Sulforaphane prevents type 2 diabetes-induced nephropathy via AMPK-mediated activation of lipid metabolic pathways and Nrf2 anti-oxidative function

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Abstract

Sulforaphane (SFN) prevents diabetic nephropathy (DN) in type 2 diabetes (T2D) by up-regulating nuclear factor (erythroid-derived 2)-like 2 (Nrf2). AMP-activated protein kinase (AMPK) can attenuate the pathogenesis of DN by improving renal lipotoxicity along with the activation of Nrf2-mediated anti-oxidative signaling. Therefore, we investigated whether AMPKα2, the central subunit of AMPK in energy metabolism, is required for SFN protection against DN in T2D, and whether potential crosstalk occurs between AMPKα2 and Nrf2. AMPKα2 knockout (Ampkα2−/−) mice and wild-type mice were fed a high-fat diet (HFD) or a normal diet (ND) to induce insulin resistance, followed by streptozotocin injection to induce hyperglycemia, as a T2D model. Both T2D and control mice were treated with SFN or vehicle for three months. At the end of the three-month treatment, all mice were maintained only on HFD or ND for an additional three months without SFN treatment. Mice were sacrificed at 6th month after T2D onset. Twenty-four-hour urine albumin at 3rd and 6th months was significantly increased as renal dysfunction, along with significant renal pathological changes and biochemical changes including renal hypertrophy, oxidative damage, inflammation, and fibrosis in wild-type T2D mice, which were prevented by SFN in certain extends, but not in Ampkα2−/− T2D mice. SFN prevention of T2D-induced renal lipotoxicity was associated with AMPK-mediated activation of lipid metabolism and Nrf2-dependent anti-oxidative function in wild-type mice, but not in SFN-treated Ampkα2−/− mice. Therefore, SFN prevention of DN is AMPKα2-mediated activation of probably both lipid metabolism and Nrf2 via AMPK/AKT/GSK3β/Fyn pathways.

Keywords: Sulforaphane, Diabetic Nephropathy, AMPK, Nrf2, Renal Lipotoxicity
1. Introduction

Diabetic nephropathy (DN) is the leading cause of end-stage renal disease worldwide [1]. Although hyperglycemia and insulin resistance are generally considered to be the primary causative factors for DN, mounting evidence suggests that lipid accumulation is a crucial contributor to DN, especially in type 2 diabetes (T2D) [2]. Renal lipid accumulation in the patients with T2D, which is caused by increased lipid uptake and decreased lipid oxidation, is associated with renal inflammation, oxidative stress, fibrosis, and deterioration of renal function. Identifying the mechanisms of T2D-induced renal lipid accumulation and lipotoxicity may identify additional therapeutic targets to delay development and progression of glomerulosclerosis in DN [2, 3].

Sulforaphane (SFN), a natural isothiocyanate compound isolated from cruciferous vegetables such as broccoli and cabbage, is a nuclear factor (erythroid-derived 2)-like 2 (Nrf2) activator. Our prior study showed that SFN protection against DN in T2D is partially dependent on Nrf2-mediated suppression of oxidative stress [4]. However, several other studies showed that SFN alleviated clinical diabetic complications related to improvement of lipid profile and insulin resistance independent of the antioxidant response [5]. In addition, SFN was also found to inhibit adipogenesis accompanied with activation of AMP-activated protein kinase (AMPK) in recent study [6], suggesting that SFN could play an important role in improving dyslipidemia probably through AMPK signaling. These studies indicated that SFN may alleviates diabetic complications through multiple mechanisms, including Nrf2-dependent and -independent pathways, and noticed us a potential role for interactions between SFN and AMPK-mediated lipid metabolism. Therefore, we sought to determine whether SFN could activate AMPK-mediated lipid metabolism pathways to protect against kidney damage in T2D in the current work, which is as yet unknown.
AMPK, a pivotal fuel sensor of glucose and lipid metabolism, has two active isoforms (α1 and α2) with differential downstream effects and expression patterns. Previous studies identified distinctly different physiological effects for AMPKα1/α2 subunits in regulation of systemic energy metabolism and insulin sensitivity. Ablation of AMPKα2 causes metabolic defects including glucose intolerance, impaired insulin secretion, insulin resistance, and defective glycogen synthesis, all of which are vital characteristics of T2D, while deletion of the AMPKα1 gene (Ampkα1−/−) did not induce these metabolic defects [7]. Besides, AMPKα2 is more highly expressed than AMPKα1 in the kidney, and endoplasmic reticulum stress-induced renal inflammation and fibrotic injury is more pronounced in Ampkα2−/− mice than in Ampkα1−/− mice [8-10]. Taken together, these findings suggest that AMPKα2 subunit seems to be a crucial role in renal energy metabolism and injury. The activation of AMPK reduces adipogenesis and uptake of fatty acid while promoting subsequent fatty acid oxidation (FAO), therefore AMPK is a potential target for the treatment of various metabolic syndromes related with lipid metabolism disorder in this context [6].

Further, prior study suggested that AMPK is a potential target for treatment of oxidative stress [11]. Besides, mounting evidence also indicated that increased AMPK activity is associated with increased Nrf2 protein abundance and transcriptional function, which decreases oxidative DNA damage in db/db mice, which is a model of leptin deficiency [12, 13]. Further, there is growing evidence that the increased AMPK activity can activate Nrf2 through up-regulating phosphorylation of glycogen synthase kinase (GSK)-3β mediated by activation of Akt [14]. However, it remains unknown whether this mechanism is also responsible for the prevention by SFN of DN.

Thus, the present study sought to (1) determine whether SFN prevents T2D-induced renal lipotoxicity and DN via AMPK activation; (2) determine whether AMPKα2 is indispensable for
SFN-mediated activation of the Nrf2 antioxidant pathway and lipid metabolic pathway in DN; and (3) investigate the lasting, post-treatment protective effects of SFN against DN after cessation of SFN therapy in T2D.

2. Materials and Methods

2.1. Animals

Global AMPKα2 knockout (AMPKα2<sup>−/−</sup>) mice and their wild-type (WT) C57BL6/J mice were purchased from Jackson Laboratories (Bar Harbor, ME). The gene nomenclature of AMPKα2 was Prkaa2 and full name of knockout (KO) mice was Prkaa2<sup>tm1.1Sjm/J</sup>. To make it easier, we used AMPKα2<sup>−/−</sup> to replace Prkaa2<sup>tm1.1Sjm/J</sup> in this context. Lack of renal AMPKα2 expression in Ampkα2<sup>−/−</sup> (KO) mice compared to WT mice was confirmed by western blot (Supplementary Figure 2E). Mice were housed at the research resource center of the University of Louisville with an ambient temperature of 22°C, a 12/12-hour light/dark cycle, and free access to standard rodent chow and tap water. All studies were conducted in accordance with the National Institutes of Health's Guide for Care and Use of Laboratory Animals (NIH publication No. 85-23, revised 1996). All experimental procedures were approved by the Institutional Animal Care and Use Committee of the University of Louisville, which is accredited by the American Association for Accreditation of Laboratory Animal Care.

For T2D model, both eight-week old male WT and KO mice were fed with a high-fat diet (HFD, 60.3 kcal% fat; 5.1 kcal/g, TD.09766, Teklad Custom Research Diets, Envigo) or a normal diet (ND, 16.8 kcal% fat; 3.3 kcal/g, TD.120455, Teklad Custom Research Diets, Envigo) for three months to induce insulin resistance (Supplementary Figure 2A, B). These insulin-resistant mice at age of 20...
weeks were received one intraperitoneal injection of streptozocin (STZ, Sigma Aldrich, St. Louis, MO, USA; 100 mg/kg) freshly dissolved in sodium citrate (0.1 M, pH 4.5) to induce mild hyperglycemia, as outlined in Supplementary Figure 1A, B while ND-fed mice were injected with vehicle (0.1 M sodium citrate). Seven days after STZ injection, 21-week old mice with hyperglycemia (3-h fasting blood glucose≥250 mg/dl) were defined as T2D [15]. HFD/STZ mice with blood glucose <250 mg/dl were excluded from the analysis. Once diabetes was confirmed, both diabetic and age-matched control mice were injected subcutaneously with either 0.5 mg/kg SFN (Sigma Aldrich, St. Louis, MO) or vehicle (1% DMSO in PBS) for five days every week over the course of three months with continuous HFD or ND feeding (at 33-weeks old), as outlined in Supplementary Figure 1C. The SFN dosage in the present study was based on our previous studies [4, 16] and other studies [17, 18]. In addition, there were two previous studies [19, 20] where SFN was given mice either by oral at the dose of 110 µmol/kg [19] or intraperitoneally at the dose of about 63.8 mg/kg [20] and their plasma peak concentrations of SFN were 6.66 µM at 2 hr or 210 µM at 1 hr, respectively. Based on these two studies, we estimated that the serum peak concentration of the SFN used here should be 0.17 – 1.65 µM. Since we treated diabetic mice for three months, the purpose of SFN treatment is to preserve the normal level of SFN (0.1 – 2.5 µM) to overcome diabetes-induced systemic reduction of antioxidant capacity via preserving SFN-stimulation of Nrf2 function in normal level. Then in order to investigate the lasting effects of SFN, the treatment of SFN in T2D was ceased and all mice were maintained on HFD or ND for an additional three months without SFN treatment till sacrificed (at 45 weeks old). Mice were intraperitoneally anesthetized with Avertin (2,2,2-tribromoethanol, 350 mg/kg) to collect kidneys for weighing and performing pathological and biochemical examinations.

