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Metallothionein plays a prominent role in the prevention of diabetic nephropathy by sulforaphane via up-regulation of Nrf2

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Abstract

Sulforaphane (SFN) prevents diabetic nephropathy (DN) in type 1 diabetes via up-regulation of nuclear factor (erythroid-derived 2)-like 2 (Nrf2). However, it has not been addressed whether SFN also prevents DN from type 2 diabetes or which Nrf2 downstream gene(s) play(s) the key role in SFN renal protection. Here we investigated whether Nrf2 is required for SFN protection against type 2 diabetes-induced DN and whether metallothionein (MT) is an Nrf2 downstream antioxidant using Nrf2 knockout (Nrf2-null) mice. In addition, MT knockout mice were used to further verify if MT is indispensable for SFN protection against DN. Diabetes-increased albuminuria, renal fibrosis, and inflammation were significantly prevented by SFN, and Nrf2 and MT expression was increased. However, SFN renal protection was completely lost in Nrf2-null diabetic mice, confirming the pivotal role of Nrf2 in SFN protection from type 2 diabetes-induced DN. Moreover, SFN failed to up-regulate MT in the absence of Nrf2, suggesting that MT is an Nrf2 downstream antioxidant. MT deletion resulted in a partial, but significant attenuation of SFN renal protection from type 2 diabetes, demonstrating a partial requirement for MT for SFN renal protection. Therefore, the present study demonstrates for the first time that as an Nrf2 downstream antioxidant, MT plays an important, though partial, role in mediating SFN renal protection from type 2 diabetes.

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No competing financial interests exist.

Keywords

Diabetes; Kidney; Oxidative stress; Inflammation; Fibrosis

Introduction

Diabetic nephropathy (DN) is the leading cause of end-stage renal failure worldwide but still cannot be effectively treated [1, 2]. Oxidative stress is considered to be its main cause [3]. Nuclear factor (erythroid-derived 2)-like 2 (Nrf2) plays a central role in antioxidant–redox signaling [4, 5]. By binding antioxidant response element (ARE) within the promoter of its downstream genes, such as heme oxygenase 1 and NAD (P) H-quinone oxidoreductase 1 (NQO1), Nrf2 turns on the transcription of these antioxidants [6–8]. Therefore, inducing Nrf2 expression and function is a promising strategy for preventing or slowing down the progression of DN.

Sulforaphane (SFN), a potent Nrf2 activator [9, 10], protects against diabetes-induced renal oxidative damage that eventually leads to DN [11, 12]. SFN modifies specific cysteine residues in Keap1, enabling Nrf2 to escape from Keap1-mediated ubiquitination and degradation [13, 14]. As a result, Nrf2 translocates into the nucleus to activate transcription of downstream antioxidants [13, 14]. Streptozotocin (STZ)-induced type 1 diabetic mice with global deletion of Nrf2 gene (Nrf2-null) completely lost SFN protection from DN [12], suggesting the pivotal role of Nrf2 in SFN protective effect. It is notable that as a nuclear factor, Nrf2 functions through induction of downstream antioxidants, rather than itself protecting from oxidative stress and damage. However, which Nrf2 downstream gene(s) plays the key role in SFN protection against DN has not been addressed.

As a potent antioxidant, metallothionein (MT) has been reported to protect against DN [15–17]. An analysis of liver gene expression using a 4967-oligonucleotide microarray and real-time PCR revealed that SFN drastically induced MT gene expression (up to 10-fold) [18], suggesting that MT might play an important role in the protective effect of SFN on the oxidative stress and damage. However, whether and how MT is involved has never been addressed.

Therefore, the present study aimed to define (1) whether SFN could up-regulate MT expression in the diabetic kidney; (2) whether MT acts as an Nrf2 downstream antioxidant; (3) whether SFN functions entirely through Nrf2 in type 2 DN; and (4) whether the induction of MT is required for SFN protection against DN. To these ends, mice with global deletion of either nrf2 gene (Nrf2-null) or mt gene (MT-null) and their associated wild types (WT), C57BL/6J and 129S1 mice, were used to induce type 2 diabetes by a high-fat diet (HFD) followed by injection of STZ. As speculated, both MT and Nrf2 could be up-regulated by SFN under diabetic and nondiabetic conditions, and MT played an important role in protection against DN by the SFN-induced Nrf2 pathway.

