Sulforaphane, a natural constituent of broccoli, prevents cell death and inflammation in nephropathy☆,☆☆

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Abstract

Cisplatin (*cis*-diamminedichloroplatinum II, CIS) is a potent and widely used chemotherapeutic agent to treat various malignancies, but its therapeutic use is limited because of dose-dependent nephrotoxicity. Cell death and inflammation play a key role in the development and progression of CIS-induced nephropathy. Sulforaphane (SFN), a natural constituent of cruciferous vegetables such as broccoli, Brussels sprouts, etc., has been shown to exert various protective effects in models of tissue injury and cancer. In this study, we have investigated the role of prosurvival, cell death and inflammatory signaling pathways using a rodent model of CIS-induced nephropathy, and explored the effects of SFN on these processes. Cisplatin triggered marked activation of stress signaling pathways [p53, Jun N-terminal kinase (JNK), and p38-α mitogen-activated protein kinase (MAPK)] and promoted cell death in the kidneys (increased DNA fragmentation, caspases-3/7 activity, terminal deoxynucleotidyl transferase-mediated uridine triphosphate nick-end labeling), associated with attenuation of various prosurvival signaling pathways [e.g., extracellular signal-regulated kinase (ERK) and p38-β MAPK]. Cisplatin also markedly enhanced inflammation in the kidneys [promoted NF-κB activation, increased expression of adhesion molecules ICAM and VCAM, enhanced tumor necrosis factor-α (TNF-α) levels and inflammatory cell infiltration]. These effects were significantly attenuated by pretreatment of rodents with SFN. Thus, the cisplatin-induced nephropathy is associated with activation of various cell death and proinflammatory pathways (p53, JNK, p38-α, TNF-α and NF-κB) and impairments of key prosurvival signaling mechanisms (ERK and p38-β). SFN is able to prevent the CIS-induced renal injury by modulating these pathways, providing a novel approach for preventing this devastating complication of chemotherapy.
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1. Introduction

Cisplatin (*cis*-diamminedichloroplatinum II, CIS) is a widely used, potent antineoplastic agent to treat various forms of cancer; however, its therapeutic utility is limited by the development of dose-dependent nephrotoxicity in about 30% of the patients treated with this drug [1]. The mechanisms of the CIS-induced nephropathy are complex and may involve increased DNA damage, apoptotic and necrotic cell death, mitochondrial dysfunction and inflammation [1–17].

Multiple studies have demonstrated that the activation of various stress signaling pathways [e.g., mitogen-activated protein kinases (MAPKs) and p53], which are key regulators of proliferation, differentiation and cellular survival, contributes to the development and progression of CIS-induced nephropathy [18–22]. Emerging recent evidence also supports a key role of the inflammation in this process, which involves enhanced infiltration of leukocytes [8,23,24], increased expression of adhesion molecules [intercellular and

vascular adhesion molecules (ICAM and VCAM)] and increased secretion of various inflammatory mediators by inflammatory or parenchyma cells ([e.g., monocyte chemoattractant protein-1, ED-1, and tumor necrosis factor-α (TNF-α)] [9,12,25–27].

Sulforaphane (SFN) is a naturally occurring isothiocyanate produced by the enzymatic action of the myrosinase on glucophar-anine, a glucosinolate contained in cruciferous vegetables such as broccoli, brussel sprouts, cabbage, etc. [28,29]. Experimental studies have demonstrated that SFN protects against renal fibrosis [30], kidney and intestinal ischemic-reperfusion injuries [31,32], cytokine- and streptozotocin-induced beta-cell injury [33], bacterial lipopoly-saccharide-induced inflammatory damage in human vascular endo-thelial cells [34], and inhibits angiogenesis [35]. Furthermore, increasing evidence based on various in vitro studies using cancer cell lines and rodent tumor models, demonstrating antiproliferative and proapoptotic effects of SFN in cancer cells or tumors, coupled with epidemiological studies suggesting that dietary intake of cruciferous vegetables is associated with lower cancer risk in bladder, lung, prostate, breast, kidney, ovarian cancers and lymphoma, supports a potential benefit of SFN in various cancers and their prevention (reviewed in Refs. [36,37]). In this study, we have explored the interplay of prosurvival, cell death and inflammatory signaling pathways using a rodent model of CIS-induced nephropathy and explored the effects of SFN on these processes.

2. Materials and methods

2.1. Animals and treatment

Male Wistar rats with an initial body weight of 230–260 g were used. The animals were maintained under 12-h light/dark cycles at controlled temperature, having free access to water and food. Experimental work was approved by the local ethical committee (Comité Institucional para el cuidado y uso y de animales de Laboratorio approval ID 002/10) and followed the Guide for the Care and Use of Laboratory Animals, published by the US National Institutes of Health, as well as the guidelines of Mexican Official Norm Guide (NOM) for the use and care of laboratory animals (NOM-062-ZOO-1999) and for the disposal of biological residues (NOM-087-ECOL-1995).

