Sirt1, an enzyme belonging to the family of sirtuins. They are highly conserved NAD+-dependent deacetylases that regulate cellular energy and lifespan in mammals through histone H3 deacetylation [44], and thereby modulate the promoter activities of its target genes [33]. Sirt1 over-expression can inhibit HG-induced p66Shc expression through Ac-H3, which decreases oxidative stress and improves endothelial functions [50]. These data would indicate that Sirt1-mediated p66Shc expression regulation via Ac-H3 may be related to oxidative injury in DN. In light of these findings, we investigated the expression of p66Shc, Sirt1 and Ac-H3 in renal biopsy tissue specimens of patients with DN. We noted significantly decreased Sirt1 expression while there was an increased expression of p66Shc and Ac-H3 (Fig. 1B). Furthermore, we noted a correlation between p66Shc expression and tubulo-interstitial damage, as well as a correlation between Sirt1 and Ac-H3 expression (Fig. 1F–H), suggesting that Sirt1/Ac-H3 modulates p66Shc expression, which most likely would result in renal oxidative injury in DN. To verify whether targeting p66Shc attenuates renal oxidative damage in DN and to determine whether the Sirt1/Ac-H3 pathway is involved in this process, probucol, an antioxidant, was administered to diabetic mice and its effects in in vitro cell culture studies were also assessed.

Among various strains of mice, C57BL/6 mice are the most widely used for hyperglycemia-induced renal injury. The glomerular injury and tubulo-interstitial fibrosis can be induced by daily intraperitoneal injection of low-dose STZ or by high-fat and high-cholesterol diet administration [3,27]. Probuloc treatment may exert therapeutic effects by inhibiting oxidative injury in STZ-induced diabetic rats [24]. Likewise, we observed that after hyperglycemia induction via STZ injection, the mice exhibited high glucose levels, increased proteinuria (Fig. 2B and C), and enhanced expression of oxidative stress markers, such as, urinary H_{2}O_{2}, TRARS, serum MDA and LDL-c (Fig. 2D–G). Additionally, p66Shc mRNA expression was also increased in the kidneys of diabetic mice (Fig. 3H). We also observed a notable increase in renal fibrosis in
STZ-induced diabetic mice (Fig. 3A). However, all of these changes were reversed by probucol treatment. Furthermore, analysis showed that p66Shc mRNA expression correlated with H2O2 and LDL-c levels (Fig. 2I and J) and is altered expression was associated with the extent of glomerular damage (Fig. 2O). We therefore surmised that probucol reduces oxidative damage by inhibiting p66Shc expression. Our results also support the findings of previous studies, which suggest that probucol is a cholesterol-lowering drug with antioxidant properties that

![Image of renal Sirt1, Ac-H3, p66Shc and p-AMPK expression in STZ-induced diabetic mice following probucol treatment. Panel A-B: Western blot analysis of p-AMPK and total AMPK (A), Sirt1 (B, upper panel), Ac-H3 & Total H3 (B, middle panel) and p66Shc (B, bottom panel) protein expression in the kidneys of various groups. Panel C: Densitometric analyses of the Western blotting results. C-F: Each bar graph represents the ratios of p-AMPK to total AMPK (C), Sirt1 to β-actin (D), Ac-H3 to H3 (E) and p66Shc-to-β-actin (F). G-H: The relative associations among pAMPK, Sirt1 and Ac-H3 protein expression: R (p-AMPK/Ac-H3) = -0.811, R(Ac-H3/Sirt1) = -0.895, and R (p-AMPK/Sirt1) = 0.909 (G) and R (Ac-H3/p66Shc) = 0.789, R (Ac-H3/Sirt1) = -0.895, and R (Sirt1/p66Shc) = -0.726 (p < 0.01) (H). I-J: Bar graphs representing the levels of p-AMPK (I) and Sirt1 activity (%) in the renal cortices of mice in various groups (J). K: Immunohistochemical staining of renal tissues with anti-Sirt1 antibody (upper panel, a–d), anti-AcH3 antibody (middle panel, e–h) and anti-p66Shc antibody (lower panel, i–l). Values are the mean ± SEM (n = 15). *P < 0.01 vs. Control; R: correlation coefficient, #P < 0.01 vs. STZ groups. (magnification ×200).
Fig. 5. p-AMPK, SIRT1, AcH3 and p66Shc expression in HK-2 cells exposed to High glucose (HG) conditions and probucol treatment. Panel A–C: Representative Western blots and densitometric analyses of p-AMPK, Sirt1, Ac-H3 and p66Shc expression in HK-2 cells. High glucose (HG) reduced p-AMPK expression in HK-2 cells in a time-dependent manner (A). This effect was reversed by probucol treatment (B). HG notably decreased Sirt1 expression and increased the ratio of Ac-H3/H3 and p66Shc expression. These changes were reversed following probucol treatment (C). Densitometric analyses represent four independent experiments and depict the ratios of p-AMPK/total AMPK (A and B), SIRT1/β-actin (D), p66Shc/β-actin (F) and AcH3/H3 (E). G: Immunofluorescence shows decreased Sirt1 expression in HK-2 cells exposed to HG compared to that in LG. This effect was partially blocked by probucol treatment (upper panel). Contrasting results were observed regarding p66Shc expression (middle panel). No changes were observed in DMSO-treated cells. H: ELISA assess showed that the p-AMPK concentrations in HK-2 cells in various groups. I: Sirt1 activity in HK-2 cells. Values are the mean ± SEM (n = 5). *P < 0.01 vs. LG; #P < 0.05 vs. HG; & P < 0.01 vs. HG.
Fig. 6. Effects of AICAR, Dorsomorphin and EX-527 on p66Shc mRNA expression and MDA levels under HG ambience in HK-2 cells treated with probucol. A: Real-time PCR showing p66Shc mRNA expression in HK-2 cells under HG conditions treated with or without probucol and AICAR, Dorsomorphin or EX-527. B: MDA content in the medium of HK-2 cells. Values are the mean ± SE (n = 4), *P < 0.01 vs. LG; #P < 0.01 vs. HG; $P < 0.01 vs. HG + Probucol; @ P < 0.01 vs. HG + Probucol + Ex-527. C: Cellular immunofluorescence with anti-p-AMPK, anti-Sirt1 and anti-p66Shc antibodies. High levels of p-AMPK were observed under LG conditions and were accompanied by Sirt1 expression. HG reduced this fluorescence. This effect was reversed by probucol treatment and Dorsomorphin and EX-527 treatment but was increased by AICAR treatment. Contrasting results were observed regarding p66Shc and DHE staining (lower panel). D–F: Representative Western blots (D) and densitometric analysis of Sirt1 (E) and p66Shc protein expression (F) in HK-2 cells exposed to HG conditions with or without treatment with various reagents. Quantitative analyses of the Western blotting results are included, and the data were normalized to β-actin and are presented as the mean ± SEM (n = 4). *P < 0.01 versus LG; #P < 0.01 vs. HG; $P < 0.01 vs. HG + Probucol; @ P < 0.01 vs. HG + Probucol + Ex-527.
protect against free radical-induced damage in a wide variety of scenarios, including doxorubicin-induced cardiotoxicity [7], HG-induced podocyte injury [49] and tubular epithelial cell injury [6]. The mechanism by which probucol protects against oxidative injury may involve increase in antioxidant gene mRNA expression and decrease in apoptosis via improving mitochondrial oxidative phosphorylation and energy production [1]. Previously studies has showed that the podocyte injury such as cytoskeletal rearrangement and podocyte effacement plays a key role in development of proteinuria of DN [29]. Probucol can reduce ROS production and attenuated podocyte injury in db/db mice [49], which may relative to its effect on inhibiting Ang II receptor expression [23]. Here, we demonstrate that probucol is not only exerts its beneficial anti-diabetic effect by regulating lipid metabolism but also can eliminating oxygen radicals in the kidneys of STZ-induced diabetic mice by p66Shc, a novel pathway, and thus the p66Shc may play a role in these processes as the molecular targets of probucol.