For mouse grouping, WT mice were divided into four groups (n = 5/group): ND + vehicle (Ctrl),
ND + SFN (Ctrl/SFN), HFD/STZ + vehicle (T2D), and HFD/STZ + SFN (T2D/SFN). Based on our previous studies [4, 16], we did not find any significant side effects of SFN on non-diabetic kidneys.

In addition, SFN effect on nondiabetic kidney is included in WT mice, therefore, KO mice were only divided into three groups (KO-Ctrl, n = 4; KO-T2D, n = 7; KO-T2D/SFN, n = 6): ND + vehicle (KO-Ctrl), HFD/STZ + vehicle (KO-T2D), and HFD/STZ + SFN (KO-T2D/SFN), as illustrated in Supplementary Figure1A, B.

During the 6-month experimental period, tail vein blood glucose was measured for five times to confirm T2D status (Supplementary Figure 2C, D). In addition, 24-hour urine albumin was measured by collecting 24-hour urine for each of mice in all groups with metabolic cages at the 3rd and 6th month (before euthanized).

2.2. Glucose tolerance

After three months of HFD or ND feeding, an intraperitoneal glucose tolerance testing (IPGTT) was performed. After 6h of fasting, we measured tail vein blood glucose, which served as the 0-min time point. Glucose (1.5 mg/g body weight) was then injected IP, followed by 15, 30, 60 and 120-min tail vein blood glucose measurements. The area under the curve (AUC) using the trapezoid rule was calculated to determine glucose tolerance.

2.3. Urine albumin analysis

Urine albumin concentration was assessed by measuring 24-h urine, which was centrifuged at 3000 xg for 15 minutes. The urine albumin level was detected using a Mouse Albumin ELISA Quantification Kit (Bethyl Laboratories, Montgomery, TX) according to the manufacturer’s instructions.

2.4. Histology and immunohistochemical staining
Kidney tissue was immediately fixed in 10% formalin solution buffer, embedded in paraffin, and sectioned into 5-µm slices. Sections were processed for periodic Acid-Schiff (PAS), Sirius Red, and immunohistochemical (IHC) staining as described previously [21]. Antibodies against fibronectin (FN, 1:100 dilution), transforming growth factor β1 (TGF-β1, 1:100 dilution), and collagen-1 (COL-1, 1:100 dilution) (all from Abcam, Cambridge, MA) were used for IHC.

2.5. Western blot analysis

Western blot was performed as described previously [22]. Primary antibodies used in the present study include 3-nitrotyrosine (3-NT, 1:2000 dilution, Millipore Corp, Temecula, CA), 4-hydroxy-2-nonenal (4-HNE 1:3000, Abcam, Cambridge, MA), plasminogen activator inhibitor-1 (PAI-1, 1:1000 dilution, BD Biosciences, San Jose, CA), peroxisome proliferator-activated receptor-α (PPARα, 1:1000 dilution, Thermo fisher), sirtuin1 (SIRT1, 1:1000 dilution, Santa Cruz Biotechnology, Santa Cruz, CA), peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α, 1:1000 dilution, Santa Cruz Biotechnology) and β-actin (1:2000 dilution, Santa Cruz Biotechnology) as an internal control. For the primary antibodies against fibronectin (FN), TGF-β1, tumor necrosis factor-α (TNFα), carnitine palmitoyl transferase-1α (CPT-1α), sterol regulatory element-binding protein 1 (SREBP-1), stearoyl-CoA desaturase-1 (SCD-1), Nrf2, AMPKα, all were purchased from Abcam (Cambridge, MA) and used at a dilution of 1:1000. The anti-Nrf2 antibody used in our study targets Nrf2 protein with molecular weight of about 100kDa (Abcam, Cat.#: ab62352). For the primary antibodies against phospho-AMPKα (p-AMPKα (Thr172)), AMPKα, phospho-Acetyl-CoA Carboxylase (p-ACC (Ser79)), ACC, phospho-Akt (p-Akt (Ser473)), Akt, phospho-GSK3β (p-GSK3β (Ser9)), GSK3β, phosphor-nuclear factor kappa-light-chain-enhancer of activated B cells p65 (p-NF-κB (Ser536)), NF-κB p65, Fyn, and Histone H3, all were purchased from
Cell Signaling Technology (Beverly, MA) and used at a dilution of 1:1000. Appropriate secondary antibodies (Cell Signaling Technology) were used.

2.6. Nitric oxide analysis

The level of nitric oxide (NO) in kidney tissue was measured according to manufacturer’s instructions using the QuantiChromTM Nitric Oxide Assay kit from BioAssay Systems.

2.7. Oil Red O staining

Renal lipid accumulation was detected by Oil Red O staining as described previously [23]. Kidney cryosections were fixed in formalin buffer and rinsed with running water. The slides were then soaked in isopropanol and incubated with Oil Red O working solution (saturated oil red O isopropanol solution diluted to 60% in isopropanol, Sigma-Aldrich).

2.8. Real-time qPCR

Real-time RT-PCR was performed as described previously [24] using primers from Life Technologies (Grand Island, NY) for NAD(P)H: quinone oxidoreductase (Nqo1, Mm01253561_m1 catalog# 4448892), heme oxygenase 1 (Hmox1, Mm00516005_m1 catalog# 4453320), catalase (Cat, Mm00437992_m1 catalog# 4453320), Superoxide dismutase 2 (Sod2, Mm01313000_m1 catalog# 4453320), β-actin (Actb, Mm01205647_g1 catalog# 4453320), phospholipaseA2 (YWHAZ, Mm01158416_g1 catalog# 4448892) and glyceraldehyde 3-phosphate dehydrogenase (Gapdh, Mm99999915_g1 catalog# 4453320).

2.9. Nuclear extraction

The renal nuclear were extracted according to manufacturer’s instructions using the nuclear extraction kit from Abcam.

2.10. Statistical analysis
Data were collected from each group (n = 4–7 per group) and were expressed as mean ± standard deviation (SD). Comparisons among three or more groups were performed by one-way analysis of variance (ANOVA) followed by Tukey's test. All statistical analyses were performed using Prism 6 (GraphPad Software Inc, San Diego, CA, USA). A P-value < 0.05 was considered to be statistically significant.

3. Results

3.1 General characteristics of WT and Ampkα2−/− T2D mice

T2D was induced in Ampkα2−/− and WT mice (Supplementary Figure 1), reflected by increased AUC of the IPGTT curve and fasting blood glucose. Comparing two strains of mice, there was no significant difference for the AUC was between HFD-fed Ampkα2−/− ice and HFD-fed WT mice before diabetes onset (Supplementary Figure 2A, B). Once diabetes onset, the dynamic blood glucose levels were significantly increased in both diabetic WT and Ampkα2−/− mice, the increased glucose level in Ampkα2−/− mice is higher at the early time point (first two months, Supplementary Figure 2C, D). SFN treatment did not significantly affect blood glucose levels.

Lack of renal Ampkα2 gene in Ampkα2−/− mice was confirmed by almost undetectable protein expression compared with AMPKα2 expression in WT mice (Supplementary Figure 2E). Body weight was significantly higher in T2D mice than Ctrl mice only in WT mice (Figure 1A). However, T2D induced the increase of kidney weight similarly in both diabetic WT mice and Ampkα2−/− mice, which was significantly prevented by SFN only in WT diabetic mice (Figure 1B). To evaluate the changes in renal function during the development of DN and the effect of SFN treatment, 24-h urine albumin were detected at the 3rd month of T2D and the end of SFN treatment (3M) as well as at the 6th month.
of T2D and three months after cessation of SFN treatment (6M) (Figure 1C, D). 24-h urine albumin was significantly increased in both WT and Ampkα2−/− T2D mice at both 3M and 6M, while SFN treatment significantly prevented the elevation of 24-h albumin only at 6M and only in WT mice. These results suggest that SFN significantly attenuated T2D induced kidney hypertrophy and dysfunction only in WT, but not Ampkα2−/− T2D mice.

3.2 The prevention of T2D-induced renal pathological abnormalities and fibrotic responses by SFN in WT mice was lost in Ampkα2−/− mice.

Histological examination with PAS staining revealed glomerulopathy, including glomerular hypertrophy and increased mesangial matrix, similarly in both diabetic WT and Ampkα2−/− mice, which were significantly alleviated by SFN in WT mice but not in Ampkα2−/− mice (Figure 2A-C). Furthermore, all these changes were slightly severe in Ampkα2−/− mice compared to that in WT mice in term of the fold changes between two strain diabetic mice relative to controls (Figure 2D), suggesting that AMPK deficiency may potentially exacerbate T2D-induced glomerular hypertrophy and increased mesangial matrix expansion. The renal fibrosis, which was defined by increased collagen accumulation, was analyzed by Sirius Red staining. The result showed that renal collagen disposition was markedly increased both in diabetic WT and Ampkα2−/− mice, but SFN treatment reduced diabetes-induced collagen accumulation only in diabetic WT mice. However, although collagen deposition in the SFN-treated group was significantly lower than that in the T2D group, it was still notably higher than Ctrl group. Therefore, SFN cannot completely prevent diabetes-induced renal fibrosis, but ameliorate it (Figure 2E, F).