Materials and methods

Animals

Nrf2-null (Nrf2^{-/-}) male mice with a C57BL/6J background were obtained through breeding of homozygote (Nrf2^{-/-}) with heterozygote (Nrf2^{+/-}). MT-null (MT^{-/-}) male mice with a 129S1 background were obtained by breeding homozygote (MT^{-/-}) with homozygote (MT^{-/-}). Mating followed the system suggested by Jackson Laboratory. Eight-week-old WT C57BL/6J (Nrf2^{+/+}) and 129S1 (MT^{+/+}) male mice were purchased from Jackson Laboratory (Bar Harbor, ME).

Type 2 diabetes was induced by feeding HFD (Research Diets, New Brunswick, NJ, No. 12492, 60% kcal from fat) for 3 months while age-matched control mice were given normal diet (ND, Research Diets, New Brunswick, NJ, No. 12450B, 10% kcal from fat) for 3 months. Compared with ND fed mice, 3-month HFD feeding induced insulin resistance without hyperglycemia (fasting glucose level < 250 mg/dl), as described previously [19]. These insulin-resistant mice were injected intraperitoneally once with STZ (dissolved in sodium citrate, pH 4.5) at 100 mg/kg body weight, and ND-fed control mice were injected with vehicle (sodium citrate only). Five days later, HFD/STZ-treated mice that showed hyperglycemia (fasting blood glucose levels > 250 mg/dl) were considered as insulin-defective stage of type 2 diabetes [19].

Diabetic and control mice were further injected subcutaneously with either SFN (Sigma-Aldrich, St. Louis, MO) at 0.5 mg/kg or vehicle five days a week for four months with continual feeding with HFD and ND, respectively. At the end of the study, the diabetic mice continued to demonstrate significant insulin resistance along with elevated levels of blood glucose, insulin, triglyceride, and cholesterol, as previously reported [19]. All animal-related experimental protocols were approved by the Institutional Animal Care and Use Committee of the University of Louisville, and conformed to National Institutes of Health standards.

Kidney function analysis

Kidney function was determined by urinary albumin-to-creatinine ratio (UACR). Urinary albumin and creatinine were measured on spot urine samples with a mouse albumin ELISA kit (Bethyl Laboratories Inc., Montgomery, TX) and a QuantiChrom Creatinine Assay Kit (BioAssay Systems, Hayward, CA) according to the manufacturer's procedures and expressed as urinary albumin (μg) per urinary creatinine (mg).

Histopathological examination and immunohistochemical staining

Kidney tissues were fixed immediately in 10% buffered formalin solution after harvesting, embedded in paraffin, and sectioned into 5-μm-thick sections on glass slides. Sections were processed for hematoxylin-eosin (H&E), Sirius-Red, and IHC staining as previously described [11]. Antibodies against transforming growth factor beta 1 (TGF-β1, Cell Signaling, Beverly, MA, 1:100 dilution), vascular cell adhesion molecule-1 (VCAM-1, Santa Cruz Biotechnology, Dallas, TX, 1:100 dilution), 3-nitrotyrosine (3-NT, Millipore Corp., Temecula, CA, 1:100 dilution), MT (Dako, Carpintería, CA, 1:100 dilution), and Nrf2 (Santa Cruz Biotechnology 1:100 dilution) were used for IHC staining.

Western blotting

MT expression was detected using a modified western blot protocol, as previously described [20], using antibody against MT (Dako, 1:500 dilution). A regular western blot protocol was performed as described in our previous studies [11, 21]. Primary antibodies used included TGF- β 1 (Cell Signaling, 1:500 dilution), connective tissue growth factor (CTGF, Santa Cruz Biotechnology, 1:500 dilution), plasminogen activator inhibitor-1 (PAI-1, BD Biosciences, San Jose, CA, 1:1,000 dilution), VCAM-1 (Santa Cruz Biotechnology, 1:1000 dilution), 3-NT (Millipore, 1:1000 dilution), 4-hydroxy-2-nonenal (4-HNE, Alpha Diagnostic Int., San Antonio, TX, 1:3000 dilution), Nrf2 (Santa Cruz Biotechnology, 1:1000 dilution), Keap1 (Santa Cruz Biotechnology, 1:500 dilution), Histone H3 (H3, Santa Cruz Biotechnology, Dallas, TX, 1:500 dilution), and β -actin (Actin, Santa Cruz Biotechnology, 1:2000 dilution). Appropriate secondary antibodies (Santa Cruz Biotechnology) were used.