It is known that the tubulo-interstitial fibrosis can occur in DN and it may occur independent of glomerular injury [37,52]. p66Shc is mainly expressed in the proximal tubules, and its up-regulation correlates with tubular damage in biopsy specimens of DN patients, as described in our previous publications [42]. Thus, we investigated the effects of probucol on tubulo-interstitial injury in STZ-induced diabetic mice. We observed a notably reduced tubular damage and interstitial fibrosis, as well as decreased oxidative damage and apoptosis, in STZ-induced diabetic mice treated with probucol (Fig. 3A–C). Correlation analyses showed that p66Shc mRNA expression was associated with tubulo-interstitial damage scores, oxidative stress and apoptosis (Fig. 3D). In addition, probucol treatment in STZ-induced diabetic mice also

Fig. 7. The effects of Probucol on Sirt1/Ac-H3 binding to the p66Shc promoter in HK-2 cells. Panels A–B: The HK-2 cells were pretreated with probucol, AMPK siRNA and Scramble siRNA, respectively, and then exposed to HG for 24 h. Sirt1 and Ac-H3 protein expression were analyzed by Western blot. C: The bar graphs represent the immunoprecipitated genomic DNA amplified by real-time PCR using different primers corresponding to specific areas of the p66Shc promoter region. D–E: Chromatin immunoprecipitation (ChIP) was carried out in HK-2 cells using normal rabbit IgG antibodies, anti-Sirt1 antibodies or anti-Ac-H3 antibodies, as indicated. Precipitated DNA was analyzed by amplifying the PCR product spanning the regions of potential Sirt1/Ac-H3 binding sites in the p66Shc promoter region (−508 bp to −250 bp). IgG served as a negative control. Data were normalized by total input DNA, SIRT1/input (D, bottom graph) and AcH3/input (E, bottom graph). Values are the mean ± SEM, n = 4. *P < 0.01 vs. LG; #p < 0.01 vs. HG; $p < 0.01 vs. HG + Probucol. @ p < 0.01 vs. HG + AMPK siRNA.
attenuated renal oxidative stress and apoptosis, and these changes correlated with p66Shc mRNA expression (Fig. 3E–I). These findings indicated that p66Shc over-expression causes renal oxidative stress, which plays a key role in DN progression, and also indicated that the probucol exerts an anti-oxidative and anti-apoptotic effect on the kidneys of diabetic mice that may be facilitated by p66Shc inhibition.

We subsequently attempted to elucidate the mechanisms by which p66Shc transcription is regulated in the kidneys of diabetic mice following probucol treatment. Abnormal Sirt1 and Ac-H3 expression has been noted in patients with DN, and the expression of these proteins has been correlated with p66Shc up-regulation (Fig. 1). It has been demonstrated that HG-induced oxidative stress and apoptosis are associated with AMPK/SIRT1 signaling. AICAR, an AMPK activator, activates Sirt1 and prevents oxidative stress and apoptosis in cultured mesangial cells exposed to HG conditions [21,47]. In addition, AMPK phosphorylation is decreased in STZ-induced diabetic [13] and db/db mice [15], as well as in cultured mesangial cells exposed to HG ambience [14]. Furthermore, Wu et al. found that probucol treatment prevents nonalcoholic steatohepatitis development by up-regulating p-AMPK expression [40], which may be facilitated by probucol-enhanced AMPK-Thr172 phosphorylation though hepatic kinase B1 (LKB1) phosphorylation at Ser428 [30].