To explore the fibrotic pathways, the protein expression of TGF-β1 a major fibrotic initiator was examined by Western blot (Figure 3A), which was significantly increased in both WT and...
Ampkα2—/— diabetic groups and that was preventable only in WT, but not in Ampka2—/—, diabetic mice.

To further define the fibrotic response, FN as one of fibrotic end-products was examined for its expression (Figure 3B) and renal structural location (Figure 3C) was examined by Western blot and immunohistochemical stain and showed the significant increase in both WT and Ampka2—/— diabetic groups, which was preventable only in WT, but not in Ampka2—/—, diabetic mice. Similarly, another fibrotic end-product Col-1 was also showed significant increase in T2D kidney and its prevention by SFN (Figure 3D). Importantly FN mainly accumulates in glomeruli while COL-1 mainly accumulates in renal interstitium (Figure 3C, D).

3.3 AMPKα2 deletion abolished SFN prevention of diabetes-induced renal inflammation and oxidative damage.

Because inflammation and oxidative damage play critical roles in diabetes-induced renal pathogenesis, we used Western blot to quantify expression of three important inflammatory cytokines, PAI-1 and TNF-α (Figure 4A), NF-κB p65 (Figure 4B) as well as two oxidative stress markers, 3-NT (a marker of nitrosative stress) and 4-HNE (a marker of lipid peroxidation) (Figure 4C, D). The results indicated that T2D significantly increased renal protein levels of PAI-1, TNFα, the activity of NF-κB p65, and the expression of 3-NT as well as 4-HNE both in WT and Ampka2—/— mice. SFN improved diabetes-increased these protein levels in WT mice, but not in Ampka2—/— mice. Considering protein nitration is caused by peroxynitrite formation, which is derived from the interaction of NO with superoxide, we also revealed the increased level of nitric oxide in T2D group, an effect that can be improved by SFN in WT mice, but not in Ampka2—/— mice (Figure 4E).

3.4 SFN prevented diabetes-induced renal lipid accumulation by preserving AMPK activation and its downstream target regulation while AMPKα2 deletion abolished the prevention of
SFN against renal lipotoxicity.

AMPK alleviates cardiac and renal complications of T2D by preserving lipid metabolism and alleviating lipotoxicity [25, 26]. Therefore, we determined whether SFN reduced lipid accumulation using Oil Red O staining, and assessed AMPK activation by measuring phosphorylation of AMPK at Thr172 as well as phosphorylation and/or abundance of its downstream targets. Oil Red O staining revealed an increase renal lipid accumulation in both diabetic WT and Ampkα2−/− mice. Interestingly, SFN markedly decreased renal lipid accumulation only in diabetic WT mice, but not in Ampkα2−/− mice (Figure 5A). Further, Western blot analysis demonstrated that the ratio of p-AMPK to AMPK was significantly decreased in T2D kidney, and that SFN attenuated this decrease in WT mice (Figure 5B). Afterwards, we then assessed activation and protein levels of AMPK targets using Western blot both in WT and Ampkα2−/− mice. In T2D group of WT and Ampkα2−/− mice, PPARα, p-ACC, CPT-1α (Figure 5C-E), SIRT1, and PGC-1α (Figure 6A, B), which were involved in FAO [27], were markedly down-regulated. SFN significantly reversed this down-regulation only in diabetic WT mice, but not in diabetic Ampkα2−/− mice. On the other side, the levels of fatty acid synthesis proteins SREBP-1 and SCD1 (Figure 6C, D) were significantly increased in DN and these increases were also inhibited by SFN only in WT mice, but not in diabetic Ampkα2−/− mice. All of these findings suggested that SFN prevented diabetic renal injury by alleviating renal lipotoxicity, which was dependent on AMPKα2.

3.5 AMPKα2 deletion abolished the up-regulation of nuclear Nrf2 protein abundance and transcription activation by SFN in diabetic kidney.

SFN activation of Nrf2, which is a crucial antioxidant transcriptional factor, plays an important role in its antioxidant capacity and protection of DN [16]. In the present study, the expression of nuclear Nrf2 was significantly decreased both in WT T2D group and Ampka2−/− T2D group, which was
improved by SFN treatment only in diabetic WT mice, but not in Ampka2/− mice (Figure 7A). To
determine whether the decline of nuclear Nrf2 expression is accompanied by the impairment of its
function and confirm SFN activated Nrf2 in an AMPKα2-dependent manner, we further measured
mRNA levels of Nrf2 target genes, including Nqo1, Cat, Hmox1 and Sod2 relative to the genome of 3
different housekeeping genes including β-actin, YWHAZ and Gapdh (Figure 7B-I) by real-time PCR
assay. Consistently, they also showed significant decrease in WT T2D group (Figure 7B-E) and
Ampka2/− T2D group (Figure 7F-I), which was also notably up-regulated by SFN treatment only in
diabetic WT mice, but not in Ampka2/− mice. These results suggested that SFN-induced Nrf2
activation was also AMPKα2-dependent.

3.6 Nrf2 activation induced by SFN is mediated through AMPK/Akt/GSK3β/Fyn pathway.

To explore the potential mechanism for AMPK-mediated induction of Nrf2 activation in diabetic
kidney, activation of the Akt, GSK3β and nuclear Fyn was examined both in WT mice and Ampka2/−
mice by using Western blot. The results showed that the phosphorylation of AKT at Ser473 residue
(Figure 8A) and GSK3β phosphorylation at Ser9 residue (Figure 8B) were down-regulated
accompanied with the increased nuclear Fyn expression (Figure 8C) in diabetic kidney. SFN treatment
notably up-regulated AKT/GSK3β/Fyn pathway in diabetic WT mice but not in diabetic Ampka2/−
mice.

4. Discussion

The present study focuses on the specific role of AMPK in SFN renal protection and the
potentially long-term post-treatment protective effects of SFN against DN by using HFD/STZ induced
T2D mouse model. In the present study, T2D mice exhibited significant insulin resistance
accompanied with increases in blood glucose (Supplementary Figure 2A-D), body weight, and kidney weight (Figure 1A, B). The renal dysfunction (Figure 1C, D) and structural damage (Figure 2) along with significant increases in renal inflammation, oxidative damage and fibrosis (Figure 3, 4) were all observed in diabetic mice and could be ameliorated by SFN treatment only in WT mice but not in Ampkα2−/− mice. We also revealed for the first time that SFN treatment only at the first three months offered a renal prevention of DN persistently seen at three months after SFN withdrawal.

Mechanistically, we also firstly demonstrated that SFN-mediated renal protection involved in both improvement of renal lipid accumulation and activation of the Nrf2 anti-oxidative response, both were all dependent on AMPKα2, as illustrated in Figure 9.

Hyperglycemia, obesity and insulin resistance are well-known primary causes of T2D-induced DN [1]. In clinical studies, however, subjects with obesity and dyslipidemia are more likely to develop DN [28]. Emerging basic research also identified an important role of lipid accumulation as an initiator for diabetic renal damage [26]. The present study confirmed the therapeutic potential of SFN in improving renal lipid metabolism and preventing associated lipotoxicity in the kidney of T2D mice.

SFN was reported to ameliorate obesity, diabetes, nonalcoholic fat liver disease, diabetic cardiomyopathy and related disorders along with significant reduction of lipid accumulation [5, 6, 25, 29]. SFN attenuates obesity and diabetic cardiomyopathy closely accompanied with the activation of AMPK [5, 25], which is a crucial regulator of lipid metabolic pathways [6]. These results indicated a potential role of SFN in activating AMPK-mediated lipid metabolism to prevent T2D-induced DN. Therefore, the present study primarily focused on the activity of AMPK in diabetic WT kidneys with or without SFN treatment to confirm the preventive effect of SFN on T2D-induced DN, and then compared it in WT with Ampka2−/− to define its pivotal role in SFN-mediated prevention of DN. The
Main finding of this study was that AMPK activity was lower in the diabetic kidney, which manifested as decreased AMPK phosphorylation, together with impaired renal structure and function. SFN preserved AMPK function (Figure 5, 6) with significant alleviation of renal damage and dysfunction in T2D WT, but not in Ampkα2−/− mice (Figures 1-4).

Activated AMPK plays lipid-lowering effects in the diabetic kidney through two distinct mechanisms: Up-regulation of FAO mediated by PPARα, ACC, CPT-1α, SIRT-1 and PGC-1α, and down-regulation of fatty acid synthesis mediated by SREBP-1 and SCD-1 (Figure 5C-E and Figure 6).

