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Original Article

The transcription factor Nrf2 might be involved in the process of renal aging

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Abstract: Increased inflammation and oxidative stress are associated with aging-related renal damage. We aimed to explore the role of Nrf2 (an important transcription factor involved in antioxidant responses) in the process of renal aging and to identify the potential mechanism. In *in vivo* experiments, mice were divided into three groups: the young group (3 months), the old group (23-24 months), and the sulforaphane (SFN) treatment group (23-24 months). We compared the metabolic characteristics, kidney morphology changes (PAS and Masson's trichrome staining), SA-β-gal staining, the expression of p16 and Nrf2 (Western blot) among the three groups. Real-time quantitative polymerase chain reaction was used to detect the expression of Nrf2 downstream genes and pro-oxidant and pro-inflammatory markers. The results showed that the expression of Nrf2 was lower in the older mice than in the younger mice. Compared with the old group, the level of serum urea nitrogen and the urine protein/urine creatinine ratio were lower, and the kidney weight/body weight was higher in the SFN treatment group. SFN upregulated Nrf2 target genes in the old mice while reducing interleukins (IL-1β, IL-6) and inducible nitric oxide synthase (iNOS). In *in vitro* experiments, SFN was used to stimulate the renal residential cells isolated from old mice, and Western blot was used to detect the expression of p16, Nrf2 and iNOS at different time points. We found that as the SFN intervention time was extended in the renal residential cells, expression of Nrf2 increased while iNOS decreased. Thus, Nrf2 plays an important role in renal aging, and Nrf2 activation by SFN could alleviate renal damage related to aging via antioxidant and anti-inflammatory effects.

Keywords: Renal aging, Nrf2, SFN, antioxidation, anti-inflammation

Introduction

Aging in most species is associated with impaired adaptive and homeostatic mechanisms, leaving an individual more susceptible to environmental and internal stress with increasing rates of morbidity and mortality [1]. The kidney is a typical target organ of such age-associated tissue damage, and the increased incidence of chronic kidney disease in the elderly is a public health problem worldwide [2]. Presently, the molecular basis of such renal aging is not fully understood.

Nuclear erythroid 2 p45-related factor-2 (Nrf2) is a basic leucine zipper redox-sensitive transcription factor that regulates the expression of several cellular antioxidant and cytoprotective genes. Nrf2-mediated transcriptional responses have been shown to be protective against various experimental diseases, including LPS-induced sepsis, oxidative lung injury and fibrosis, asthma, smoking-induced emphysema, and brain IRL [3]. Additionally, it has been reported that Nrf2 may be involved in organ aging [4, 5].

In this study, we sought to explore the role of Nrf2 in the process of renal aging and the potential mechanism, which may provide new ideas for anti-aging therapies.

Material and methods

Animals

All experiments were performed using C57Bl/6 mice at the age of 3 months (young group) or

Table 1. Genes, primer, and conditions for RT-PCR amplification

Gene product	Forward Primer	Reverse primer
Nrf2	5' TGGACGGGACTATTGAAGGCTG 3'	5' GCCGCCTTTTCAGTAGATGGAGG 3'
NQO1	5' ATTGTACTGGCCCATTCAGA 3'	5' GGCCATTGTTTACTTTGAGC 3'
HO-1	5' TGCTCAACATCCAGCTCTTTGA 3'	5' GCAGAATCTTGCACTTTGTTGCT 3'
SOD1	5' ATCCACTTCGAGCAGAAG 3'	5' TTCCACCTTTGCCCAAGT 3'
SOD2	5' AGCGGTCGTGTAAACCTCA 3'	5' AGACATGGCTGTCAGCTTC 3'
CAT	5' AATCCTACACCATGTCGGACA 3'	5' CGGTCTTGTAATGGAACTTGC 3'
iNOS	5' CAAGAGTTTGACCAGAGGACC 3'	5' TGGAACCACTCGTACTTGGGA 3'
IL-1β	5'-TTGACGGACCCCAAAGAGTG-3'	5'-ACTCCTGTACTCGTGGAAGA-3
IL-6	5' GAGGATACCACTCCCAACAGACC 3'	5' AAGTGCATCATCGTTGTTCATACA 3'
β-Actin	5' TGTTTGAGACCTTCAACACC 3'	5' CGCTCATTGCCGATAGTGAT 3'

23-24 months (old group). Mice were purchased from the Si Bei Fu Laboratory Animal Company (Beijing, China). The experimental protocol was carried out in accordance with the approved guidelines of the Institutional Animal Care and Use Committee at the Chinese PLA General Hospital.