Four groups of rats were studied: (1) the control group (CT) was injected via jugular vein with DMSO/isotonic saline solution and then with isotonic saline solution by intraperitoneal injection; (2) SFN group (LKT Laboratories, St. Paul, MN): SFN suspended in DMSO/isotonic saline solution was injected at a dose of 500 μg/kg as previously described [31] via jugular vein two times (24 h before and 24 h after isotonic saline solution injection); (3) the cisplatin group (CIS) (Sigma-Aldrich, St. Louis, MO) received a solution of CIS dissolved in isotonic saline solution by a single intraperitoneal injection (7.5 mg/kg) [38]. (4) In the last group (CIS+SFN), SFN was
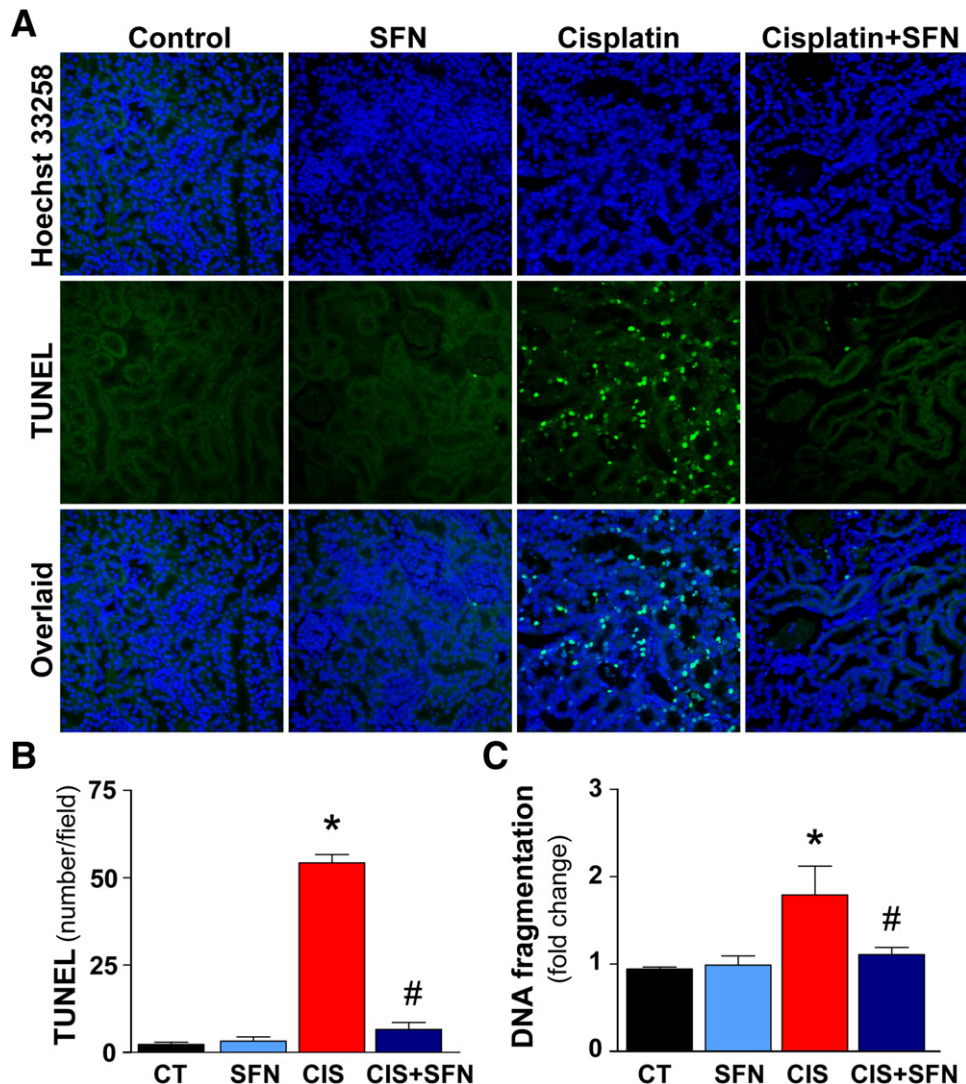

Fig. 1. SFN treatment prevents CIS-induced cell death. (A–B) Late apoptotic cell death in kidney was evaluated by fluorescent TUNEL staining. As shown in representative images (original magnification ×200), CIS markedly increased the number of TUNEL-positive cells (green) in the kidneys. (Middle) Nuclear staining with Hoechst (blue) and (right) colocalization with TUNEL. The number of TUNEL-positive cells was attenuated by SFN treatment. (C) CIS markedly increased DNA fragmentation; SFN treatment prevented this DNA damage. Results are means±S.E.M., n=5. *P<.001 vs. CT and SFN; #P<.001 vs. CIS (B), *P<.05 vs. CT and SFN; #P<.05 vs. CIS (C).

suspended in DMSO/isotonic saline solution and injected at a dose of 500μg/kg via jugular vein two times (24 h before and 24 h after 7.5 mg/kg CIS injection). Animals were sacrificed 72 h after CIS or vehicle injection, and kidneys were collected.