Previous studies have demonstrated that activated AMPK phosphorylates and inhibits ACC to regulate the potent CPT-1α inhibitor malonyl-CoA [30]. CPT-1α is a rate-limiting enzyme for mitochondrial uptake of fatty acid and subsequent FAO, and CPT-1α deficiency is associated with lipotoxicity [25, 26]. Inactivation of ACC reduces CPT-1α deficiency and promotes mitochondrial uptake of fatty acids in the kidney [31]. Up-regulation of PPARα, another crucial FAO regulator downstream of AMPK [32], could also up-regulate CPT-1α to promote fatty acid catabolism [33]. In the present study, SFN significantly increased ACC phosphorylation, the expression of PPARα and CPT-1α only in diabetic WT kidneys, but not in diabetic Ampkα2−/− kidneys (Figure 5C-E). These results suggest that SFN may increase mitochondrial uptake of fatty acids via the AMPK/ACC/CPT-1α and AMPK/PPARα/CPT-1α pathway to prevent T2D-induced lipotoxicity. In addition, our results also revealed that SFN might improve mitochondrial function and alleviate lipotoxicity by activating the AMPK/SIRT1/PGC1α pathway (Figure 6A, B) [34] and SFN inhibit aberrant fatty acid synthesis by suppressing expression of lipogenesis-associated enzymes such as SREBP-1 [35] and SCD1 (Figure 6C, D) [36].

A recent study reported that AMPK was implicated in the activation of Nrf2-mediated antioxidant pathway [13], which was supported by our the present study: SFN markedly activated
Nrf2 and its downstream genes only in diabetic WT mice (Figure 7), but not in Ampkα2<sup>−/−</sup> mice. This suggests that the preventive effect by SFN of T2D-induced nephropathy by reducing lipid accumulation and activating Nrf2-mediated antioxidants was AMPKα2-dependent, which was reported for the first time. Furthermore, how AMPK mediates Nrf2 mechanistically is also studied in our present study. A previous study demonstrates that AMPK activates AKT by phosphorylating Ser473 in DN [37]. Consistently, we also found the phosphorylation of AKT at Ser473 was down-regulated in diabetic kidneys, which could be up-regulated by SFN in AMPK-dependent manner. Subsequently, the activated AKT inactivated its downstream effector protein GSK3β by phosphorylating it at Ser9 residue (Figure 8A, B), which was also demonstrated by another study [14]. Fyn, a well-known negative regulator of Nrf2, promotes the degradation of Nrf2 by entering into nucleus to export nuclear Nrf2 to the cytoplasm while the nucleus translocation of Fyn is mediated by GSK-3β activation [38]. The present study suggested that SFN-mediated AMPKα2 activation stimulates Akt phosphorylation and, subsequently, GSK-3β phosphorylation to prevent Fyn entering nuclei to export Nrf2, resulting in an increase in nuclei Nrf2 accumulation as illustrated in Figure 9.

Nrf2, which is a crucial factor to defense against oxidative stress, regulates intracellular antioxidants such as NQO1, Hmox1, SOD and glutathione S-transferase to reduce oxidative stress in diabetic kidney [39]. Additionally, the critical role of activation of Nrf2 in preventing against diabetes-induced renal fibrosis through regulating the related pro-fibrogenic factors [40, 41]. These two studies confirmed that SFN-induced protection against renal fibrosis is Nrf2 dependent: Nrf2-mediated protection against STZ-induced DN was achieved through inhibiting TGF-β1 (a major profibrotic mediator of DN) and its downstream effectors including FN and collagen IV, which were significantly abrogated by treatment with SFN only in the Nrf2<sup>+/+</sup> mice but not in the Nrf2<sup>−/−</sup> mice.
that had even severer renal fibrosis than \( \text{Nrf2}^{+/+} \) mice [40, 41]. Consistently, we also proved that SFN’s protection against T2D-induced renal fibrotic effect is also Nrf2-dependent [4]. Using the same T2D model here we further confirmed the pivotal role of AMPK\( \alpha_2 \) in SFN prevention of T2D-induced renal fibrosis and DN and further defined the dependence of AMPK\( \alpha_2 \) for Nrf2 activation in response SFN, therefore, we propose that SFN prevents T2D-induced renal fibrosis and dysfunction by activating AMPK\( \alpha_2 \)-dependent activation of Nrf2-mediated antioxidant function and lipid metabolism, as illustrated in Figure 9.

Previous studies revealed that Nrf2 activation resulted in inactivation of NF-\( \kappa \)B, a family of transcription factor that have been proved to mediate pro-fibrotic mediators and pro-inflammatory reactions [42-44]. Phosphorylation of the canonical NF-\( \kappa \)B subunit p65 transferred to the nuclear at Ser536 is thus considered as a hallmark for NF-\( \kappa \)B activation [42]. Since in general NF-\( \kappa \)B function is negatively related to the activation of Nrf2 [43], in the present study we also tested the relationship between Nrf2 activation and NF-\( \kappa \)B-mediated renal pro-inflammatory and pro-fibrotic responses by examining the ratio of p-p65 to p65 in the nuclear by Western blot (Figure 4B). The suppression Nrf2 activation in diabetic kidney was accompanied by NF-\( \kappa \)B activation. Reportedly NF-\( \kappa \)B stimulates fibrosis through up-regulating inflammatory and pro-fibrotic mediators [42, 44]. Consistently, we also found that the decrease of Nrf2 activity under diabetic condition was associated with the increase of NF-\( \kappa \)B activity along inflammatory and profibrotic responses, effects that could be significantly inhibited after Nrf2 activation by SFN. The other previous and our present studies suggest that AMPK/Nrf2/NF-\( \kappa \)B anti-inflammation and fibrosis pathways play important roles in SFN renal protection from DN.

Prior studies have suggested that SFN ameliorates DN by alleviating oxidative stress through
Nrf2 activation in both T2D and T1D models [4, 16, 40]. Unlike their studies on acute SFN efficacy, we mainly evaluated DN severity and downstream signaling three months after SFN withdrawal, thereby allowing an evaluation of the long-term and sustained protective effects of SFN. In the WT mice, we proved that SFN treatment significantly recovered impairment of AMPK and Nrf2 function, and improved renal structure and function in T2D-induced DN even after three months of withdrawal. This may be related to “metabolic memory” of SFN, a term often used to describe the beneficial effects of immediate intensive treatment of hyperglycemia on microvascular complications, regardless of glycemic control later in the course of disease [45].

SFN was shown to improve cancer and chronic diseases through epigenetic modifications including DNA methylation, histone modifications, and regulation of microRNA expression. Although we cannot identify the specific epigenetic modification by SFN in the present study, the impact of SFN on microRNA expression levels and associated target gene expressions may explain its effect on AMPK activation. For example, miR-34a, which could be inhibited by SFN [46], can aggravate lipotoxicity by inhibiting AMPK pathway in renal tubular epithelial cells and proximal renal tubular [47,48]. Similarly, miR-21 that could be inhibited by SFN [48] was able to increase fibrosis and inflammatory response accompanied with AMPK inactivation in white adipose tissue [49,50]. Therefore, SFN may up-regulate AMPK-mediated lipid metabolism by regulating microRNAs expression, which still needs to be further confirmed in diabetic kidneys in the future studies.

In addition to the animal experiments, clinical studies have also found the important role of SFN in treatment of diabetes patients. Highly concentrated SFN provided as broccoli sprout extracts was proved to ameliorate fasting glucose and HbA1c in obese patients with dysregulated T2D (HbA1c above 50 mmol/mol) without reported adverse effect, which revealed the therapeutic effect of SFN on
obese T2D patients is similar to metformin but with a different mechanism [51]. Considering that metformin, a most clinically-used anti-diabetic drug, is also a well-known AMPK activator. Metformin stimulates AMPK activity in insulin-sensitive tissue notably, which is related to alleviation of endotoxemia, and also activates the Nrf2 anti-oxidative system. Both endotoxemia and oxidative stress are important causal factors of insulin resistance and interruption of insulin signaling [52]. Therefore, this effect of metformin plays an important role in glycemic control and prevention of diabetic complications. Considering the similar effects of SFN and metformin in AMPK and Nrf2 activation accompanied with the higher safety of SFN as a plant extract, we postulated that SFN may have the similar potential to be used for diabetic patients as an alternative of metformin for improving diabetes and preventing diabetic complications.

In summary, our study revealed that SFN prevented renal injury in T2D by improving renal lipotoxicity and the Nrf2-mediated antioxidant pathway, which are both dependent on AMPKα2. This is the first study to demonstrate that: (1) SFN prevents DN through activation of AMPKα2; (2) SFN-activated Nrf2-transcriptional function is mediated by AMPKα2 via Akt/GSK3β/Fyn pathway; and (3) SFN-mediated renal protection persisted even after three months of SFN withdrawal. Taken together, these findings suggest that SFN is an effective and safe strategy for the treatment of DN, and may be applicable in the clinic. This study further proposed and elucidated a novel mechanism for SFN treatment of DN, laying the groundwork for future clinical application of SFN.
Clinical perspectives

Derangements in lipid metabolism play the crucial role in the development and progression of diabetic nephropathy (DN). SFN can improve lipotoxicity accompanied with the activation of AMPK pathway. This study was conducted to define whether SFN can be used as an activator of AMPK to prevent DN.

The present study indicates that SFN prevented renal injury in T2D by improving renal lipotoxicity and Nrf2 function, which are both dependent on AMPKα2. More importantly, the protective effect by SFN can persist even for three 3 months after stopping SFN treatment.

This study reveals a novel mechanism by which SFN prevents DN. Clinically, the long-time and sustained AMPK activation by SFN maybe not only a promising therapeutic strategy for DN prevention, but also one of alternatives of metformin, a clinic drug that significantly increases AMPK function.