Real-time RT-PCR

Real-time RT-PCR was performed as previously described [22, 23] using primers for NQO1, MT1, Nrf2, Keap1, and Actin (Life Technologies, Grand Island, NY).

Isolation of nuclei

The renal nuclei were isolated using a nuclei isolation kit (Sigma-Aldrich). Kidney tissue (30 mg) from each mouse was homogenized for 45 s in 150 μ l of cold lysis buffer containing 0.5 μ l of dithiothreitol (DTT) and 0.1% Triton X-100. After that, 300 μ l of cold 1.8 M Cushion solution (sucrose Cushion solution: sucrose Cushion buffer: DTT = 900:100:1) was added to the lysis solution. The mixture was transferred to a new tube preloaded with 150 μ l of 1.8 M sucrose Cushion solution followed by centrifugation at 13,000 rpm for 45 min. The supernatant, containing cytosolic component and nuclei, was visible as a thin pellet at the bottom of the tube.

Statistical analyses

Data were collected from each group of mice ($n = 6$ per group) and expressed as mean $\pm$ SD. Image Quant 5.2 (GE Healthcare Bio-Sciences, Pittsburgh, PA) was used to analyze western blots. Comparisons were performed by one-way ANOVA between different groups, followed by post hoc pairwise repetitive comparisons using Tukey's test with Origin 8.6 data analysis and graphing software Lab (OriginLab, Northampton, MA). In addition, we performed a χ^2 test to compare the amount of decrease by SFN between WT 129S1 diabetic mice and MT-null diabetic mice. Statistical significance was considered to be $p < 0.05$.

Results

Sulforaphane ameliorated diabetes-induced albuminuria and renal remodeling

A type 2 diabetes model was induced by feeding C57BL/6J mice with HFD followed by STZ injection. Both diabetic and nondiabetic mice were treated with SFN for 4 months as described previously [19]. SFN slightly decreased the blood glucose level in diabetic mice [19]. Kidney dysfunction was determined by the increased UACR. SFN markedly prevented diabetes-increased UACR (Fig. 1A) and the ratio of kidney weight to tibia length as an

index of renal hypertrophy or swelling was lower (Fig. 1B). H&E (Fig. 1C) and Sirius-Red (Fig. 1C) staining were performed to evaluate renal morphological changes. The diabetic group showed significant increase in glomerular area and tubular dilation (Fig. 1C) and fibrosis (Fig. 1D), all of which were diminished by SFN (Fig. 1C,D).

Sulforaphane markedly ameliorated diabetes-induced renal fibrosis, inflammation, and oxidative damage

To further explore fibrotic response and its associated inflammation, IHC staining (Fig. 1E) for TGF- β 1, VCAM-1, and 3-NT, as indices of fibrosis, inflammation, and oxidative damage, was applied and showed their expression predominantly in diabetic renal tubules. These effects were diminished by SFN (Fig. 1E). Furthermore, the pathological changes observed were confirmed by increased protein levels of TGF- β 1, CTGF, PAI-1, VCAM-1, 3-NT, and 4-HNE with western blotting. All were increased by diabetes and significantly decreased by SFN (Figs. 2A–2F).

Sulforaphane enhanced renal Nrf2 expression and function along with increasing renal metallothionein expression under both diabetic and nondiabetic conditions

SFN is known to protect against DN through Nrf2 in STZ-induced type 1 diabetic mice [12]. To test whether SFN induces Nrf2 function in type 2 diabetic kidneys, total and nuclear Nrf2 protein and NQO1 mRNA (a result of Nrf2 activity) were evaluated. Diabetes decreased total and nuclear Nrf2 protein (Figs. 3A, 3B) and NQO1 mRNA expression (Fig. 3C), all of which were increased by SFN in both diabetic and nondiabetic kidneys compared with diabetes and controls. Neither diabetes nor SFN treatment affected Keap1 mRNA (Fig. 3D) or protein (Fig. 3E) expression in nondiabetic or diabetic mice.