Mice were housed under specific pathogen-free conditions in the Experimental Animal Center of the Academy of Military Medical Sciences: 22 ± 1°C, 40% humidity, 12/12-h light/dark cycle, and free access to water. Mice were allocated randomly into three groups (n = at least 6 per group): young, old, and sulforaphane (SFN) groups. For the SFN group, SFN (Sigma-Aldrich, St. Louis, MO, USA) was injected subcutaneously at 0.5 mg/kg for 5 days per week for 3 months at age 20-21 months. As SFN was dissolved in dimethyl sulfoxide (DMSO) and diluted in phosphate-buffered saline (PBS), mice serving as vehicle controls were given the same volume of PBS (1% DMSO).

Histological examinations

Kidney slices were fixed in 10% formalin solution overnight. After automated dehydration through a graded alcohol series, transverse kidney slices were embedded in paraffin wax, sectioned at 4 μm, and stained with periodic acid-Schiff (PAS) and Masson's trichrome. Histological examinations were performed independently in a blinded fashion by two observers. Quantitative analyses of glomerular and fibrosis-positive areas were made with the Image-Pro software (Media Cybernetics Inc., Silver Springs, MD, USA) from 20 random fields per mouse at a magnification of × 200.

Senescence-associated β-galactosidase staining

Cryostat sections (4 μm) were mounted on glass slides and fixed in 0.2% glutaraldehyde and 2% formaldehyde at room temperature for 15 min. Sections were washed in PBS and incubated in freshly prepared senescence-associated β-galactosidase (SA-β-gal) staining solution (1 mg/mL X-gal, 40 mM citric acid/sodium phosphate, pH 6.0, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 150 Mm NaCl, and 2 mM $MgCl_2$) overnight at 37°C. Tissue sections were counterstained with eosin and examined under a microscope. An investigator who was blinded to sample identity performed the image analysis. Quantitative analysis of SA-β-gal staining positive area was performed with the Image-Pro software from 20 random fields per mouse at a total magnification of × 200.

Western blot analysis

Protein concentration was determined with the Pierce BCA assay kit (Thermo Fisher Scientific, Waltham, MA, USA). In total, 50-100 μg protein was separated by 6-16% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to a nitrocellulose membrane, blocked with blocking buffer for 1 h at room temperature, and incubated with primary antibodies (p16, Nrf2, iNOS) at 4°C overnight. Blots were subsequently incubated with secondary immunoglobulins conjugated with horseradish peroxidase. Immunoreactive bands were visualized by enhanced chemiluminescence, and densitometry was performed using Quantity One software (Bio-Rad Laboratories, Hercules, CA, USA). Band intensities were quantified with ImageJ software (NIH, Bethesda, MD, USA).

RNA extraction and real-time quantitative polymerase chain reaction (PCR)

Total RNA was isolated using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The high-capacity cDNA Reverse Transcription Kit (Applied