2.2. Western blot analyses

Antibodies for cleaved caspase-3, p38-α, p38-β, phospho-Jun N-terminal kinase (JNK), JNK, phospho-extracellular signal-regulated kinase (ERK), ERK, p53, phospho NF-κB (p65) and NF-κB (p65) were obtained from Cell Signaling Technology Inc. (Danvers, MA). Antibodies for TNF-α, ICAM and VCAM were obtained from R&D Systems Inc. (Minneapolis, MN) and β-actin antibody was obtained from Chemicon (Temecula, CA). Protein was extracted from tissue homogenate using TREP lysis buffer, containing protease inhibitor cocktail set III and phosphatase inhibitor cocktail set I (Calbiochem, EMD Biosciences, San Diego, CA). The kidney protein samples were mixed in Laemmli buffer, boiled for 7 min, and then equal amounts (40 μg per lane) were fractionated on Criterion Bis-Tris gel and transferred onto Hybond-C Extra nitrocellulose membrane (GE Healthcare Bio-Sciences Corp., Piscataway, NJ) using a semidry transfer apparatus (Bio-Rad, Hercules, CA). The blocking was carried out for 2 h in 5% nonfat dry milk in PBS. The primary antibodies were added as per the manufacturer's recommended dilution in PBS buffer containing 0.1% Tween 20 and 5% bovine serum albumin overnight at 4°C. After three washes in PBS containing 0.1% Tween 20, secondary HRP conjugate (Pierce Biotechnology, Inc. Rockford, IL) was added, followed by three washes with PBS containing 0.1% Tween 20. The blots were detected with Supersignal West Pico chemiluminescent substrate (Pierce Biotechnology, Inc. Rockford, IL) and developed using Kodak Biomax film (PerkinElmer, Wellesley, MA).

2.3. Detection of apoptosis by terminal deoxynucleotidyl transferase-mediated uridine triphosphate nick-end labeling, renal DNA fragmentation and caspase 3/7 activity assays

Paraffin sections were dewaxed; and in situ detection of apoptosis in the renal tissues was performed by terminal deoxynucleotidyl transferase-mediated uridine triphosphate nick-end labeling (TUNEL) assay according to the instructions of the manufacturer (Roche Diagnostics, Indianapolis, IN) and the number of apoptotic cells, as defined by chromatin condensation of nuclear fragmentation (apoptotic bodies), was counted. After TUNEL labeling, nuclei were labeled with Hoechst 33258 (Molecular Probes, Invitrogen) and the TUNEL-positive kidney cells were observed using an Olympus IX81 fluorescence microscope with a 20× objective at 2048×2048 resolution. The morphometric examination was performed by two independent, blinded investigators. The average number of apoptotic cells in each group was calculated by taking the average of TUNEL-positive apoptotic cells in 10 fields from each kidney sample with 200× magnification.

Caspase 3/7 activity in tissue lysate was measured using the Apo-One Homogenous Caspase-3/7 assay kit (Promega Corp., Madison, WI). An aliquot of caspase reagent was added to each well and mixed on a plate shaker for 1 h at room temperature shielded from light, and the fluorescence was measured.

The DNA fragmentation assay was based on measuring the amount of mono- and oligonucleosomes in the cytoplasmic fraction of tissue extracts using a commercially available kit (Roche Diagnostics) according to the manufacturer's instructions [9].

2.4. Myeloperoxidase staining and intercellular adhesion molecule-1 immunostaining

Kidney tissues were fixed in 4% buffered formalin. Paraffin-embedded sections were cut, deparaffinized, and hydrated in descending gradations of ethanol, followed by antigen retrieval with citrate buffer. Next, sections were incubated in 0.3% H₂O₂ in PBS to block endogenous peroxidase activity. The sections were then incubated with anti-myeloperoxidase (MPO) (Biocare Medical, Concord, CA), or anti-intercellular adhesion molecule-1 (ICAM-1) (R&D Systems Inc.) overnight at 4°C in a moist chamber. Biotinylated secondary antibodies and ABC reagent were added as per the kit's instructions (Vector Laboratories, Burlingame, CA). Color development was induced by incubation with a DAB kit (Vector Laboratories) for 3–5 min, and specific staining was visualized by light microscopy as described [9,39–41]. After MPO staining and ICAM-1 immunostaining, nuclei were contrast with Nuclear Fast Red H-3403 (Vector Laboratories).