To determine whether SFN can up-regulate MT, the expression of MT1 mRNA and MT protein was determined; both were decreased by diabetes. Treatment with SFN significantly increased, in the nondiabetic group, and preserved, in the diabetic group, the MT expression (Figs. 3F, 3G). IHC staining showed that diabetic kidneys had less expression of Nrf2 and MT, which were increased in SFN-treated diabetic and nondiabetic kidneys (Fig. 3H). Furthermore, the predominant localization of both Nrf2 and MT was in renal tubules (Fig. 3H), which coincided with the localization of renal fibrosis, inflammation, and oxidative damage (Fig. 1E). These results demonstrated that SFN enhanced Nrf2 expression and function along with increasing MT expression.

Nrf2-null mice completely lost sulforaphane protection from diabetic nephropathy and induction of metallothionein

Deletion of the Nrf2 gene resulted in complete abolition of SFN renal protection against STZ-induced type 1 diabetes [12]. However, whether Nrf2 accounts for the entire protection by SFN from DN in type 2 diabetes has not been addressed. Therefore, Nrf2-null mice and WT C57BL/6J mice were examined. Diabetes significantly increased UACR and renal protein expression of CTGF, VCAM-1, 3-NT, and 4-HNE in WT mice (Figs. 4A–4E), and all these changes were more severe in Nrf2-null mice than in WT mice (see Fig. 4F, the fold changes between two diabetic models). SFN treatment significantly reduced diabetes-increased changes in WT mice (Figs. 4A–4E, left panels) but did not affect any of these

changes in Nrf2-null mice (Figs. 4A–4E, right panels). These results demonstrate that SFN functions completely through Nrf2 to protect from type 2 DN.

Given that MT was also up-regulated by SFN under both diabetic and nondiabetic conditions (Figs. 3F, 3G), we evaluated MT expression in Nrf2-null mice to test whether Nrf2 deletion influenced MT expression. First, renal Nrf2 expression and function were tested in Nrf2-null mice and their control, WT C57BL/6J mice, to confirm the effect of Nrf2 gene deletion. In WT mice, SFN reversed the decrease of Nrf2 protein as a result of diabetes (Fig. 5A, left panel). However, Nrf2 protein was not detectable in Nrf2-null mice (Fig. 5A, right panel), which confirmed the deletion of the Nrf2 gene. NQO1 mRNA, MT1 mRNA, and protein levels were all in line with Nrf2 expression in WT mice (Figs. 5B–5D, left panels), but could no longer be up-regulated by SFN in the absence of Nrf2 (Figs. 5B–5D, right panels). These results show that both NQO1 and MT were dependent on Nrf2, demonstrating MT as a downstream antioxidant of Nrf2.

Metallothionein deletion resulted in partial loss of sulforaphane protection against diabetic nephropathy without alteration in sulforaphane-induced Nrf2 function

Although these results confirmed that SFN could up-regulate renal MT expression, whether MT is really required for the protective effect of SFN on DN is unknown. Therefore, type 2 diabetes was induced in MT-null and their WT 129S1 mice as mentioned. Diabetes increased UACR, CTGF, VCAM-1, 3-NT, and 4-HNE in both WT and MT-null mice (Figs. 6A–6E, left panels), but these pathological changes were significantly higher in MT-null diabetic mice than in WT diabetic mice (Fig. 6F). SFN drastically diminished these indicators induced by diabetes in WT mice but much less in MT-null mice (Fig. 6F). This indicates an important, though partial, role for MT in SFN-mediated renal protection.

Deletion of the MT gene was confirmed by evaluation of MT protein in kidneys of MT-null mice (Fig. 7A, right panel). Diabetes decreased MT protein in 129S mice and this effect was reversed by SFN treatment (Fig. 7A, left panel). Both 129S and MT-null diabetic mice had lower Nrf2 mRNA levels than their respective controls (Fig. 7B). SFN could up-regulate Nrf2 mRNA in both mice under either nondiabetic or diabetic conditions (Fig. 7B). We next evaluated the effect of SFN on Nrf2 function. Nuclear Nrf2 protein and NQO1 mRNA were decreased under diabetic conditions but increased by SFN in the diabetic and nondiabetic kidneys of 129S1 and MT-null mice (Figs. 7C, 7D), which was consistent with the observations in WT C57 mice (Figs. 5A, 5B, left panels). These results suggest that SFN-induced Nrf2 expression and function are not dependent on MT.