Based on these findings, we hypothesized that the probucol protects against renal oxidative damage by inhibiting p66Shc expression though the AMPK/Sirt1/Ac-H3 signaling pathway. To confirm this hypothesis, we investigated the relevance of two important metabolic sensors and stress regulators in the kidneys of STZ-induced diabetic mice. As shown in Fig. 4, probucol administration restored p-AMPK expression and activity, as well as of Sirt1 expression and activity. In addition, Ac-H3 expression, which was notably increased in STZ-induced diabetic mice, was decreased by probucol treatment (Fig. 4A–F). Further analysis showed that p66shc mRNA expression was negatively correlated with p-AMPK and Sirt1 expression and positively correlated with Ac-H3 expression (Fig. 4G and H). Similar results were noted regarding p-AMPK and Sirt1 activity. These results were also supported by both IHC and Western blot analysis with respect to protein expression. These findings indicate that probucol-mediated p66Shc mRNA suppression most likely occurs via the AMPK/Sirt1 pathway.

To confirm these findings, we performed in vitro studies using HK-2 cells, a human proximal tubular cell line. Increased apoptosis has been observed in the proximal and distal tubular epithelial cells of patients with diabetes [25]. Also, apoptosis is seen in the proximal tubular epithelial cells of kidney tissues exposed to HG conditions [35]. In addition, probucol has been shown to attenuate methylguanidineduced HK-cell apoptosis [39]. In this study, we observed that HG treatment significantly decreased p-AMPK expression and activity which was partially ameliorated by probucol treatment. Similar results were observed for Sirt1 expression. Furthermore, probucol treatment of HK-2 cells reversed HG-mediated Ac-H3 up-regulation (Fig. 5). Additional studies were performed using Dorsomorphin, a cell-permeable and reversible ATP-competitive AMPK inhibitor, and also Ex527, a Sirt1 inhibitor. Both the agents reversed the effects of probucol on p66Shc expression. Concomitant treatment with probucol and AICAR, an activator of AMPK, augmented the above-mentioned decreases in p66Shc mRNA expression in HK-2 cells exposed to HG ambience. Sirt1 and p66Shc exhibited contrasting changes in their protein expression patterns, and also MDA and ROS revealed contrasting changes in their levels as well (Fig. 6). These findings suggest that probucol inhibits p66Shc expression and thus modulates renal tubular oxidative injury in diabetes via the AMPK-SIRT signaling pathway.

The next question that we addressed in this study was the epigenetic regulation of p66Shc. The p66Shc increase has been described to be associated with increased histone acetylation [45], which is necessary for p66Shc transcription [22]. Promoter analysis showed that the highest transcription levels in the p66Shc promoter were localized to a region between –434 and –101 bp upstream of ATG upon transcription of cells lacking endogenous p66Shc [38]. Another interesting point described in the literature is that epigenetic p66Shc gene expression regulation is modulated by histone modifications that are dependent on Sirt1 binding to the p66Shc promoter, and as a result there is an aberrant acetyl-HistoneH3 (Lys9) expression [45]. In addition, it has been shown that SIRT1 affects the p66Shc promoter in hyperglycemia-induced endothelial dysfunction through histone H3 modification [50]. Recent studies by Bock et al. demonstrated that the coagulation pro-tease activated protein C (aPC) exerts cyto-protective effects in DN. The mechanism by which it reverses glucose-induced hyperacetylation of the p66Shc promoter in podocytes may involve the suppression of HG-induced p66Shc expression and the reduction of glucose-induced mitochondrial p66Shc translocation, as well as mitochondrial ROS production [2]. We thus explored the epigenetic factors which may be involved in the regulation of p66Shc expression in HK-2 cells treated with probucol. As shown in Fig. 7A and B, significantly decreased Sirt1 expression was observed in HK-2 cells treated with HG. Sirt1 expression was restored by pretreatment with probucol but was partially blocked by AMPK siRNA co-transfection. Contrasting results were noted regarding Ac-H3 expression, as determined by Western blot analysis. Furthermore, to delineate the Sirt1 binding region in the p66Shc gene promoter, ChIP assay was performed in HK-2 cells after nuclear chro-matin immunoprecipitation with normal rabbit IgG or antibodies against Sirt1. Within the p66Shc gene promoter (−1641 to + 45 bp), only one region (−508 to −250 bp) could be amplified by Chip-PCR. These results are consistent with the observations made by Zhou et al. [50], indicating that Sirt1 affects the p66Shc promoter through Ac-H3 modification in HK-2 cells and that the effects of probucol may be modulated by inhibiting p66Shc transcription under HG conditions. It has been demonstrated that H3 acetylation within the p66Shc promoter is induced by glucose [2]. Similar results were observed in cells treated with AICAR, but not following AMPK siRNA treatment. In contrast, Ac-H3 binding was dramatically increased under HG ambience and was reduced by probucol treatment (Fig. 7), indicating that probucol regulates p66shc transcription by facilitating Sirt1 activity and modulation of Ac-H3 acetylation.