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Competing interests

The authors declare that they have no competing interests.

Authors’ contributions

H. G, L.N.M., Z.G.Z. and L.C. originally designed the project. Z.L. and H.G. performed experiments, analyzed data, and wrote the manuscript draft. Z.L., J.L., T.J.M. and S.S.Z. performed partial
experiments and data collection. L.C. was responsible for scientific review and manuscript editing.

L.N.M. and L.C. monitored the project progression, modified the experimental designs and manuscript revision. All authors approved the final version of the manuscript.

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Appendix A. Supplementary data

Supplementary data to this article can be found here. Supplementary Materials. pdf

Abbreviations

SFN, sulforaphane; DN, diabetic nephropathy; T2D, type 2 diabetes; Nrf2, nuclear factor (erythroid-derived 2)-like 2; AMPK, AMP-activated protein kinase; WT, wild type; HFD, high-fat diet; STZ, streptozotocin; ND, normal diet; IP, intraperitoneal; IPGTT, intraperitoneal glucose tolerance test; AUC, the area under the curve; PAS, periodic acid-Schiff; IHC immunohistochemical; PCR, polymerase chain reaction; FN, fibronectin; TGF-β1, transforming growth factor beta-β1; COL-1, collagen-1; TNFα, tumor necrosis factor-α; CPT-1α, carnitine palmitoyl transferase-1α; SREBP-1, sterol regulatory element-binding protein 1; SCD-1, stearoyl-CoA desaturase-1; 3-NT, 3-nitrotyrosine; 4-HNE, 4-hydroxy-2-nonenal; PAI-1, plasminogen activator inhibitor-1; ACC, Acetyl-CoA
Carboxylase; PPARα, peroxisome proliferator-activated receptor-α; SIRT1, sirtuin1; PGC-1α, peroxisome proliferator-activated receptor gamma coactivator 1-alpha; GSK, glycogen synthase kinase; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; Nqo1, NAD(P)H: quinone oxidoreductase; Hmox1, heme oxygenase 1; Cat, catalase; Sod2, Superoxide dismutase 2; YWHAZ, phospholipaseA2; Gapdh, glyceraldehyde 3-phosphate dehydrogenase; SD, standard deviation; FAO, fatty acid oxidation;
References

[1] Kang YS, Lee MH, Song HK, Ko GJ, Kwon OS, Lim TK, et al. (2010) CCR2 antagonism improves insulin resistance, lipid metabolism, and diabetic nephropathy in type 2 diabetic mice. *Kidney international*. **78**, 883-94, https://doi.org/10.1038/ki.2010.263

[2] Lieben L. (2017) Diabetic nephropathy: Lipid toxicity drives renal disease. *Nature reviews Nephrology*. **13**, 194, https://doi.org/10.1038/nrneph.2017.22

[3] Herman-Edelstein M, Scherzer P, Tobar A, Levi M, Gafter U. (2014) Altered renal lipid metabolism and renal lipid accumulation in human diabetic nephropathy. *Journal of lipid research*. **55**, 561-72, https://doi.org/10.1016/j.jlr.P040501

[4] Wu H, Kong L, Cheng Y, Zhang Z, Wang Y, et al. (2015) Metallothionein plays a prominent role in the prevention of diabetic nephropathy by sulforaphane via up-regulation of Nrf2. *Free radical biology & medicine*. **89**, 431-42, https://doi.org/10.1016/j.freeradbiomed.2015.08.009

[5] Carolina Guerini de Souza aLLdM, c Adrian Martimbianco de Assis, c Anderson Rech,c Ricardo Bruch,d Fábio Klamtc and Diogo Onofre Souzac. (2016) Sulforaphane ameliorates the insulin responsiveness and the lipid profile but does not alter the antioxidant response in diabetic rats. *Food Function*. **7**, 2060-5, https://doi.org/10.1039/C5FO01620G

[6] Choi KM, Lee YS, Kim W, Kim SJ, Shin KO, Yu JY, et al. (2014) Sulforaphane attenuates obesity by inhibiting adipogenesis and activating the AMPK pathway in obese mice. *The Journal of nutritional biochemistry*. **25**, 201-7, https://doi.org/10.1016/j.jnutbio.2013.10.007

[7] Viollet B, Andreelli F, Jørgensen SB, Perrin C, Flamez D, Mu J, Wojtaszewski JF, Schuit FC, et al. (2003) Physiological role of AMP-activated protein kinase (AMPK): insights from knockout mouse models, *Biochem Soc Trans*. **31**, 216-9, https://doi.org/10.1042/bst0310216

[8] Feng Y, Wang S, Zhang Y, Xiao H. (2017) Metformin attenuates renal fibrosis in both AMPKalpha2-dependent and independent manners. *Clinical and experimental pharmacology & physiology*. **44**, 648-55, https://doi.org/10.1111/1440-1681.12748

[9] Qiu S, Xiao Z, Piao C, Zhang J, Dong Y, Cui W, et al. (2015) AMPKalpha2 reduces renal epithelial transdifferentiation and inflammation after injury through interaction with CK2beta. *The Journal of pathology*. **237**, 330-42, https://doi.org/10.1002/path.4579

[10] Yang D, Livingston MJ, Liu Z, Dong G, Zhang M, Chen JK, et al. (2018) Autophagy in diabetic
kidney disease: regulation, pathological role and therapeutic potential. *Cellular and molecular life sciences: CMLS*. **75**, 669-88, https://doi.org/10.1007/s00018-017-2639-1

[11] Qu LL, Yu B, Li Z, Jiang WX, et al. (2016) Gastrodin Ameliorates Oxidative Stress and Proinflammatory Response in Nonalcoholic Fatty Liver Disease through the AMPK/Nrf2 Pathway. *Phytotherapy research: PTR*. **30**, 402-11, https://doi.org/10.1002/ptr.5541

[12] Hong YA, Lim JH, Kim MY, et al. (2018) Extracellular Superoxide Dismutase Attenuates Renal Oxidative Stress Through the Activation of Adenosine Monophosphate-Activated Protein Kinase in Diabetic Nephropathy. *Antioxidants & redox signaling*. **28**, 1543-61. https://doi.org/10.1089/ars.2017.7207

[13] Habib SL, Yadav A, Kidane D, Weiss RH, Liang S. (2016) Novel protective mechanism of reducing renal cell damage in diabetes: Activation AMPK by AICAR increased NRF2/OGG1 proteins and reduced oxidative DNA damage. *Cell cycle*. **15**, 3048-59, https://doi.org/10.1080/15384101.2016.1231259

[14] Wang L, Zhang S, Cheng H, Lv H, et al. (2016) Nrf2-mediated liver protection by esculentoside A against acetaminophen toxicity through the AMPK/Akt/GSK3beta pathway. *Free radical biology & medicine*. **101**, 401-12, https://doi.org/10.1016/j.freeradbiomed.2016.11.009

[15] Wang Y, Zhang Z, Guo W, et al. (2014) Sulforaphane reduction of testicular apoptotic cell death in diabetic mice is associated with the upregulation of Nrf2 expression and function. *Am J Physiol Endocrinol Metab*. **307**, E14-23, https://doi.org/10.1152/ajpendo.00702.2013

[16] Cui W, Bai Y, Miao X, Luo P, Chen Q, Tan Y, et al. (2012) Prevention of diabetic nephropathy by sulforaphane: possible role of Nrf2 upregulation and activation. *Oxidative medicine and cellular longevity*. **2012**, 821936, https://doi.org/10.1155/2012/821936

[17] Shawky NM, Pichavaram P, Shehatou GS, Suddek GM, et al. (2016) Sulforaphane improves dysregulated metabolic profile and inhibits leptin-induced VSMC proliferation: Implications toward suppression of neointima formation after arterial injury in western diet-fed obese mice. *Nutr Biochem*. **32**, 73-84, https://doi.org/10.1016/j.nutbio.2016.01.009

[18] Yang Mu, Tai-Lang Yin, Xiao-Xuan Huang, et al. (2019) Sulforaphane ameliorates high-fat diet-induced spermatogenic deficiency in mice. *Biol Reprod*. **101**, 223-234, https://doi.org/10.1093/biolre/ioz067
[19] John D Clarke, Anna Hsu, David E Williams, et al. (2011) Metabolism and tissue distribution of sulforaphane in Nrf2 knockout and wild-type mice. Pharm Res. 28, 3171-9, https://doi.org/10.1007/s11095-011-0500-z

[20] Jin-Sun Kong, Seung-Ah Yoo, Hyun-Sook Kim, et al. (2010) Inhibition of synovial hyperplasia, rheumatoid T cell activation, and experimental arthritis in mice by sulforaphane, a naturally occurring isothiocyanate. Arthritis Rheum. 62, 159-70, https://doi.org/10.1002/art.25017

[21] Cui W, Wang Y, Chen Q, Sun W, Cai L, Tan Y, et al. (2013) Magnolia extract (BL153) ameliorates kidney damage in a high fat diet-induced obesity mouse model. Oxidative medicine and cellular longevity. 2013, 367040, https://doi.org/10.1155/2013/367040