2.5. Data analysis

Results were expressed as mean±S.E.M. Data were analyzed by ANOVA followed by Dunnett's multiple comparisons test, using software Prism 5 (GraphPad, San Diego, CA). A P value < .05 was considered significant. N represents number of separate experiments/rats.

3. Results

3.1. SFN prevents cisplatin-induced cellular death

Cellular death was evaluated by TUNEL staining, DNA fragmentation, renal caspase-3 cleavage and caspase-3/7 activity assays (Figs. 1 and 2). As shown in Fig. 1, the TUNEL-positive
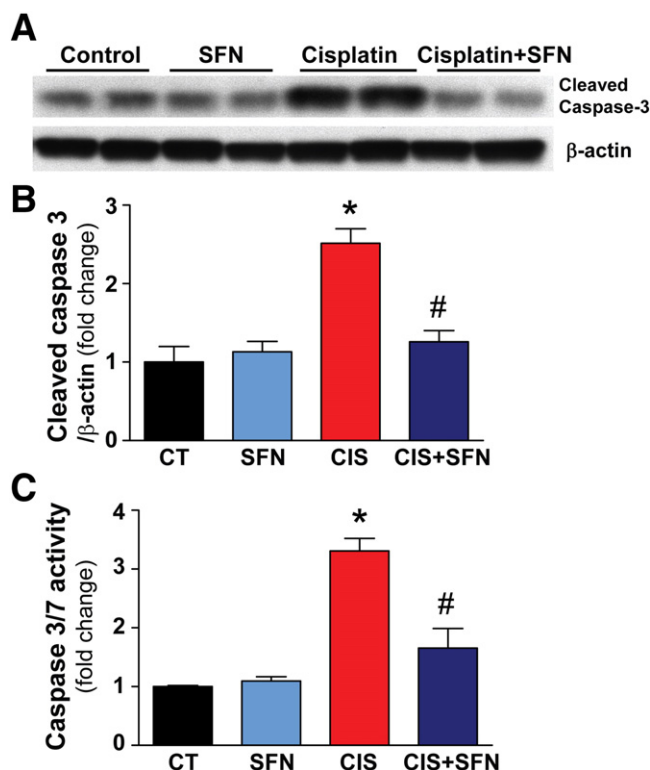

Fig. 2. SFN treatment prevents CIS-induced cell death by decreasing caspase-3/7 activity and caspase-3 cleavage. Caspase-3 cleavage and caspase-3/7 activity in kidney homogenates were markedly increased in CIS-treated group. SFN treatment prevented significantly these increases. Results are means±S.E.M., n=4 (B), n=5 (C). *P<.001 vs. CT and SFN; #P<.001 vs. CIS (B and C).
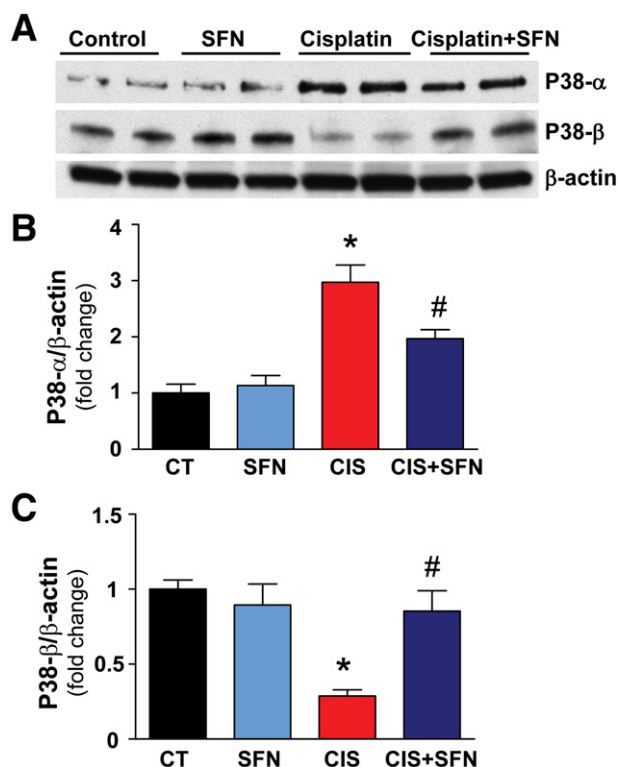