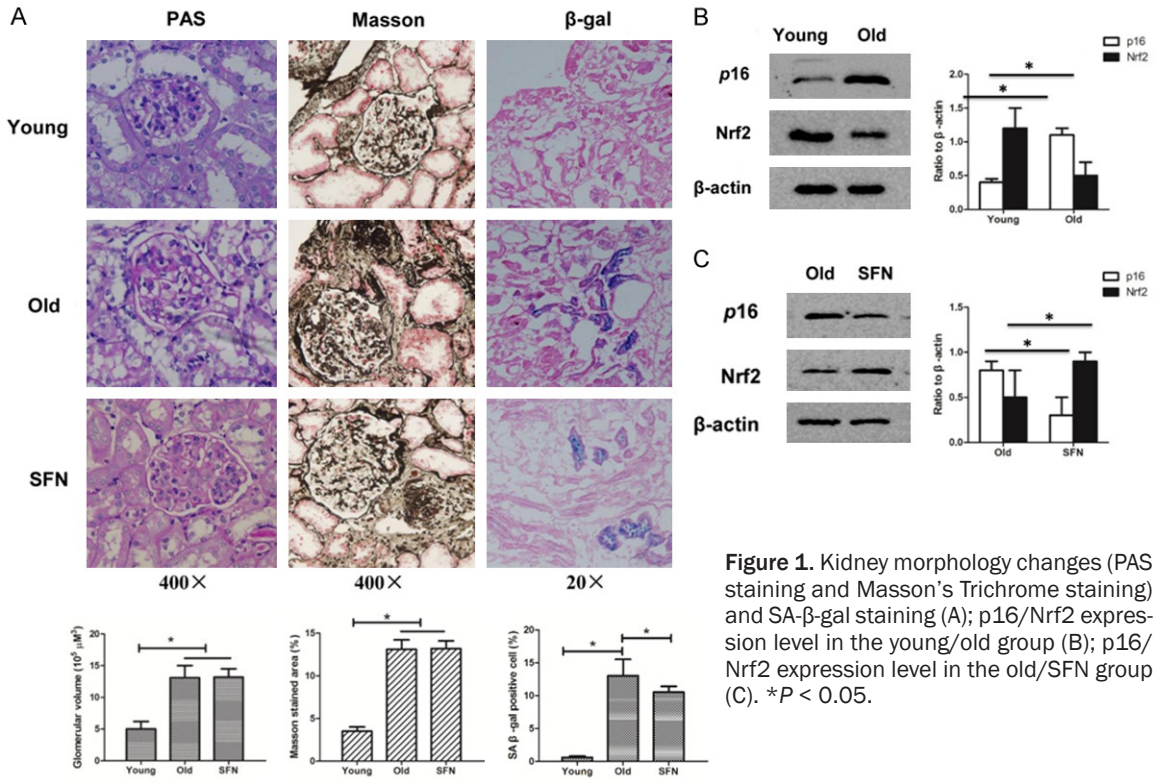

Figure 1. Kidney morphology changes (PAS staining and Masson's Trichrome staining) and SA-β-gal staining (A); p16/Nrf2 expression level in the young/old group (B); p16/Nrf2 expression level in the old/SFN group (C). *$P < 0.05$.

Biosystems, Foster City, CA, USA) and a GeneAmp PCR System 9700 (Applied Biosystems) were used to generate cDNA. Gene expression analysis used quantitative real-time PCR, with the Taqman Master mix and specific probes and primers (as shown in **Table 1**). PCR was performed on a 7500 Real-time PCR System (Applied Biosystems), and the results were analyzed using the $2^{-\Delta\Delta CT}$ method with normalization against glyceraldehyde 3-phosphate dehydrogenase expression ($n = 6$ for each group).

Isolation of primary renal residential cells

Primary mesangial cells of renal glomeruli, from male mice at the age of 23-24 mouths, were isolated and cultured as described previously. Cells were grown in RPMI-1640 medium containing 10% fetal bovine serum. The experimental group was treated with 2.5 μM SFN in 0.1% BSA for 0, 3, 9, or 24 h. Cell protein extraction was performed according to a standardized protocol.

Statistical analysis

All data analyses were performed using SPSS software (ver. 18.0; SPSS, Chicago, IL, USA). Data are expressed as the means ± SD. Multi-comparison among groups was performed by an ANOVA test. P values < 0.05 were considered to indicate statistical significance.

Results

Nrf2 expression in kidney tissues

As Nrf2 might be involved in renal aging, we first detected the expression of Nrf2 in kidney tissues (old & young). We found that the expression of Nrf2 decreased significantly in kidney tissues of old rats compared with young rats ($P < 0.001$) (**Figure 1B**). Nrf2 activator SFN could increase the expression of Nrf2 in the old mice ($P < 0.001$) (**Figure 1C**). Thus, we speculated that Nrf2 might be involved in renal aging. The Nrf2 activator SFN can be used during *in vivo* and *in vitro* experiments to understand the role of Nrf2 in renal aging.