Our study showed that there was significantly positive correlation between p66Shc expression and renal tubulo-interstitial damage in DN patients. Probucol administration could effectively alleviate renal tubular oxidative injury by inhibiting p66Shc expression, accompany with enhanced the expression of p-AMPK and Sirt1, and decreased Ac-H3 expression both in vivo and in vitro. Further analysis showed that p66shc expression was negatively correlated with p-AMPK and Sirt1 expression while positively correlated with Ac-H3 expression. Furthermore, both AMPK inhibitor and Sirt1 inhibitor could reverse the anti-oxidative injury effects of probucol and the regulation of p66Shc expression, while AMPK activator could enhance the reduction of p66Shc and Ach3 expression. Moreover, ChIP analysis showed that probucol could inhibit p66Shc transcription by reducing Ac-H3 binding to p66Shc promoter (−508 to −250 bp) via facilitating Sirt1 activity and modulating of Ac-H3 acetylation. Take together, these findings indicated that probucol suppressed HG-induced tubular oxidative injury, fibrosis and apoptosis by inhibiting p66Shc expression via activating the AMPK-SIRT1-Ach3 pathway (shown in Fig. 8).

In summary, we demonstrated that probucol ameliorates oxidative stress in kidneys of STZ-induced diabetic mice and it suppresses HG-induced tubular oxidative injury by inhibiting p66Shc expression, and it seems that these effects are mediated by activating the AMPK/Sirt1 pathway. Because probucol has other effects, such as, alleviation of renal vasoconstriction [19] and reduction of inflammatory responses [28], additional studies are needed to determine if probucol also exerts such beneficial therapeutic in the amelioration of DN.

4. Innovation

Probucol ameliorates renal damage in DN by epigenetically...
suppressing p66Shc expression via the AMPK-SIRT1-AcH3 pathway.

5. Materials and methods

5.1. Cell lines and reagents

Human proximal tubular epithelial cells (HK-2) were purchased from ATCC (USA). The mouse monoclonal IgG antibody directed against Shc/p66 was purchased from LifeSpan BioSciences. This antibody reacts with p66Shc protein, but not with p46/p52 isoforms. Polyclonal anti-SIRT1 antibody, rabbit recombinant monoclonal AMPK alpha 1 antibody (Y365), anti-histone H3 antibody (ab61251), and anti-fibronectin antibody (ab2413) were purchased from Abcam (UK). Anti-Histone H3 acetylated rabbit polyclonal peptide antibody was purchased from EMD Millipore Corp (USA). Probucol was purchased from Sigma-Aldrich (USA). Cell-permeable activator of AMP-activated protein kinase (AICAR), the selective and reversible AMP-kinase inhibitor Dorsomorphin and the selective SIRT1 inhibitor EX-527 were purchased from Abcam (UK). Secondary antibodies used for immunoblot analyses were obtained from ZSGB-BIO (Beijing, China). Other secondary antibodies used for immunofluorescence microscopy were purchased from Jackson Immuno Research (USA). 4′,6-diamidino-2-phenylindole (DAPI) and dihydroethidium (DHE) were obtained from Sigma Aldrich (USA). A DAB kit was purchased from CWBIO (Beijing, China). Other materials, including an In situ BrdU-Red DNA Fragmentation (TUNEL) Assay Kit (ab66110) and anti-β-actin antibody (ab8226), were purchased from Abcam (UK). A CycLex® AMPK Kinase Assay Kit and low-glucose DMEM medium, bovine serum albumin (BSA) and trypsin were obtained from GIBCO (USA).

5.2. Kidney morphological analysis

Human kidney biopsy tissues were obtained from patients with a 10–15-years history of class III DN (n = 15). These patients were confirmed to have pathological and clinical findings consistent with DN (including both type 1 and type 2 diabetic nephropathy), and an equal number of patients of non-diabetic renal disease (NDRD, n = 15) with minimal change nephropathy as control were recruited for this study. Renal biopsy tissue sections were subjected to hematoxylin-eosin (H & E), periodic acid-Schiff (PAS), periodic acid-silver methenamine (PASM) and Masson’s trichrome staining. Glomerular damage and tubulo-interstitial lesion indices were assessed using a semi-quantitative scoring system, as described previously [46].

5.3. Animal experimental design

C57BL/6 mice (~ 20 g B.W) were purchased from Better Biotechnology Co., Ltd, Nanjing, China, and provided by the Second Xiangya Hospital Animal Center. The animals were housed at 22 °C under a 12 h light-dark cycle, and they were allowed free access to food and water. A total of 60 adult C57BL/6 mice aged 8 weeks were used, and they were divided into 4 groups of 15 animals. The first group was injected with sodium citrate buffer (25 mM/L pH 4.0) only and served as a control group. The second group was injected intraperitoneally (i.p.) with 40 mg/kg body wt. streptozotocin (STZ, Sigma-Aldrich, St. Louis, MO). The solution was freshly made in sodium citrate buffer and injected for 5 consecutive days. Tail vein blood glucose levels were tested 1 week after the last STZ injection, and mice with glucose levels > 250 mg/dl were considered diabetic. The third group of STZ-induced diabetic mice received DMSO (10 ml/kg). The fourth group of STZ-induced diabetic mice received probucol every other day for 20 weeks. For probucol-treated mice, a probucol dose of 10 mg/kg body wt IP was calculated based on average daily liquid ingestion, as described previously [51]. All animals were fed a high-fat high-cholesterol Western diet (WD,TD88137) from Harlan-Teklad (Madison, WI) after the onset of diabetes, and they were sacrificed at 20 weeks following STZ administration. Their sera and kidneys were saved for various biochemical and morphological studies. All animal experiments were performed in accordance with the guidelines for animal care and use established by the National Institutes of Health. The study was approved by the Institutional Animal Care and Use Committee of the Second Xiangya Hospital, Central South University.
performed in accordance with the regulations established by the Institutional Committee for Care & Use of Laboratory Animals at Central South University, China.