Fig. 3. SFN treatment prevents CIS-mediated increase in p38-α and decrease in p38-β MAPK. CIS-induced p38-α and p38-β expression in rat kidneys was prevented significantly by SFN treatment. Results are means±S.E.M., n=4. *P<.001 vs. CT and SFN; #P<.05 vs. CIS (p38-α); *P<.001 vs. CT and SFN; #P<.05 vs. CIS (p38-β).
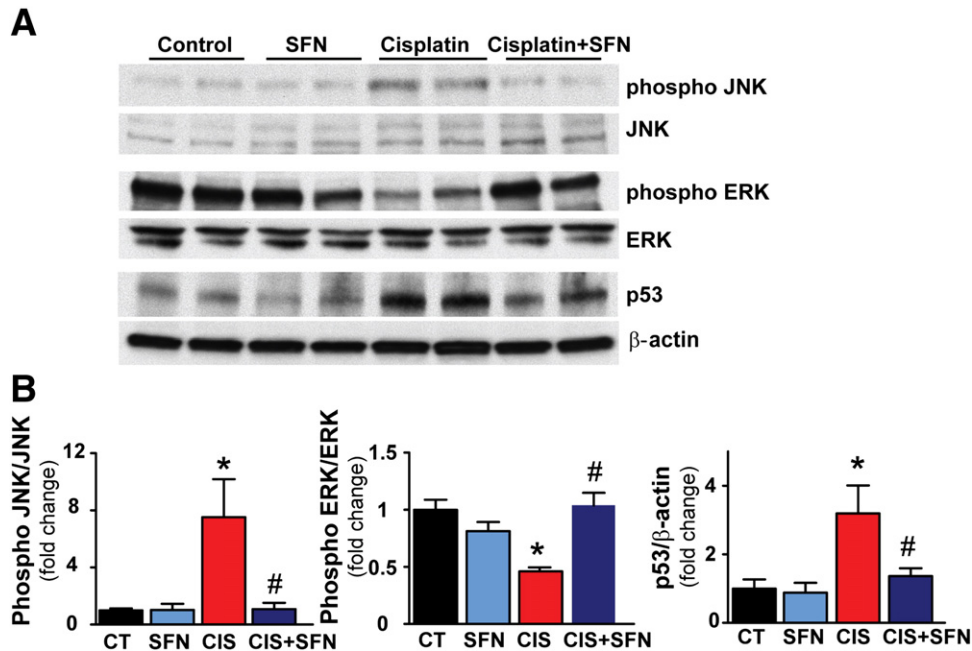
Fig. 4. CIS-induced MAPK activation is attenuated by SFN treatment. CIS-induced marked phosphorylation of JNK and p53, and dephosphorylation of ERK was attenuated by SFN treatment in CIS+SFN group. Results are means±S.E.M., n=4. *P<.05 vs. CT and SFN; #P<.05 vs. CIS (phospho-JNK); *P<.001, *P<.05 vs. CT and SFN, respectively; #P<.05 vs. CIS (phospho-ERK); *P<.05 vs. CT and SFN; #P<.05 vs. CIS (p53).

(late apoptotic) cell number and DNA fragmentation were markedly increased in kidneys of CIS-treated rats (Fig. 1A–C). SFN pretreatment prevented the CIS-induced enhanced cell death (Fig. 1A–C). Caspase-3/7 activity and caspase-3 cleavage in kidney homogenates from CIS-treated rats were also markedly increased (Fig. 2A–C), which could be largely attenuated by SFN pretreatment. SFN had no effect in control (CT) animals on cell death (Figs. 1 and 2).

3.2. SFN prevents cisplatin-mediated increase in p38-α and decrease in p38-β MAPK

As shown in Fig. 3, SFN significantly attenuated the CIS-induced increased expression of the proapoptotic p38-α in the kidneys while prevented the abolished expression of the antiapoptotic p38-β (Fig. 3A–C). SFN had no effect in control (CT) animals on p38-α or β (Fig. 3A–C).

3.3. Cisplatin-induced MAPK activation is attenuated by SFN treatment

CIS treatment induced marked phosphorylation of renal phospho-JNK and induction of p53 (7.52- and 3.19-fold, respectively, as compared with CT group) and dephosphorylation of phospho-ERK (decreased to 0.46-fold vs. CT) (Fig. 4). SFN treatment significantly attenuated these alterations (Fig. 4). SFN had no effect in control (CT) animals on any of these variables (Fig. 4).

3.4. SFN treatment prevents inflammatory response in rat kidneys

CIS triggered markedly increased inflammatory response in the kidneys (elevated TNF-α levels and NF-κB activation) (Fig. 5). SFN treatment significant reduced these proinflammatory changes (Fig. 5). SFN had no effect in control (CT) animals on any of these variables (Fig. 5).