Discussion

Using Nrf2-null mice, the present study shows that SFN protection against DN in a type 2 diabetes mouse model is completely dependent on the induction of Nrf2 expression and its function. SFN also up-regulates renal MT expression in the presence of Nrf2 but loses this function in the absence of Nrf2, demonstrating that MT is an Nrf2-dependent antioxidant. SFN lacks some renal protection but retains the ability to induce Nrf2 expression in the absence of MT, indicating that MT is required for partial SFN renal protection and Nrf2 expression is independent of MT, as summarized in Fig. 8.

It is known that oxidative stress is the main cause for DN and approaches to activating antioxidant genes have become attractive [12, 16, 24]. Among these, Nrf2 is an emerging therapeutic target to up-regulate phase II antioxidant components [25, 26]. Through functionally acting on Keap1 [13], SFN has been shown to be a potent Nrf2 activator in various disease models [19, 27, 28], including DN [12, 24]. Previous studies of SFN protection from DN focused on STZ-induced type 1 diabetes models. Even though SFN has multiple effects [29], Nrf2 deletion resulted in complete abolition of SFN protective effects on either high-glucose-treated human kidney tubular cells (HK11) [24] or DN in type 1 diabetic mice [12], indicating that the Keap1/Nrf2 pathway plays the predominant role in the prevention of DN. However, the effect and protective mechanism of SFN on DN in type 2 diabetes has not been addressed. The present study provides the first evidence that Nrf2 plays a pivotal role in SFN protection from type 2 diabetes-induced DN.

As a nuclear transcription factor, Nrf2 cannot function directly as an antioxidant to protect from oxidative damage. Thus, which Nrf2 downstream gene(s) plays the key role in SFN protection from DN needs to be defined. An early finding that SFN could drastically induce MT gene expression [18] implied that MT may play an important role in SFN protection. In the present study, we demonstrate for the first time that SFN up-regulates renal MT expression under both diabetic and nondiabetic conditions, which plays a partial but important role in SFN renal protection from diabetes. The partial protection offered by MT is in agreement with the notion that other Nrf2 downstream antioxidants, such as NQO1, may also contribute to SFN renal protection.

Whether MT is an Nrf2 downstream gene has not been well addressed. Nrf2 has been proven to bind the ARE of the MT-1 gene, leading to its expression in rat astrocytes [30]. More recently, a study by Song et al. demonstrated that Nrf1 and Nrf2 contributed equally to MT-1 transcription by copper in mouse liver fibroblasts [31]. The study by Wu et al. showed that the mRNA of MT-1 and MT-2 were higher in the liver of the Keap1-hepatocyte knockout mice than that of WT mice (5-fold and 11-fold, respectively), indicating that MT-1 and MT-2 are potential Nrf2 target genes in vivo. However, following cadmium treatment, MT-1 and MT-2 were not Nrf2-dependent in mouse liver [32]. In another study, Ohtsui et al. showed that Nrf1, rather than Nrf2, activated MT transcription in mouse hepatocytes, although Nrf2 bound the MT-1 ARE with comparable affinity [33]. Thus, controversies exist in defining whether MT is a downstream gene of Nrf2, and might be explained by the different diseases and cell types. Here we show that SFN-induced MT up-regulation is Nrf2-dependent in the kidneys of diabetic and nondiabetic mice, since MT can no longer be up-regulated by SFN in the absence of Nrf2 under either condition. To date, this is the first in vivo study that demonstrates MT as an Nrf2 downstream antioxidant that ameliorates DN.

In the present study, both NQO1 and MT serve as Nrf2 downstream antioxidants. However, unlike NQO1, MT is down-regulated in the Nrf2-null diabetic group regardless of SFN treatment (Figs. 5C, 5D, right panels). This result suggests that Nrf2 is not the sole transcription factor to activate MT transcription, which supports the notion that other transcription factors, such as metal-responsive-element-binding transcription factor 1 (MTF-1), may also regulate MT transcription [34]. It is well established that zinc activates MTF-1, which binds the metal-responsive element of the MT promoter, leading to the

transcription of MT [35–38]. Moreover, diabetics develop zinc deficiency [39–43], which may result in lower expression of MT through inactivation of MTF-1. Further studies are needed to explore the mechanism by which MT expression is down-regulated in kidneys of type 2 diabetic mice. Nevertheless, the fact that SFN can no longer up-regulate MT in the absence of Nrf2 demonstrates that Nrf2 exclusively mediates SFN induction of MT regardless of diabetic condition.