The impact of Nrf2 on metabolic index

We first compared the difference between the young group and the old group. As shown in **Table 2**, compared with the young group, in the old group, the body weight ($P = 0.002$), serum

Table 2. Metabolic parameters and renal functions in three groups

Parameter	Young group	Old group	Sulforaphane group
Body weight (g)	19.35 ± 2.56	39.58 ± 7.58*	32.78±5.64*
Kidney weight/body weight (g/100 g of body wt)	0.72 ± 0.08	0.42 ± 0.12*	0.65 ± 0.11#
Serum urea nitrogen (mg/dL)	3.85 ± 0.65	6.45 ± 1.55*	5.12 ± 2.10#
Serum creatinine (mg/dL)	31.25 ± 5.99	33.68 ± 7.44	32.21 ± 5.35
Triglyceridies (mmol/L)	0.64 ± 0.22	1.52 ± 0.53*	1.10 ± 0.21*
Cholesterol (mmol/L)	1.75 ± 0.42	2.22 ± 0.42	2.09 ± 0.52
Serum albumin (g/L)	20.32 ± 5.23	24.71 ± 2.99	22.97 ± 3.12
Total protein (g/L)	1.62 ± 4.20	1.89 ± 6.87	1.72 ± 5.42
Urine protein/urine creatinine ratio (mg/mmol)	94.56 ± 5.64	356.91 ± 35.48*	210.76 ± 32.44*,#

*$P < 0.05$, compared with young group; #$P < 0.05$, compared with old group.

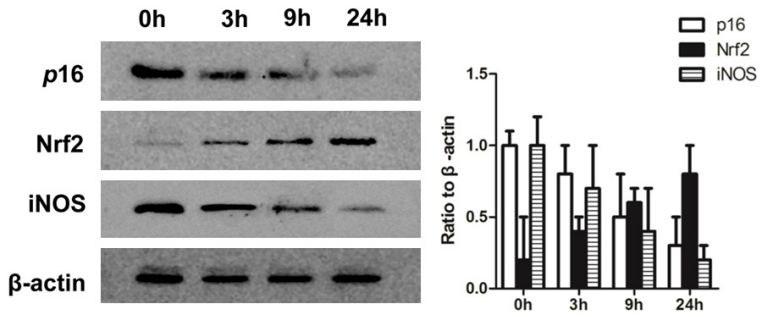

Figure 2. The expression level of p16, Nrf2 and iNOS at different time point in old renal residential cells.

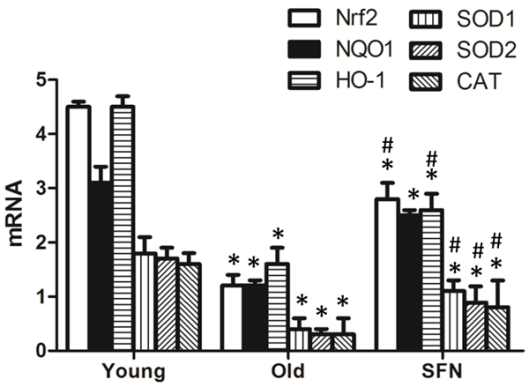

Figure 3. mRNA level of Nrf2, NQO1, NO-1, SOD1, SOD2 and CAT in the three group. *Compared with the young group $P < 0.05$; #Compared with the old group $P < 0.05$.

urea nitrogen ($P = 0.027$), triglyceride levels ($P = 0.032$), and urine protein/urine creatinine ratio ($P < 0.001$) were higher, and the kidney weight/body weight ($P = 0.029$) was lower. Compared with the old group, in the SFN group, the level of serum urea nitrogen ($P = 0.031$) and the urine protein/urine creatinine ratio ($P = 0.033$) were lower, and the kidney weight/body weight ($P = 0.025$) was higher.

Kidney morphological changes

Renal tissues were examined with routine PAS and Masson's trichrome staining. Compared with the young group, the number of normal glomeruli decreased with age, whereas the glomerular volume ($P = 0.023$) and degree of fibrosis ($P = 0.012$) increased in the old group. However, there was no significant difference between the old group and SFN group (**Figure 1A**).

Senescence marker changes in the kidney

p16 and β-gal are both senescence markers in aging related research. We found that the expression of p16 increased significantly in old versus young kidney tissues ($P = 0.022$) (**Figure 1B**), while it declined significantly in the SFN group versus the old group ($P = 0.032$) (**Figure 1C**). As shown in **Figure 1A**, the positive rate of SA-β-gal staining of kidney was markedly higher in the old than the young group ($P < 0.001$); however, SFN decreased the number of SA-β-gal positive cells ($P = 0.028$).