3.5. SFN treatment prevents cisplatin-induced enhanced renal adhesion molecule expression and renal inflammatory cell infiltration

As shown in Fig. 6A–B, CIS treatment triggered increased expression of adhesion molecules ICAM-1 and VCAM-1 (6.8- and
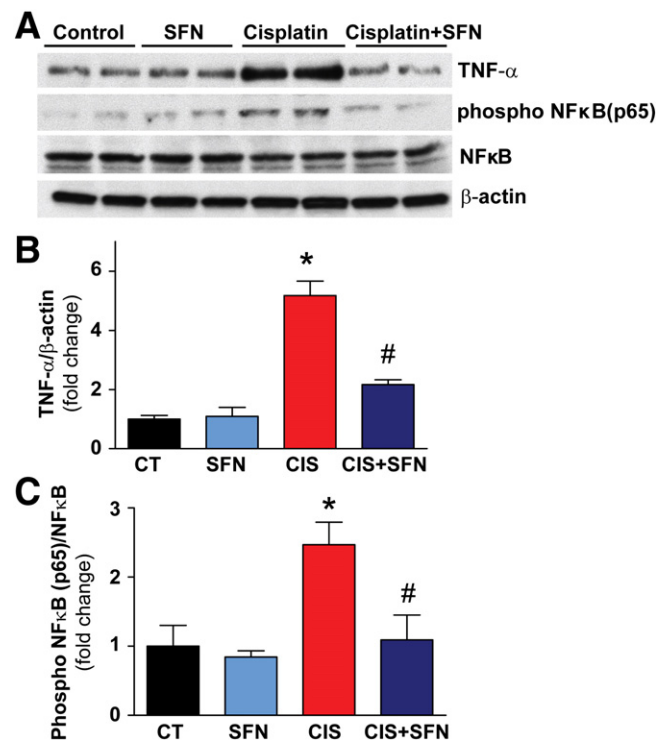

Fig. 5. SFN treatment prevents inflammatory response in rat kidneys. CIS-induced marked phosphorylation of TNF-α and phospho NF-κB (p65) was prevented by SFN treatment in CIS+SFN group. Results are means±S.E.M., n=4. *P<.001 vs. CT and SFN; #P<.001 vs. CIS (TNF-α); *P<.001 vs. CT and SFN; #P<.05 vs. CIS (phospho NF-κB (p65)).
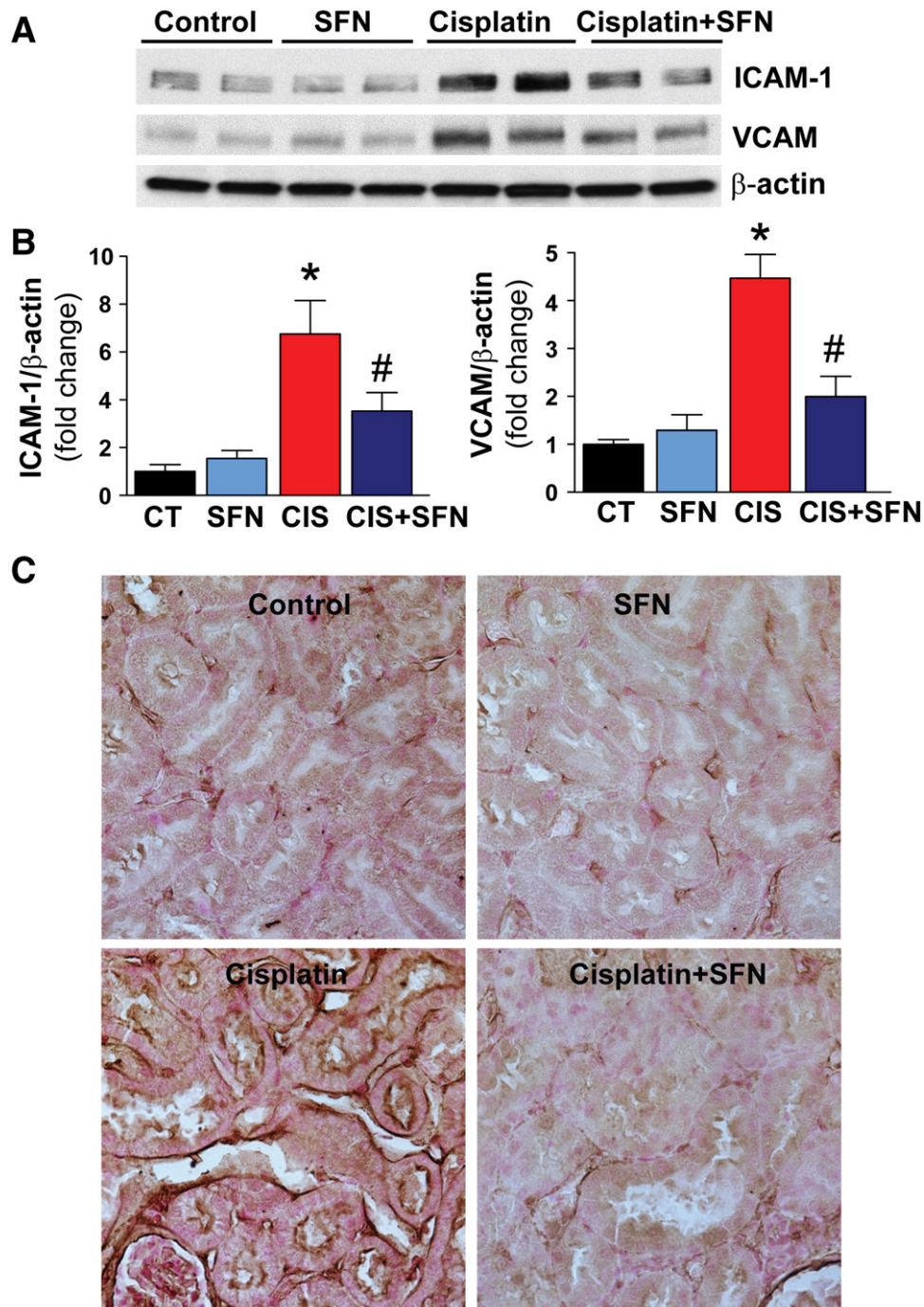
Fig. 6. SFN treatment prevents CIS-induced enhanced renal adhesion molecule (ICAM-1, VCAM-1) expression. (A–B) CIS-induced ICAM-1 and VCAM-1 expression in rat kidneys was prevented significantly by SFN treatment in CIS+SFN group. Results are means±S.E.M., $n=4$. *$P<.001$ vs. CT and SFN; #$P<.05$ vs. CIS (ICAM-1); *$P<.001$ vs. CT and SFN; #$P<.001$ vs. CIS (VCAM-1). (C) ICAM-1 expression is evident by an increased intensity of staining in kidney tissue in CIS-treated groups. This expression was prevented by SFN treatment in CIS+SFN group ($n=5$).