5.4. Morphological studies

Kidney tissues were fixed with 4% buffered paraformaldehyde and embedded in paraffin, and 4 μm-thick sections were prepared. The sections were then subjected to hematoxylin-eosin (H & E), Periodic acid-Schiff (PAS) and Masson's trichrome staining. A semi-quantitative scoring system was used to evaluate tubulo-interstitial injury severity, as previously described [36].

5.5. Serum biochemistry

Blood glucose levels were measured using a blood glucose monitor (Boehringer Mannheim, Germany). Serum triglyceride and cholesterol levels were measured by an automated biochemical analyzer (OLYMPUS AU100, Japan).

5.6. Urinary and blood chemistry

Mices were placed in individual metabolic cages for 24 h urine collection. Urine samples were centrifuged at 2000g for 5 min, and the supernatants were saved. Urine albumin was measured by enzyme-linked immunosorbent assay (ELISA; Bethyl Laboratories, USA) [36]. Urine creatinine (Cr) concentrations were determined using an ELISA kit from Exocell (Philadelphia, USA). Urinary thio-barbituric acid reactive substances (TBARS) and H2O2 levels were measured using kits from Thermo Fisher Scientific (Rockford, USA) and Bioassay Systems (Hayward, USA), respectively.

5.7. Kidney AMPK activity

Activated AMPKα1 levels in renal cortical tissues were measured using an enzyme-linked immunosorbent assay (ELISA) kit (R & D systems, USA & D systems, UR & D systems, USA), according to manufacturer's instructions [48].

5.8. SIRT1 deacetylase activity assay

Renal tissue and HK-2 cell Sirt1 deacetylase activity was determined using the Sirt1 deacetylase activity assay kit (Genmed Sciences Inc., USA), according to the manufacturer's instructions [43]. Activity was expressed as a percentage of the control.

5.9. Renal tissue immunohistochemistry (IHC) and apoptosis assay

Human and mouse kidney tissue samples were used for IHC analysis. Tissue Section (3 μm) thick were prepared, mounted on glass slides, de-paraffinized, rehydrated and blocked with 3% H2O2 solution. Following antigen retrieval in a microwave oven, the sections were then incubated with antibodies against p66Shc (1:50 dilution), SIRT1 (1:100 dilution), β-actin (mouse) (sense, 5′- AGACAGCAGCTTCAGCTACGCTTCTGGGTATGGAATC-3′ and antisense, 5′- AGAAGTCGAAAGGGTCAAGCCTAGAG-3′), and antisense, 5′-GATGCTCCAGCTACAFCT CA-3′) and β-actin (mouse) (sense, 5′- CCACTGTTGTTGATGGAATC-3′, and antisense, 5′- AGCAGTGGTGGCCA TAGAGT-3′). The cycling conditions were as follows: 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min.

5.11. Western blotting procedure

Western blot analyses were carried out as described in our previous publications [9]. Briefly, samples (50 μg protein) were subjected to SDS-PAGE. After the proteins were transferred onto a nitrocellulose membrane (Amersham, USA), the blots were probed with the following antibodies: mouse monoclonal IgG anti-Shc/p66 (p66: 1:1000, it reacts with the N-terminus of p66Shc protein and not with the p46/p52 isoforms), anti-SIRT1 (1:1000), anti-AcH3 (1:1000), anti-H3 (1:1000), anti-AMPK (1:500), anti-p-AMPK (1:1000) and anti-β-actin (1:2000). The membrane blots were then immersed in solutions containing secondary antibodies. Autoradiograms were prepared using an ECL kit (Amersham, USA), in accordance with the manufacturer’s protocol.

5.12. Cell culture and treatment

Human proximal tubular epithelial cells (HK-2) were purchased from ATCC and cultured in Dulbecco's modified Eagle medium (DMEM)-F12 medium supplemented with 10% fetal bovine serum (FBS). After the cells achieved 80% confluence, the medium was changed to FBS-free DMEM, and the culture was maintained in an atmosphere of 5% CO2/95% air at 37 °C. Four sets of experiments involving the following cells were performed: cells maintained in 5 mM o-glucose (LG), cells maintained in 30 mM o-glucose (HG), cells treated with HG plus 20–80 μM probucol, and cells maintained in LG medium and DMSO. Dorosomorphin, AICAR and EX-527 were added individually to the culture medium at concentrations of 20 μM, 1 mM and 1 μM, respectively, before HG treatment at different time points.

5.13. Cells immunofluorescence

Cells were grown on coverslips, washed three times with PBS, fixed in 4% paraformaldehyde for 20 min, permeabilized with 0.1% Triton X-100 and then incubated in blocking buffer. They were subsequently incubated in primary antibody solution overnight. Then, the cells were incubated with FITC or Rhodamine-conjugated secondary antibodies and examined with a Zeiss fluorescence microscope equipped with a UV epi-illumination [36].

5.14. Cellular AMPK measurement

HK-2 cells were subjected to various treatments, as indicated. Cell lysates were prepared and centrifuged, and the supernatants were collected for AMPK ELISA. AMPK concentrations were measured using an ELISA kit, according to the manufacturer's instructions [48].