Unlike MT, NQO1 expression is completely dependent on Nrf2, as evidenced by the present study (Fig. 5B) and Zheng et al.'s work [12] showing that NQO1 mRNA does not change in the absence of Nrf2, under either diabetic or nondiabetic conditions. Our result further confirms that NQO1 is a complete Nrf2-directed downstream target, the expression of which depends solely on Nrf2 expression.

It is worth mentioning here that diabetes significantly decreased both Nrf2 and MT expression in the kidneys of type 2 diabetic mice at the seventh month after HFD feeding and the fourth month after one dose of STZ (Figs. 3, 5, 7), which is consistent with our own and others' previous findings, which have reported the decreased expression of Nrf2 in the late stages of diabetes [44–46]. The exact mechanism by which diabetes decreases Nrf2 remains unclear. One possible mechanism might be the loss of tubular tissues and replacement of fibrotic tissue induced by diabetes. The replacement of normal tissue with fibrotic tissue might be responsible for the decreased expression of Nrf2 and MT, which needs to be further explored.

In summary, the present study demonstrates for the first time that (1) Nrf2 is pivotal in SFN renal protection from type 2 diabetes; (2) MT, as an Nrf2 downstream antioxidant, contributes almost half of SFN's protective effects on type 2 diabetes-induced DN. Furthermore, the present study provides a deeper understanding of Nrf2 function and the protective mechanism of SFN, which may indicate induction of Nrf2 and MT expression as a potential approach to the prevention and treatment of DN in patients with type 2 diabetes.

Conclusions

The present study identified that SFN prevents DN from type 2 diabetes via increasing Nrf2 expression and function, in which MT as one of Nrf2's downstream antioxidants offers almost half of SFN renal protection.

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Highlights

1. Nrf2 is pivotal in sulforaphane (SFN) renal protection from type 2 diabetes
2. SFN can up-regulate both Nrf2 and metallothionein (MT) expression in the kidney
3. Nrf2 is indispensable for SFN-induced renal MT under both normal and diabetic conditions
4. MT as one of Nrf2 downstream antioxidants protects from diabetic nephropathy in type 2 diabetes

Fig. 1.

Effects of SFN on kidney function and pathological changes. A type 2 diabetes model of C57BL/6J mice was induced by a single dose of STZ injection with HFD feeding for 3 months, and then diabetic and age-matched control mice were treated with SFN (0.5 mg/kg) or vehicle daily, five days per week, along with continually feeding HFD for 4 months. Urinary albumin-to-creatinine ratio (UACR, A) and kidney-weight-to-tibia length (B) were determined in all mice. H&E staining was performed to present kidney remodeling (C). Sirius Red staining with semiquantification for renal fibrosis (D). Immunohistochemical

(IHC) staining (E) was performed to test localization of renal fibrosis, inflammation, and oxidative damage, using antibodies against TGF- β 1, VCAM-1, and 3-NT. Bar = 100 μ M. Data are presented as mean $\pm$ SD (n = 6 per group). * p < 0.05 vs. control group (Ctrl); # p < 0.05 vs. diabetic group (DM).

Fig. 2.

SFN significantly attenuated diabetes-induced renal fibrosis, inflammation, and oxidative damage. Western blotting was performed to evaluate the expressions of profibrotic mediators [TGF-β1 (A) and CTGF (B)], inflammatory cytokines [PAI-1 (C) and VCAM-1 (D)], and oxidative damage accumulation [3-NT (E) and 4-HNE (F)]. Data are presented as mean ± SD (n = 6 per group). * p < 0.05 vs. ctrl; # p < 0.05 vs. DM. Abbreviations are the same as in Fig. 1.

Fig. 3.

SFN induced Nrf2 expression and function along with metallothionein (MT) expression under both diabetic and nondiabetic conditions. Total (A) and nuclear (B) Nrf2 protein levels were measured by western blotting. NQO1 mRNA, which represents Nrf2 function, was determined by real-time PCR (C). Keap1 mRNA (D) and protein (E) levels were evaluated by RT-PCR and western blotting. MT1 mRNA (F) and protein (G), as interest targets of the present study, were also evaluated. Localization of Nrf2 and MT protein

induced by SFN was determined by IHC staining (H). Data are presented as mean $\pm$ SD (n = 6 per group). * p < 0.05 vs. ctrl; # p < 0.05 vs. DM. Abbreviations are the same as Fig. 1.