[22] Bai Y, Cui W, Xin Y, Miao X, Barati MT, Zhang C, et al. (2013) Prevention by sulforaphane of diabetic cardiomyopathy is associated with up-regulation of Nrf2 expression and transcription activation. Journal of molecular and cellular cardiology. 57, 82-95, https://doi.org/10.1016/j.yjmcc.2013.01.008

[23] Zhang Z, Chen J, Zhou S, et al. (2015) Magnolia bioactive constituent 4-O-methylhonokiol prevents the impairment of cardiac insulin signaling and the cardiac pathogenesis in high-fat diet-induced obese mice. International journal of biological sciences. 11, 879-91, https://doi.org/10.7150/ijbs.12101

[24] Wang Y, Feng W, Xue W, Tan Y, Hein DW, Li XK, et al. (2009) Inactivation of GSK-3beta by metallothionein prevents diabetes-related changes in cardiac energy metabolism, inflammation, nitrosative damage, and remodeling. Diabetes. 58, 1391-402, https://doi.org/10.2337/db08-1697

[25] Zhang Z, Wang S, Zhou S, Yan X, Wang Y, et al. (2014) Sulforaphane prevents the development of cardiomyopathy in type 2 diabetic mice probably by reversing oxidative stress-induced inhibition of LKB1/AMPK pathway. Journal of molecular and cellular cardiology. 77, 42-52, https://doi.org/10.1016/j.yjmcc.2014.09.022

[26] Kim Y, Lim JH, Kim MY, Kim EN, Yoon HE, Shin SJ, et al. (2018) The Adiponectin Receptor Agonist AdipoRon Ameliorates Diabetic Nephropathy in a Model of Type 2 Diabetes. Journal of the American Society of Nephrology : JASN. 29,1108-27, https://doi.org/10.1681/ASN.2017060627
[27] Wang S, Moustaid-Moussa N, Chen L, Mo H, Shastri A, Su R, et al. (2014) Novel insights of dietary polyphenols and obesity. *The Journal of nutritional biochemistry*. **25**, 1-18, https://doi.org/10.1016/j.jnutbio.2013.09.001

[28] Gross JL, de Azevedo MJ, Silveiro SP, Canani LH, Caramori ML, Zelmanovitz T, (2005) Diabetic nephropathy: diagnosis, prevention, and treatment. *Diabetes Care*. **28**, 164-76, https://doi.org/10.2337/diacare.28.1.164

[29] Yang G, Lee HE, Lee JY. (2016) A pharmacological inhibitor of NLRP3 inflammasome prevents non-alcoholic fatty liver disease in a mouse model induced by high fat diet. *Scientific reports*. **6**, 24399, https://doi.org/10.1038/srep24399

[30] Guebre-Egziabher F, Alix PM, Koppe L, Pelletier CC, Kalbacher E, Fouque D, et al. (2013) Ectopic lipid accumulation: A potential cause for metabolic disturbances and a contributor to the alteration of kidney function. *Biochimie*. **95**, 1971-9, https://doi.org/10.1016/j.biochi.2013.07.017

[31] Kampe K, Sieber J, Orellana JM, Mundel P, Jehle AW. (2014) Susceptibility of podocytes to palmitic acid is regulated by fatty acid oxidation and inversely depends on acetyl-CoA carboxylases 1 and 2. *American journal of physiology Renal physiology*. **306**, F401-9, https://doi.org/10.1152/ajprenal.00454.2013

[32] Jung TW, Kim HC, Abd El-Aty AM, Jeong JH. (2017) Protectin DX ameliorates palmitate- or high-fat diet-induced insulin resistance and inflammation through an AMPK-PPARalpha-dependent pathway in mice. *Scientific reports*. **7**, 1397, https://doi.org/10.1038/s41598-017-01603-9

[33] Kim HJ, Yuan J, Norris K, Vaziri ND. (2010) High-calorie diet partially ameliorates dysregulation of intrarenal lipid metabolism in remnant kidney. *The Journal of nutritional biochemistry*. **21**, 999-1007, https://doi.org/10.1016/j.jnutbio.2009.08.006

[34] Kim MY, Lim JH, Youn HH, Hong YA, Yang KS, Park HS, et al. (2013) Resveratrol prevents renal lipotoxicity and inhibits mesangial cell glucotoxicity in a manner dependent on the AMPK-SIRT1-PGC1alpha axis in db/db mice. *Diabetologia*. **56**, 204-17, http://dx.doi.org/10.1007/s00125-012-2791-y.
[35] Lin YC, Wu MS, Lin YF, Chen CR, Chen CY, Chen CJ, et al. (2019) Nifedipine Modulates Renal Lipogenesis via the AMPK-SREBP Transcriptional Pathway. *Int J Mol Sci*. 20, E1570, https://doi.org/10.3390/ijms20071570

[36] Carobbio S, Hagen RM, et al. (2013) Adaptive changes of the Insig1/SREBP1/SCD1 set point help adipose tissue to cope with increased storage demands of obesity. *Diabetes*. 62, 3697-708, https://doi.org/10.2337/db12-1748

[37] Choi SR, Lim JH, Kim MY, et al. (2018) Adiponectin receptor agonist AdipoRon decreased ceramide, and lipotoxicity, and ameliorated diabetic nephropathy. *Metabolism: clinical and experimental*. 85, 348-60, https://doi.org/10.1016/j.metabol.2018.02.004

[38] Jain AK, Jaiswal AK. (2007) GSK-3beta acts upstream of Fyn kinase in regulation of nuclear export and degradation of NF-E2 related factor 2. *The Journal of biological chemistry*. 282, 16502-10, https://doi.org/10.1074/jbc.M611336200

[39] Li B, Cui W, Tan Y, et al. (2014) Zinc is essential for the transcription function of Nrf2 in human renal tubule cells in vitro and mouse kidney in vivo under the diabetic condition. *Journal of cellular and molecular medicine*. 18, 895-906, https://doi.org/10.1111/jcmm.12239

[40] Zheng H, Whitman SA, Wu W, et al. (2011) Therapeutic potential of Nrf2 activators in streptozotocin-induced diabetic nephropathy. *Diabetes*. 60, 3055-66, https://doi.org/10.2337/db11-0807

[41] Jiang T, Huang Z, Lin Y, Zhang Z, Fang D, Zhang DD. (2010) The protective role of Nrf2 in streptozotocin-induced diabetic nephropathy. *Diabetes*. 59, 850-60, https://doi.org/10.2337/db09-1342

[42] Wu F, Zhang W, Li L, Zheng F, Shao X, Zhou J, Li H. (2011) Inhibitory effects of honokiol on lipopolysaccharide-induced cellular responses and signaling events in human renal mesangial cells. *Eur J Pharmacol*. 654, 117-21, https://doi.org/10.1016/j.ejphar.2010.11.022

[43] Ganesh Yerra V, Negi G, Sharma SS, Kumar A. (2013) Potential therapeutic effects of the simultaneous targeting of the Nrf2 and NF-kappaB pathways in diabetic neuropathy. *Redox biology*. 1, 394-7, https://doi.org/10.1016/j.redox.2013.07.005

[44] Saber S, Mahmoud AAA, Helal NS, et al. (2018) Renin-angiotensin System Inhibition
Ameliorates CCl₄-induced Liver Fibrosis in Mice Through the Inactivation of Nuclear Transcription Factor Kappa B. Can J Physiol Pharmacol. 96, 569-576,
https://doi.org/10.1139/cjpp-2017-0728

[45] Kato M, Natarajan R. (2019) Epigenetics and epigenomics in diabetic kidney disease and metabolic memory. Nature reviews Nephrology. 15, 327-45,
https://doi.org/10.1038/s41585-019-0227-8

[46] Wen F, An C, Wu X, et al. (2018) MiR-34a regulates mitochondrial content and fat ectopic deposition induced by resistin through the AMPK/PPARalpha pathway in HepG2 cells. The international journal of biochemistry & cell biology. 94, 133-45,
https://doi.org/10.1016/j.biocel.2017.11.008

[47] Loboda A, Stachurska A, Sobczak M, et al. (2017) Nrf2 deficiency exacerbates ochratoxin A-induced toxicity in vitro and in vivo. Toxicology. 389, 42-52,
https://doi.org/10.1016/j.tox.2017.07.004

[48] Lan F, Pan Q, Yu H, Yue X. (2015) Sulforaphane enhances temozolomide-induced apoptosis because of down-regulation of miR-21 via Wnt/beta-catenin signaling in glioblastoma. Journal of Neurochemistry. 134, 811-8, https://doi.org/10.1111/jnc.13174

[49] Loboda A, Sobczak M, Jozkowicz A, Dulak J. (2016) TGF-beta1/Smads and miR-21 in Renal Fibrosis and Inflammation. Mediators of inflammation. 2016, 8319283,
https://doi.org/10.1155/2016/8319283

[50] Kim S, Lee MS, Jung S, et al. (2018) Ginger Extract Ameliorates Obesity and Inflammation via Regulating MicroRNA-21/132 Expression and AMPK Activation in White Adipose Tissue. Nutrients. 10, E1567, https://doi.org/10.3390/nu10111567

[51] Axelsson AS, Tubbs E, Mecham B, Chacko S, et al. (2017) Sulforaphane reduces hepatic glucose production and improves glucose control in patients with type 2 diabetes. Sci Transl Med. 14, 9(394), https://doi.org/10.1126/scitranslmed.aah4477

[52] Zhou ZY, Ren LW, Zhan P, Yang HY, Chai DD, Yu ZW. (2016) Metformin exerts glucose-lowering action in high-fat fed mice via attenuating endotoxemia and enhancing insulin signaling. Acta pharmacologica Sinica. 37, 1063-75, https://doi.org/10.1038/aps.2016.21
Figure legends

Figure 1: Comparison for the general characteristics between WT mice and Ampkα2−/− mice and AMPKα2 deletion abolished SFN protective effects against diabetes-induced renal function damage. (A) Body weight and (B) kidney-weight-to-tibia length ratios were measured in both wild type (WT) mice and AMPKα2 knockout (Ampkα2−/−) mice. (C-D) 24-h urine albumin levels were detected at the 3rd month of T2D (the end of SFN treatment, 3M) as well as at the 6th month of T2D (3 months after cessation of SFN treatment, 6M). Data are represented as mean ± SD (n=5 in each group for WT mice; n = 4 in KO-Ctrl; n=7 in KO-T2D; n=6 in KO-T2D/SFN for Ampkα2−/− mice). One-way ANOVA: * p < 0.05 vs. WT-Ctrl, # p < 0.05 vs. WT-T2D; & p < 0.05 vs. KO-Ctrl.