Expression of genes downstream Nrf2

Compared with the young group, the expression of Nrf2 ($P = 0.015$), NQO1 ($P = 0.022$), HO-1 ($P = 0.028$), SOD1 ($P = 0.032$), SOD2 ($P = 0.026$), and CAT ($P = 0.033$) decreased in the old group (RT-PCR). Compared with the old group, the expression of Nrf2 ($P = 0.024$), NQO1

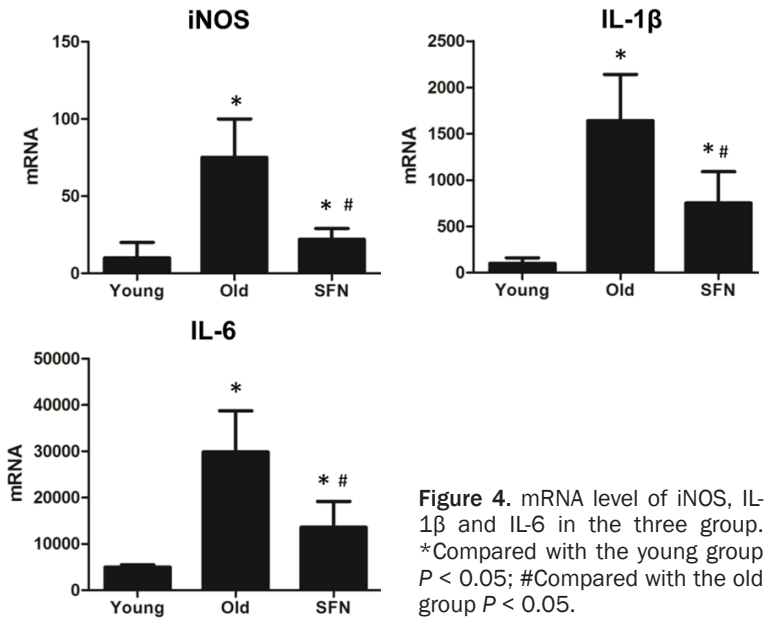

Figure 4. mRNA level of iNOS, IL-1β and IL-6 in the three group. *Compared with the young group $P < 0.05$; #Compared with the old group $P < 0.05$.

($P = 0.033$), HO-1 ($P = 0.029$), SOD1 ($P = 0.035$), SOD2 ($P = 0.031$), and CAT ($P = 0.028$) increased in the SFN group (**Figure 3**).

Expression of pro-oxidant and pro-inflammatory markers

Oxidation and inflammation are related to aging. Compared with the young group, the expression levels of inducible nitric oxide synthase (iNOS) ($P = 0.026$) and interleukins (IL1β, IL-6) ($P = 0.028$, $P = 0.033$, respectively) were higher in the old group (RT-PCR). However, SFN treatment decreased iNOS, IL1β, and IL-6 ($P = 0.028$, $P = 0.035$, $P = 0.026$) (**Figure 4**).

In vitro experiment: Nrf2 expression in old renal residential cells

As the SFN intervention time was extended, expression of Nrf2 increased, and the expression of p16 and iNOS decreased (**Figure 2**).

Discussion

The transcription factor Nrf2 is an emerging therapeutic target for several diseases, including cancer [6], neurodegenerative diseases [7], pulmonary fibrosis [8], diabetes [9], and diabetic nephropathy [10]. Nrf2 regulates the expression of numerous genes through antioxidant response elements (AREs) in their promoters to neutralize free radicals and accelerate the removal of environmental toxins. Prior to cell stress, activation of Nrf2 with low-toxicity compounds, such as SFN from cruciferous vegetables, can delay disease onset or improve prognosis [11]. In this study, we demonstrated that Nrf2 was involved in renal aging both *in vivo* and *in vitro*. Compared with the young group, body weight, serum urea nitrogen and triglyceride levels, and the urine protein/urine creatinine ratio were higher, and the kidney weight/body weight lower, in the old group. Moreover, the number of normal glomeruli was reduced with age, whereas the glomerular volume and degree of fibrosis increased. Furthermore, the expression of p16 and SA-β-gal increased, whereas the expression of Nrf2 decreased, in the old group. We showed the importance of SFN-induced Nrf2 expression in protection against renal aging. This was also reflected by the significant reductions in serum urea nitrogen, the urine protein/urine creatinine ratio, and the increase in kidney weight/body weight. *In vitro*, as the SFN intervention time was extended, the expression of p16 decreased, whereas the expression of Nrf2 increased, in the old renal residential cells. Additionally, SFN-induced Nrf2 expression could reduce renal oxidative damage and inflammation both *in vivo* and *in vitro*. This evidence suggests that Nrf2 is involved in renal aging and that high expression of Nrf2 may inhibit renal aging.