5.15. Intracellular ROS and malondialdehyde (MDA) measurements

Intracellular ROS generation was assessed by dihydroethidium (DHE) staining, as described previously [41]. Cellular lipid peroxidation injury was assessed by measuring malondialdehyde (MDA) content
were amplified by F1, F2, F3, F4 and F5 primer pairs. The following PCR primer sequences were used: F1 sense, 5′-GGTTCAGGAGTTCTCCTTGGC-3′; and antisense, 5′-CACACCTCTTGAGTGAGCTCCTGTTG-3′; F2 sense, 5′-TCTTATCCCTTTCGTCGCT-3′; and antisense, 5′-AGAGAGAAGGAGATAGGAGC-3′; F3 sense, 5′-TTTCTAGTATGGGATGAGCTTGGTG-3′; and antisense, 5′-ACGGAAAGGAGAGATTGGGGAGGA-3′; F4 sense, 5′-GTGCTGATTGTGTGGCTG-3′; and antisense, 5′-GTACCAAGGTTCTGTGACTCCTG-3′. The PCR products were visualized on a 1.5% agarose gel. Ct values were calculated using an internal control (β-actin) to normalize gene expression levels. The relative mRNA expression levels were calculated using the 2−ΔΔCt method.

5.17. Statistical analysis

Statistical analyses were carried out using SPSS 16.0 software. The experimental results are expressed as the mean ± SD, and one-way analysis of variance (ANOVA) was carried out. Correlation analyses were carried out using Spearman's correlation coefficient. p < 0.05 was considered statistically significant.

Author disclosure statement

Authors have nothing to disclose.

Acknowledgments

This work was supported by grants from the Creative Research Group Fund of the National Foundation Committee of Natural Sciences of China (81470960, 81500558, 81270812, 81300600 and 81370832), Free Explore Plan of Central South University (2012QNZT146), and a grant from the NIH, USA (DK60635). Strategy-oriented Special Project of Central South University (ZLXD2016003).