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Fig. 4. Nrf2 deletion resulted in complete abolition of SFN renal protection from albuminuria, fibrosis, and inflammation. To define the role of Nrf2 in SFN protection from type 2 diabetes-induced DN, Nrf2 knockout (Nrf2-null) and wild-type (WT) C57BL/6J (C57) mice were induced to type 2 diabetes as described in Fig. 1. UACR (A), protein levels of CTGF (B), VCAM-1 (C), 3-NT (D), and 4-HNE (E) for renal function, fibrosis, inflammation, and oxidative stress were evaluated by western blotting in all mice. Diabetes-induced functional and pathological changes (fold) were compared between WT and Nrf2-null mice (F). To

further confirm the western blot result for renal fibrosis, morphology of these kidney tissues was examined with PAS staining (G) to evaluate glomerular area (H) and mesangial matrix expansion (I). Data are presented as mean $\pm$ SD (n = 6 per group). * p < 0.05 vs. ctrl; # p < 0.05 vs. DM. ‡ p < 0.05 vs. C57 (χ^2 test). Abbreviations are the same as in Fig. 1.

Fig. 5.

SFN lost the function of inducing NQO1 and MT expression in the absence of Nrf2. Total Nrf2 protein levels were first evaluated to confirm both the Nrf2-up-regulating effect of SFN in WT mice (A, left panel) and the Nrf2-deletion effect in Nrf2-null mice (A, right panel). NQO1 mRNA (B), MT1 mRNA (C), and MT protein (D) were also evaluated in both WT and Nrf2-null mice. Data are presented as mean $\pm$ SD (n = 6 per group). * p < 0.05 vs. ctrl; # p < 0.05 vs. DM. Abbreviations are the same as in Fig. 1.

Fig. 6.

MT deletion led to partial abolition of SFN protection against diabetes-induced albuminuria, renal fibrosis, and inflammation. To test whether MT is really required for SFN renal protection, MT-null and their WT 129S1 mice were induced to type 2 diabetes as described in Fig. 1. UACR (A), protein levels of CTGF (B), VCAM-1 (C), 3-NT (D), and 4-HNE (E) were determined by western blotting in all mice. Diabetes-induced functional and pathological changes (fold) between WT and MT-null mice and the decreased percentages of these functional and pathological changes with SFN between WT and MT-null diabetic

mice were compared (F). Data are presented as mean $\pm$ SD (n = 6 per group). * p < 0.05 vs. ctrl; # p < 0.05 vs. DM. $\ddagger$ p < 0.05 vs. 129S1 DM. $\S$ p < 0.05 vs. WT129 or WT129/DM correspondingly (χ^2 test) in all mice.

Fig. 7.

SFN retained the ability to induce Nrf2 and NQO1 expression in the absence of MT. MT protein level (A) was evaluated to confirm the effect of MT deletion. With the aim of testing if MT deletion has impact on Nrf2 expression and its ability to activate NQO1 transcription, Nrf2 mRNA (B) and nuclear Nrf2 protein (C), as well as NQO1 mRNA (D), were determined. Data are presented as mean $\pm$ SD (n = 6 per group). * p < 0.05 vs. ctrl; # p < 0.05 vs. DM. Abbreviations are the same as in Fig. 1.

Fig. 8.

Possible mechanism by which SFN ameliorates DN. Type 2 diabetes induces oxidative stress to enhance renal inflammation and fibrosis, which consequently leads to development of DN. Type 2 diabetes decreases Nrf2 and MT expression, resulting in prolonged amplification of oxidative damage. SFN functions through Nrf2 to activate the expression of downstream antioxidant MT and NQO1. MT offers almost half of SFN protection against DN in addition to other Nrf2 downstream antioxidants, such as NQO1. →, activation; ⊥, inhibition. Red arrows and question marker for SFN effect on Nrf2 indicate that since we

have found increased expression of Nrf2 mRNA by SFN in the present study, it is unclear whether SFN directly up-regulated Nrf2 expression by some uncertain mechanisms alone or in combination with the stabilization of Nrf2 content by oxidizing Keap1.

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