4.5-fold, respectively) as compared to CT group. ICAM-1 expression is evident by an increased intensity of staining in kidney tissue in CIS-treated group (Fig. 6C). These increases of expression of ICAM-1, VCAM-1 and ICAM-1 staining were significantly attenuated by SFN pretreatment (Fig. 6A–C). SFN had no effect in control (CT) animals on ICAM-1 or VCAM-1 expressions (Fig. 6).

CIS treatment significantly increased the number of MPO-positive inflammatory cells around the damaged areas in the kidneys (indicative of neutrophil infiltration) to ~33 cells/field, as compared to CT group (Fig. 7). Neutrophil infiltration was markedly attenuated

by SFN pretreatment (Fig. 7). SFN had no effect in control (CT) animals on MPO-positive infiltrating cells (Fig. 7).

4. Discussion

In this study, we demonstrate that the CIS-induced nephropathy is associated with marked activation of stress signaling and inflammatory pathways coupled with attenuation of prosurvival signaling mechanisms promoting cell death in tubular cells. We also show that SFN, a natural constituent of cruciferous vegetables
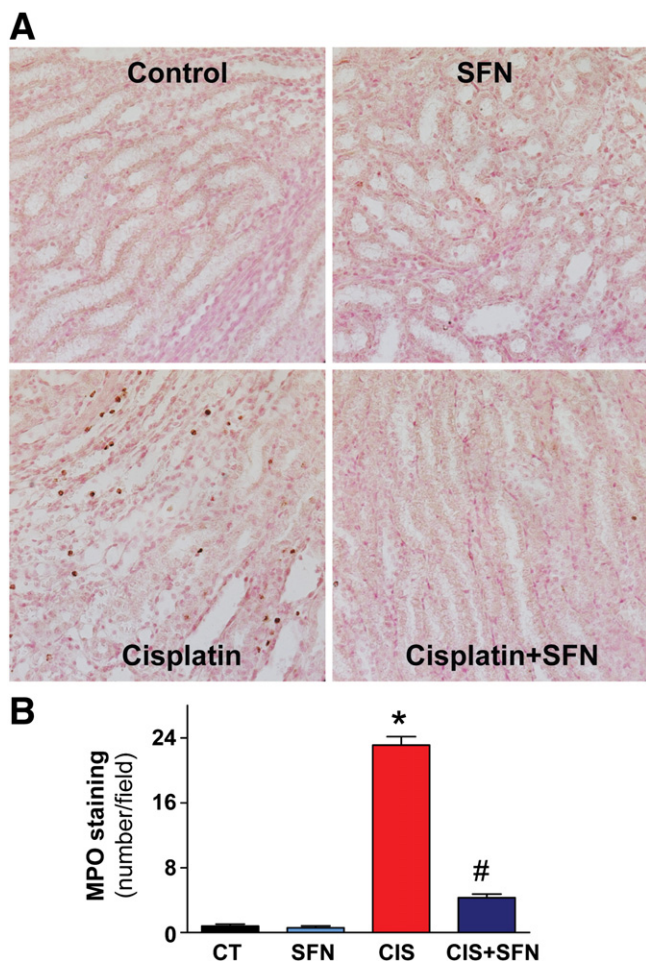
A