References

[1] Y.A. Asiri, Probucol attenuates cyclophosphamide-induced oxidative apoptosis, p53 and Bax signal expression in rat cardiac tissues, Oxid. Med. Cell. Longev. 3 (2010) 308–316.
[2] S. Bock, K. Shahzad, H. Wang, S. Stoyanov, J. Welter, W. Dong, P.G. Pellicci, M. Kashi, S. Ranjan, S. Schmidt, R. Ritzel, V. Schwenger, K.G. Reymann, C.T. Esumon, T. Madhusudhan, P.P. Nawroth, B. Isermann, Activated protein C arrests progressive mesangioproliferative glomerulonephritis in the rat, J. Am. Soc. Nephrol. 16 (2005) 201–207.
[3] M.D. Breyer, E. Bottiger, F.C. Brosius, T.M. Coffman, R.C. Harris, W.C. Heilig, K. Sharma, Amdc. mouse models of diabetic nephropathy, J. Am. Soc. Nephrol. 16 (2005) 27–45.
[4] C. Canto, Z. Gerhart-Hines, J.N. Feige, M. Lagouge, L. Noriega, J.C. Milne, P.J. Elliott, P. Puigserver, J. Auwerx, AMPK regulates energy expenditure by modulating NAD+ metabolism and SIRT1 activity, Nature 458 (2009) 1056–1066.
[5] D. Colle, D.B. Santos, J.M. Hartwig, M. Godoi, A.L. Braga, M. Farina, Succinobucol versus probucol: higher efficiency of succinobucol in mitigating 3-NP-induced brain mitochondrial dysfunction and oxidative stress in vitro, Mitochondron 13 (2013) 125–135.
[6] S.B. Duan, G.L. Liu, Y.H. Wang, J.J. Zhang, Epithelial-to-mesenchymal transdifferentiation of renal tubular epithelial cell mediated by oxidative stress and intervention effect of probucol in diabetic nephropathy rats, Ren. Fail. 34 (2012) 1244–1251.
[7] E. El-Demerdash, A.A. Ali, M.M. Sayed-Ahmed, A.M. Osman, New aspects in probucol cardioprotection against doxorubicin-induced cardiotoxicity, Cancer Chemother. Pharmacol. 52 (2003) 411–416.
[8] K. Endo, Y. Miyashita, H. Sasaki, M. Ohiya, A. Saiki, N. Koide, M. Otsuka, T. Oyama, M. Takeyoshi, Y. Ito, K. Shirai, Probucol delays progress of diabetic nephropathy, Diabetes Res. Clin. Pract. 71 (2006) 156–163.
[9] K. Endo, A. Saiki, T. Yamaguchi, K. Sakuma, H. Sasaki, N. Ban, H. Kawana, D. Nagaya, A. Nagumo, M. Ohiya, T. Oyama, T. Murano, Y. Miyashita, S. Yamamura, Y. Suzuki, K. Shirai, T. Tanigawa, Probucol suppresses initiation of chronic hemodialysis therapy and renal dysfunction related death in diabetic nephropathy patients: sakura study, J. Atheroscler. Thromb. 20. (2013) 494–502.
[10] E.R. Galimov, The role of p66shc in oxidative stress and apoptosis, Acta Nat. 2 (2011) 44–51.
[11] M. Giorgio, E. Migliaccio, F. Orsini, D. Paolucci, M. Moreni, C. Contursi, G. Pelliccia, L. Luzi, S. Minucci, M. Marcaccio, P. Pinton, R. Rizzuto, P. Bernardi, F. Paolucci, P.G. Pellicci, Electron transfer between cytochrome c and p66shc generates reactive oxygen species that trigger mitochondrial apoptosis, Cell 122 (2005) 221–233.
[12] L. Grundi, S.M. Thomas, G. Viberti, Mechanical forces in diabetic kidney disease: a trigger for impaired glucose metabolism, J. Am. Soc. Nephrol. 18 (2007) 26–223.
[13] Y. Guo, W. Yu, D. Sun, J. Wang, C. Li, R. Zhang, S.A. Babaee, Y. Li, L. Mu, M. Ma, M. Shen, C. Zeng, N. Li, W. He, Q. Zou, Y. Zhang, H. Wang, A novel protective mechanism for mitochondrial aldehyde dehydrogenase (ALDH2) in type II diabetes-induced cardiac dysfunction: role of AMPK-regulated autophagy, Biochim. Biophys. Acta 1852 (2015) 319–331.
[14] Y.N. Guo, J.C. Wang, G.Y. Cai, X. Hu, S.Y. Cui, L. Y, Z. Yin, B. Fu, Q. Hong, X.M. Chen, AMPK-mediated downregulation of connexin43 and premature senescence of mesangial cells under high-glucose conditions, Exp. Gerontol. 51 (2014) 71–81.
[15] Y.A. Hong, J.H. Lim, M.Y. Kim, T.W. Kim, Y. Kim, K.S. Yang, H.S. Park, S.R. Choi, S. Chang, H.W. Kim, H.W. Kim, B.S. Choi, Y.S. Chang, C.W. Park, Fenofibrate improves renal lipotoxicity through activation of AMPK-PGC-1alpha in db/db mice, PLoS One 9 (2014) e96147.
[16] M. Ishihara, S.D. Sharma, S. Okada, Probucol as a potent inhibitor of oxygen radical-induced lipid peroxidation and DNA damage: in vitro studies, Redox Rep. 9 (2004) 163–172.
[17] Y.S. Jiang, J.A. Lei, F. Feng, Q.M. Liang, F.R. Wang, Probucol suppresses human glioma cell proliferation in vitro by ROS production and LKB1-AMPK activation, Acta Pharmacol. Sin. 35 (2014) 1556–1565.
[18] Y.S. Kanwar, L. Sun, P. Xie, F.Y. Liu, S. Chen, A. Nishiyama, H. Kawachi, F. Shimizu, M.T. Quinn, D.J. Lambeth, S. Kagami, Addition of the antioxidant probucol to angiotensin II type I receptor antagonists arrests progressive mesangio proliferative glomerulonephritis in the rat, J. Am. Soc. Nephrol. 17 (2006) 783–794.
[19] D. Koya, K. Hayashi, M. Kitada, A. Kashiwagi, R. Kikukawa, M. Haneda, Effects of antioxidants in diabetes-induced oxidative stress in the glomeruli of diabetic rats, J. Am. Soc. Nephrol. 14 (2003) 2520–2525.
[20] D. Kumar, S. Robertson, K.D. Burns, Evidence of apoptosis in human diabetic kidney, Mol. Cell. Biochem. 259 (2004) 67–70.
[21] M. Kuzuya, F. Kuzuya, Probucol as an antioxidant and antithrombotic agent. Free Radic. Biol. Med. 14 (1993) 67–77.
[22] E.H. Leiter, Differential susceptibility of BALB/c sublines to diabetes induction by multi-dose streptozotocin treatment, Curr. Top. Microbiol. Immunol. 122 (1985) 78–85.
[23] T. Li, W. Chen, F. An, H. Tian, J. Zhang, J. Peng, Y. Zhang, Y. Guo, Probucol attenuates inflammation and increases stability of vulnerable atherosclerotic plaques in rabbits, Tohoku J. Exp. Med. 225 (2011) 23–34.
[24] J.S. Liu, Y. Shi, H. Peng, X. Shen, S. Thomas, Y. Wang, L.D. Truong, S.E. Dryer, Z. Hu, J. Xu, Loss of PTEN promotes podocyte cytoskeletal rearrangement, aggravating diabetic nephropathy, J. Pathol. 236 (2015) 30–40.
[25] J.J. Marin, M.J. Monti, A.G. Blazquez, R.J. Macias, M.A. Serrano, O. Briz, The role of reduced intracellular concentrations of active drugs in the lack of response to lapatinib in breast cancer chemothera, Acta Pharmacol. Sin. 35 (2014) 1–10.
[26] K.S. Modi, G.F. Schreiner, M.L. Parkerson, S. Klahr, Effects of probucol in renal function and structure in rats with subtotal kidney ablation, J. Lab. Clin. Med. 120 (1992) 310–317.
[27] M. Nishimura, T. Sasaki, A. Oishi, M. Oishi, S. Kono, Y. Totani, Y. Kato, Y. Noto, Redox Biology 13 (2017) 482–497

496
S. Misaki, K. Higashi, F. Shimada, H. Wakasugi, K. Inoue, Y. Hoshiyama, K. Yamada, Angiotensin-converting enzyme inhibitors and probucol suppress the time-dependent increase in urinary Type IV collagen excretion of Type II diabetes mellitus patients with early diabetic nephropathy, Clin. Nephrol. 56 (2001) 96–103.

[33] N. Poulose, R. Raju, Sirintu regulation in aging and injury, Biochim. Biophys. Acta 1852 (2015) 2442–2455.

[34] P. Song, S. Yang, L. Xiao, X. Xu, C. Tang, Y. Yang, M. Ma, J. Zhu, F. Liu, L. Sun, PKCdelta promotes high glucose induced renal tubular oxidative damage via regulating activation and translocation of p66Shc, Oxid. Med. Cell. Longev. 2014 (2014) 746531.