Figure 2: The prevention of T2D-induced renal pathological abnormalities and fibrotic responses by SFN in WT mice was lost in Ampkα2−/− mice. (A) Renal mesangial matrix expansion and glomerular hypertrophy were assessed by periodic Acid-Schiff (PAS) staining (scale bar = 50 µm). The outline of the glomerular tuft was marked by a polygon (green). The representative mesangial matrix expansion was marked by red arrows in a diabetic glomerular. (B-C) Quantitative analysis of (B) glomerular area and (C) glomerular mesangial matrix based on PAS staining. (D) The fold changes of diabetes-induced glomerular hypertrophy and mesangial matrix expansion between WT mice and Ampkα2−/− mice relative to controls. (E) Renal fibrosis, determined by Sirius Red staining of collagen accumulation (collagen is red; scale bar =100 µm). The outline of the glomerular tuft was marked by a polygon (red). (F) Quantitative analysis of Sirius Red staining for collagen accumulation. Data are represented as mean ± SD (n=5 in each group for WT mice; n = 4 in KO-Ctrl; n=7 in KO-T2D; n=6 in KO-T2D/SFN for Ampkα2−/− mice). One-way ANOVA: * p < 0.05 vs. WT-Ctrl, # p < 0.05 vs. WT-T2D; & p < 0.05 vs. KO-Ctrl.
0.05 vs. WT-T2D; & p < 0.05 vs. KO-Ctrl.

Figure 3: AMPKα2 deletion abolished SFN protection against diabetes-induced renal fibrosis. (A-B) Protein levels of (A) transforming growth factor beta-1 (TGF-β1) and (B) fibronectin (FN) were quantitatively measured by Western blot. (C-D) Representative IHC staining for (C) fibronectin (FN) and (D) collagen 1 (COL-1) in kidney sections (scale bar = 50 µm). The brown color indicates the positive expression of FN and COL-1. The outline of the glomerular tuft was marked by a polygon (red). Data are represented as mean ± SD (n=5 in each group for WT mice; n = 4 in KO-Ctrl; n=7 in KO-T2D; n=6 in KO-T2D/SFN for Ampkα2−/− mice). One-way ANOVA: * p < 0.05 vs. WT-Ctrl, # p < 0.05 vs. WT-T2D; & p < 0.05 vs. KO-Ctrl.

Figure 4: AMPKα2 deletion abolished SFN protection against diabetes-induced renal inflammation, oxidative damage. (A-B) Protein levels of (A) plasminogen activator inhibitor-1 (PAI-1), tumor necrosis factor-α (TNF-α) and (B) the ratio of phospho-nuclear factor kappa-light-chain-enhancer of activated B cells p65 (p-NF-κB) to total-NF-κB p65 (t-NF-κB) in renal nuclear fraction, the phospho-residue is Ser 536, were quantitatively measured by Western blot. (C-D) 3-nitrotyrosine (3-NT), and 4-hydroxy-2-nonenal (4-HNE) were quantitatively measured by Western blot. (E) Level of nitric oxide in kidney tissues was measured with BioAssay Systems. Data are represented as mean ± SD (n=5 in each group for WT mice; n = 4 in KO-Ctrl; n=7 in KO-T2D; n=6 in KO-T2D/SFN for Ampkα2−/− mice). One-way ANOVA: * p < 0.05 vs. WT-Ctrl, # p < 0.05 vs. WT-T2D; & p < 0.05 vs. KO-Ctrl.
Figure 5: SFN prevented diabetes-induced renal lipid accumulation by preserving AMPK activation and its downstream targets regulation while AMPKα2 deletion abolished the protection of SFN against renal lipotoxicity. (A) Renal lipid accumulation was detected by Oil Red O staining (scale bar = 50 µm) in cryosections, and area of Oil Red O staining was quantified as fold of control. The red color indicates the lipid droplets. The outline of the glomerular tuft was marked by a polygon (red) and renal tubules was marked by arrows (black). (B-E) Western blot was used to measure (B) ratio of phospho AMP-activated protein kinase (p-AMPKα) to total AMP-activated protein kinase (t-AMPKα), (C) peroxisome proliferator-activated receptor-α (PPARα) protein level, (D) ratio of phospho Acetyl-CoA Carboxylase (p-ACC) to total Acetyl-CoA Carboxylase (t-ACC), (E) carnitine palmitoyl transferase-1α (CPT-1α) protein level. Data are represented as mean ± SD (n=5 in each group for WT mice; n = 4 in KO-Ctrl; n=7 in KO-T2D; n=6 in KO-T2D/SFN for Ampkα2−/− mice). One-way ANOVA: * p < 0.05 vs. WT-Ctrl, # p < 0.05 vs. WT-T2D; & p < 0.05 vs. KO-Ctrl.

Figure 6: AMPKα2 deletion abolished the improvement of SFN against T2D-induced aberrant renal lipid metabolism. (A-D) Western blot was used to measure protein levels of (A) sirtuin1 (SIRT1), (B) peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α), (C) sterol regulatory element-binding protein 1 (SREBP-1) and (D) stearoyl-CoA desaturase-1 (SCD1). Data are represented as mean ± SD (n=5 in each group for WT mice; n = 4 in KO-Ctrl; n=7 in KO-T2D; n=6 in KO-T2D/SFN for Ampkα2−/− mice). One-way ANOVA: * p < 0.05 vs. WT-Ctrl, # p < 0.05 vs. WT-T2D; & p < 0.05 vs. KO-Ctrl.

Figure 7: AMPKα2 deletion abolished the up-regulation of Nrf2 protein abundance and
transcription activation by SFN in diabetic kidney. (A) Protein levels of nuclear factor (erythroid-derived 2)-like 2 (Nrf2) in renal nuclear fraction were measured by Western blot and quantified. The nuclear Nrf2 in the western blot image is shown at around 100 kDa. The mRNA levels of Nrf2 target genes NAD(P)H: quinone oxidoreductase (Nqo1), catalase (Cat), heme oxygenase 1 (Hmox1) and superoxide dismutase 2 (Sod2) relative to the genome of 3 different housekeeping genes including β-actin, YWHAZ and Gapdh in (B-E) WT mice and (F-I) Ampkα2−/− mice were measured by real-time polymerase chain reaction (PCR). Data are represented as mean ± SD (n=5 in each group for WT mice; n = 4 in KO-Ctrl; n=7 in KO-T2D; n=6 in KO-T2D/SFN for Ampkα2−/− mice). One-way ANOVA: * p < 0.05 vs. WT-Ctrl, # p < 0.05 vs. WT-T2D; & p < 0.05 vs. KO-Ctrl.

Figure 8: Nrf2 activation by SFN is mediated through AMPK/Akt/GSK3β/Fyn pathway. (A-C) The activation of AKT in both WT and AMPKα2−/− mice were examined by Western blot for (A) the ratio of phospho-AKT (p-AKT) to total-AKT (t-AKT), the phospho-residue is Ser473, (B) glycogen synthase kinase (GSK)-3β phosphorylation at Ser9, and (C) the expression of Fyn in renal nuclear fraction. Data are represented as mean ± SD (n=5 in each group for WT mice; n = 4 in KO-Ctrl; n=7 in KO-T2D; n=6 in KO-T2D/SFN for Ampkα2−/− mice). One-way ANOVA: * p < 0.05 vs. WT-Ctrl, # p < 0.05 vs. WT-T2D; & p < 0.05 vs. KO-Ctrl.