Nrf2, as a member of the "cap 'n' collar" family, is a master regulator of cellular detoxification responses and redox status [12]. Under physiological conditions, Kelch-like ECH-associated protein 1 (KEAP1) binds to Nrf2 and sequesters it in the cytoplasm. Under basal conditions, KEAP1 mediates rapid ubiquitination and subsequent degradation of Nrf2 by the proteasome [13]. Upon exposure of cells to oxidative stress or electrophilic compounds, Nrf2 dissociates from KEAP1 and translocates into the nucleus to bind to antioxidant-responsive elements in the genes encoding antioxidant enzymes, such as NADPH: quinone oxidoreductase (NQO1), heme oxgenase-1 (HO-1), gluta-

thione S-transferase, superoxide dismutase (SOD), catalase (CAT), and γ-glutamylcysteine synthetase. These antioxidant enzymes (AOEs) play important roles in protecting cells from reactive oxygen species. It has been shown that promoting the expression of phase II detoxifying and antioxidant enzymes, such as glutathione peroxidase, quinone reductase and NQO1, facilitates the prevention of oxidative stress-induce disease and aging. It has been reported that decreased expression of AOEs is associated with aging [14]. Studies showed that the decline in Nrf2 results in the loss of glutathione synthesis in an age-dependent manner. Induction of such phase II AOE genes by Nrf2 occurs through recruitment to the AREs in their gene promoters [15]. Under oxidant conditions, Nrf2 activates a battery of antioxidant and cytoprotective genes that share a common *cis*-acting enhancer sequence, the ARE, which includes heme oxygenase-1 (HO-1). In this study, Nrf2 and its downstream genes/ARE genes were all decreased in the older mice, while SFN could increase their levels. Upregulation of these Nrf2-dependent antioxidants promotes detoxification and anti-inflammatory function [16]. *In vivo*, SFN-induced Nrf2 activation could inhibit the expression of iNOS, IL-β, and IL-6. Thus, the regulation of cellular antioxidant and anti-inflammatory machinery by Nrf2 plays a central role in the defense against oxidative stress [17, 18]. It has been reported that ablation of the Nrf2 gene causes a lupus-like autoimmune nephritis and exacerbates diabetes-induced oxidative stress, inflammation, and nephropathy in experimental animals [19]. Upregulation of the Nrf2 pathway in endothelial cells prevented hydrogen peroxide-induced oxidative toxicity and reduced inflammation by suppressing activation of the MAPK pathway. Additionally, Nrf2-mediated inhibition of p38 phosphorylation reduced inflammatory cytokines [20].

SFN is a natural product found in cruciferous vegetables, such as broccoli, Brussel sprouts, and cabbage [21]. Although the molecular targets of this molecule have not been characterized completely, the best-known effect of SFN is the induction of Nrf2-dependent gene expression [22]. Studies have shown that SFN metabolites were detected in all tissues at 2 and 6 h after gavage, with the highest concentrations being in the small intestine, prostate, lung, and kidney, suggesting that SFN is bioavailable and could be an effective dietary chemoprevention agent in these tissues [23]. SFN has gained attention as an indirect antioxidant due to its ability to induce the expression of several enzymes via the KEAP1/Nrf2 pathway [23]. It has been reported that the preventative effects of SFN are mediated by induction of Nrf2 in chemical or ischemia-induced renal damage [24, 25]. In addition, SFN has been used to downregulate macrophage activation in *in vitro* models of inflammation [26].

Collectively, our findings indicate that the therapeutic benefit of Nrf2 activation in renal aging is multifactorial. In addition to its antioxidant function, Nrf2 also negatively regulated inflammation-associated cytokine expression. These results provide experimental evidence that dietary compounds targeting Nrf2 activation could be useful therapeutically to improve metabolic disorders and relieve kidney damage induced by aging. This study lays the foundation for the clinical evaluation of and, ultimately, the development of new Nrf2 activators for potential therapeutic use to delay renal aging.

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Disclosure of conflict of interest

None.

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