[35] L. Sun, L. Xiao, J. Nie, F.Y. Liu, G.H. Ling, X.J. Zhu, W.B. Tang, W.C. Chen, Y.C. Xia, M. Zhan, M.M. Ma, Y.M. Peng, H. Liu, Y.H. Liu, Y.S. Kanwar, p66Shc mediates high-glucose and angiotensin II-induced oxidative stress renal tubular injury via mitochondrial-dependent apoptotic pathway, Am. J. Physiol. Ren. Physiol. 299 (2010) F1014–F1025.

[36] L. Sun, D. Zhang, F. Liu, X. Xiang, G. Ling, L. Xiao, Y. Liu, X. Zhu, M. Zhan, Y. Yang, V.K. Kondeti, Y.S. Kanwar, Low-dose paclitaxel ameliorates fibrosis in the remnant kidney model by down-regulating miR-192, J. Pathol. 225 (2011) 364–377.

[37] S.C. Tang, K.N. Lai, The pathogenic role of the renal proximal tubular cell in diabetic nephropathy, Nephrol. Dial. Transplant. 27 (2012) 3049–3056.

[38] A. Ventura, L. Luzi, S. Pacini, C.T. Baldari, P.G. Pelicci, The p66Shc longevity gene is silenced through epigenetic modifications of an alternative promoter, J. Biol. Chem. 277 (2002) 22370–22376.

[39] F. Wang, B. Yang, G.H. Ling, C. Yao, Y.S. Jiang, Methylguanidine cytotoxicity on HK-2 cells and protective effect of antioxidants against MG-induced apoptosis in renal proximal tubular cells in vitro, Ren. Fail. 32 (2010) 978–985.

[40] R. Wu, W. Zhang, B. Liu, J. Gao, X.Q. Xiao, F. Zhang, H.M. Zhou, X.L. Wu, X. Zhang, Probucol ameliorates the development of nonalcoholic steatohepatitis in rats fed high-fat diets, Dig. Dis. Sci. 58 (2013) 163–171.

[41] L. Xiao, X. Zhu, S. Yang, F. Liu, Z. Zhou, M. Zhan, P. Xie, D. Zhang, J. Li, P. Song, Y.S. Kanwar, L. Sun, p66Shc: a novel biomarker of tubular oxidative injury in patients with diabetic nephropathy, Sci. Rep. 6 (2016) 29302.

[42] X.X. Wang, M.H. Edelstein, U. Gafer, G protein-coupled bile acid receptor TGR5 activation inhibits kidney disease in obesity and diabetes, J. Am. Soc. Nephrol. 27 (2016) 1362–1378.

[43] T. Zhang, W.L. Kraus, SIRT1-dependent regulation of chromatinn and transcription: linking NAD(+) metabolism and signaling to the control of cellular functions, Biochim. Biophys. Acta 1804 (2010) 1666–1675.

[44] W. Zhang, W. Ji, L. Yang, Y. Xu, J. Yang, Z. Zhang, Epigenetic enhancement of p66Shc during cellular replicative or premature senescence, Toxicology 278 (2010) 189–194.

[45] L. Xiao, P. Li, L. Sun, W. Zhang, G. Ning, A.W. Minto, J. Kong, R.J. Quigg, Y.C. Li, Renoprotective role of the vitamin D receptor in diabetic nephropathy, Kidney Int. 73 (2008) 163–171.

[46] J. Zhao, S. Miyamoto, Y.H. You, K. Sharma, AMP-activated protein kinase (AMPK) activation inhibits nuclear translocation of Smad4 in mesangial cells and diabetic kidneys, Am. J. Physiol. Ren. Physiol. 308 (2015) F1167–F1177.

[47] L. Zhao, L.N. Sun, H.B. Nie, X.L. Wang, G.J. Guan, Berberine improves kidney function in diabetic mice via AMPK activation, PLoS One 9 (2014) e113398.

[48] S. Zhou, H.Z. Chen, Y.Z. Wan, Q.J. Zhang, Y.S. Wei, S. Huang, J.J. Liu, Y.B. Lu, Z.Q. Zhang, R.F. Yang, R. Zhang, H. Cai, D.P. Liu, C.C. Liang, Repression of P66Shc expression by SIRT1 contributes to the prevention of hyperglycemia-induced endothelial dysfunction, Circ. Res. 109 (2011) 639–648.

[49] H. Zhu, X. Jin, J. Zhao, Z. Dong, X. Ma, F. Xu, W. Huang, G. Liu, Y. Zou, K. Wang, H. Hu, A. Sun, J. Ge, Probucol protects against atherosclerosis through lipid-lowering and suppressing immune maturation of CD11c+ dendritic cells in STZ-induced diabetic LDLR−/− mice, J. Cardiovasc. Pharmacol. 65 (2015) 620–627.

[50] Z. Zhang, L. Sun, W. Ji, L. Yang, Y. Xu, J. Yang, Z. Zhang, K. Hu, A. Sun, J. Ge, Probucol protects against atherosclerosis through lipid-lowering and suppressing immune maturation of CD11c+ dendritic cells in STZ-induced diabetic LDLR−/− mice, J. Cardiovasc. Pharmacol. 65 (2015) 620–627.

[51] X. Zhu, X. Xiong, S. Yuan, L. Xiao, X. Fu, Y. Yang, C. Tang, L. He, F. Liu, L. Sun, Validation of the interstitial fibrosis and tubular atrophy on the new pathological classification in patients with diabetic nephropathy: a single-center study in China, J. Diabetes Complicat. 30 (2016) 537–541.