Figure 9: Putative mechanism by which SFN alleviates T2D-induced nephropathy. In type 2 diabetes (T2D), excess fatty acid uptake increases lipid droplet (LD) accumulation, which induces oxidative stress, inflammation and fibrosis, contributing to development of diabetic nephropathy (DN). Diabetes impairs activation of the AMP-activated protein kinase (AMPK) pathway and consequently...
inactivation of nuclear factor (erythroid-derived 2)-like 2 (Nrf2) function by suppressing AKT/GSK-3β/Fyn pathway. The Nrf2 inactivation is accompanied by the increased NF-κB activation, which can increase the renal inflammatory and fibrotic response. SFN treatment in T2D mice significantly decreases lipid accumulation and up-regulates the Nrf2 antioxidant pathway by activating AMPK. The activated AMPK increases lipolysis, which might degrade intracellular lipid droplets into fatty acids for subsequent oxidation. Second, activated AMPK inhibits Acetyl-CoA Carboxylase (ACC) and malonyl-CoA and up-regulates peroxisome proliferator-activated receptor-α (PPARα) to increase carnitine palmitoyl transferase-1α (CPT-1) levels. Consequently, CPT-1 mediates mitochondrial fatty acid uptake, and is a rate-limiting step of fatty acid oxidation. Finally, activated AMPK increases activity of sirtuin1 (SIRT1) and peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α), which may increase fatty acid oxidation (FAO), and reduces fatty acid synthases sterol regulatory element-binding protein 1 (SREBP-1) and stearoyl-CoA desaturase-1 (SCD-1). Furthermore, Nrf2-mediated anti-oxidative pathway is most likely mediated by stimulating AMPK-dependent activation of AMPK/AKT/GSK3β/Fyn pathway. Therefore, SFN protects T2D-induced nephropathy in AMPKα2-dependent manner, and the effects are achieved through two ways: (1) activating AMPK-mediated lipid metabolism and (2) enhancing Nrf2-mediated anti-oxidative effects. →, activation; ┤, inhibition.
Figure 1

A

Body weight (g)

WT  Ampkα2−/−

B

Kidney Weight/Tibia Length (mg/mm)

WT  Ampkα2−/−

C

24h Albumin (lg/ug)

WT  Ampkα2−/−

D

24h Albumin (lg/ug)

WT  Ampkα2−/−

3M  6M

Ctrl  Ctrl/SFN  T2D  T2D/SFN

*  &  #  &
Figure 2

A) WT-Ctrl | WT-Ctrl/SFN | WT-T2D | WT-T2D/SFN

PAS staining showing increased mesangial matrix expansion in WT-T2D/SFN compared to WT-Ctrl and WT-T2D.

B) Graph showing glomerular area (fold of ctrl) in WT and Ampkα2-/- mice. Ctrl/SFN group shows a significant increase in WT, while Ampkα2-/- shows a significant increase over Ctrl and Ctrl/SFN.

C) Graph showing mesangial matrix expansion (fold of ctrl) in WT and Ampkα2-/- mice. Similar to glomerular area, Ampkα2-/- shows a significant increase over WT and Ctrl/SFN.

D) Table comparing fold increase by DM for glomerular area and mesangial matrix expansion between WT and Ampkα2-/-.

E) Sirius red staining showing increased collagen deposition in WT-T2D/SFN compared to WT-Ctrl and WT-T2D.

F) Graph showing Sirius red staining (fold of ctrl) in WT and Ampkα2-/- mice. Ampkα2-/- shows a significant increase over WT and Ctrl/SFN.
Figure 4

A. Western blots showing PAI-1, β-Actin, TNF-α, and β-Actin in WT and Ampkα2−/− mice under control (Ctrl) and T2D/SFN conditions. Graphs depict fold change in protein expression with error bars.

B. Western blots for p-NF-κB and t-NF-κB in WT and Ampkα2−/− mice under control (Ctrl) and T2D/SFN conditions. Graphs show fold change in protein expression with error bars.

C. Western blots for 3NT and β-Actin in WT and Ampkα2−/− mice under control (Ctrl) and T2D/SFN conditions. Graphs depict fold change in protein expression with error bars.

D. Western blots for 4HNE and β-Actin in WT and Ampkα2−/− mice under control (Ctrl) and T2D/SFN conditions. Graphs show fold change in protein expression with error bars.

E. Graphs showing nitric oxide (µM) levels in WT and Ampkα2−/− mice under control (Ctrl), Ctrl/SFN, T2D, and T2D/SFN conditions. Graphs depict fold change in nitric oxide levels with error bars.

* indicates significant difference compared to WT Ctrl
# indicates significant difference compared to WT T2D
& indicates significant difference compared to Ampkα2−/− Ctrl
Figure 7

A

WT Ampkα2−/−

| Nrf2 | Histone H3 |
|------|------------|
| Ctrl | Ctrl/SFN  | T2D | T2D/SFN |
| Ctrl | T2D       |     |         |

B

NQO1 mRNA (fold of ctrl)

- NQO1/β-Actin
- NQO1/YMHAZ
- NQO1/GAPDH

C

CAT mRNA (fold of ctrl)

- CAT/β-Actin
- CAT/YMHAZ
- CAT/GAPDH

D

Hmox1 mRNA (fold of ctrl)

- Hmox1/β-Actin
- Hmox1/YMHAZ
- Hmox1/GAPDH

E

SOD2 mRNA (fold of ctrl)

- SOD2/β-Actin
- SOD2/YMHAZ
- SOD2/GAPDH

F

NQO1 mRNA (fold of ctrl)

- NQO1/β-Actin
- NQO1/YMHAZ
- NQO1/GAPDH

G

CAT mRNA (fold of ctrl)

- CAT/β-Actin
- CAT/YMHAZ
- CAT/GAPDH

H

Hmox1 mRNA (fold of ctrl)

- Hmox1/β-Actin
- Hmox1/YMHAZ
- Hmox1/GAPDH

I

SOD2 mRNA (fold of ctrl)

- SOD2/β-Actin
- SOD2/YMHAZ
- SOD2/GAPDH
Figure 8

A. Western blot analysis of p-AKT and t-AKT levels in WT and Ampkα2−/− mice treated with different diets.

B. Western blot analysis of p-GSK-3β and t-GSK-3β levels in WT and Ampkα2−/− mice treated with different diets.

C. Western blot analysis of Fyn and Histone H3 levels in WT and Ampkα2−/− mice treated with different diets.

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SUPPLEMENTARY MATERIALS

Sulforaphane prevents type 2 diabetes-induced nephropathy via AMPK-mediated activation of lipid metabolic pathways and Nrf2 anti-oxidative function

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Supplementary Figure 1: Animal experimental design. (A) 8 week-old male C57BL6/J (WT) mice and (B) \(Ampka2^{−/−}\) mice were fed a high-fat diet (HFD) or normal diet (ND) for three months, and HFD mice were then injected once with low-dose streptozotocin (STZ) (100 mg/kg). In parallel, age-matched ND mice were injected with the same volume of vehicle (sodium citrate buffer) to match the injected STZ. Seven days after STZ injection, mice with hyperglycemia (3h fasting blood glucose \(\geq 250 \text{ mg/dL}\)) were defined as diabetic (T2D). Once diabetes was confirmed, both diabetic and non-diabetic mice were injected subcutaneously with either sulforaphane (SFN) or vehicle for three months. Then, all WT and \(Ampka2^{−/−}\) mice were kept for three more months without SFN treatment. Green arrow represents three months of SFN treatment after diabetes was confirmed. (C) The experimental timeline schematic.
Supplementary Figure 2

A

WT

Blood Glucose (mg/dl)

0 100 200 300 400 500

0 min 15 min 30 min 60 min 120 min

Ampkα 2−/−

Blood Glucose (mg/dl)

0 100 200 300 400 500

0 min 15 min 30 min 60 min 120 min

B

Area under the curve

(mg/dl x min)

10000 20000 30000 40000 50000

WT  Ampkα 2−/−

C

S F N treatment

Blood Glucose (mg/dl)

WT

1 m 2 m 3 m 4 m 6 m

D

S F N treatment

Blood Glucose (mg/dl)

1 m 2 m 3 m 4 m 6 m

Ampkα 2−/−

6M WT/6M Ampkα2−/− (C57BL/6J)

E

WT

Ampkα2−/−

AMPKα2

β-Actin

WT

Ampkα2−/−

AMPKα2

β-Actin

WT

Ampkα2−/−

AMPKα2

β-Actin
Supplementary Figure 2. Effects of HFD/STZ on blood glucose and glucose tolerance, and renal expression of total AMPKα2. (A) Insulin resistance of wild type (WT) mice and Ampkα2−/− mice was assessed by intraperitoneal glucose tolerance testing (IPGTT; glucose 2g/kg, body weight) at three months after normal diet (ND) or high-fat diet (HFD) (B) The integrated area under the curves (AUC) of IPGTT was also calculated to analyze the effect of HFD on glucose tolerance. Data are represented as mean ± SD (n=10 in each group for WT mice; n=4 in ND, n=13 in HFD for Ampkα2−/− mice). One-way ANOVA: * p < 0.05 vs. WT-ND, & p < 0.05 vs. KO-ND (C-D) Dynamic blood glucose of (C) WT and (D) Ampkα2−/− mice was measured. (E) Total AMPKα2 protein levels were measured by Western blot in both WT and Ampkα2−/− mice. Data are represented as mean ± SD (n=5 in each group for WT mice; n=4 in KO-Ctrl; n=7 in KO-T2D; n=6 in KO-T2D/SFN for Ampkα2−/− mice). One-way ANOVA: * p < 0.05 vs. WT-Ctrl, # p < 0.05 vs. WT-T2D; & p < 0.05 vs. KO-Ctrl.