B

Fig. 7. SFN treatment prevents CIS-induced renal inflammatory cell infiltration. CIS significantly increased the number of renal MPO-positive cells (brown staining) around the damaged tubular structures (original magnification ×400). SFN treatment prevented significantly the leukocyte infiltration in the CIS+SFN group. Results are means±S.E.M., $n=5$. *$P<.001$ vs. CT and SFN; #$P<.001$ vs. CIS.

such as broccoli, cabbage, etc., is able to provide significant protection against CIS-induced cell death by attenuating proapoptotic pathways and inflammation and by promoting activation of prosurvival mechanisms.

Several studies have demonstrated that the cisplatin-induced nephropathy is associated with markedly increased caspase-dependent apoptosis; however, the signaling mechanisms were not explored in detail [3,8,42].

p38 MAPK exists in four isoforms: p38-α, p38-β, p38-γ, p38-δ. Two of the four isomers of p38 MAPK (-α, -β) seem to play opposing regulatory roles in myocardial ischemic-reperfusion injury, diabetic cardiomyopathy and preconditioning [43]. While p38-β is antiapoptotic, p38-α promotes cell death [18,21]. Consistently with these results, CIS induced marked activation of proapoptotic p38-α in the kidneys and inactivation of prosurvival p38-β pathway. SFN treatment significantly reduced the CIS-induced enhanced p38α signaling and prevented the abolished p38-β activation. We also found that CIS induced increased activation of JNK and diminished phosphorylation of ERK, which can promote cell death [25,44]. p53 is a powerful tumor suppressor gene in human cancer and is also importantly implicated in cell death associated with CIS-induced nephrotoxicity [19,45]. Indeed, CIS-induced nephropathy was associated with increased activation of p53. The above-mentioned CIS-induced alterations in signaling processes could largely be attenuated by SFN pretreatment.

JNK and p38 signaling pathways can be activated by proinflammatory cytokines or in response to cellular stress such as oxidative, osmotic or genotoxic stress [45]. CIS induces increased renal expression of the inflammatory cytokine TNF-α [9,12,27], which can also trigger activation of NF-κB by increased reactive oxygen species generation [23]. Indeed, as also supported by our data, NF-κB activation plays an important role in CIS-induced renal injury [45]. In accordance with previous studies mentioned above, CIS induced TNF-α induction and subsequent phosphorylation of NF-κB (p65), which were largely prevented by SFN pretreatment (Fig. 5).

Inflammation plays an important physiological role in CIS-induced nephrotoxicity, and it has been demonstrated that CIS administration increases the infiltration of leukocytes and macrophages within 72 h in damaged renal tissues [8,23], as well as it increases the expression of adhesion molecules (e.g., ICAM-1 and VCAM-1), which play an important role in the pathophysiology of acute kidney injury [26,45]. We found increased expression of ICAM-1 and VCAM-1 in kidneys of CIS-treated rats (Fig. 6A–B), as well as increased number of myeloperoxydase positive inflammatory cells, which could be attenuated by pretreatment with SFN (Figs. 6 and 7).

Although our study has shown that administration of SFN was able to attenuate the CIS-induced kidney injury, it has not addressed the question of potential interference of SFN with the chemotherapeutic efficacy of CIS, which is a potential limitation. However, on the basis of multiple reports demonstrating anticancer effects of SFN by itself (comprising inhibition of histone deacetylation, cell cycle arrest, inhibition of cell growth and induction of apoptosis, among many other mechanisms) in cell culture studies of human liver, colon, prostate, breast, colon, ovarian, bladder and immune cell malignancies, and studies indicating efficacy of broccoli, broccoli sprouts and SF in animal models of cancer (reviewed in Ref. [36]), it is more likely that SFN would rather enhance the chemotherapeutic efficacy of CIS. The latter is also supported by SFN's reported antiangiogenic properties [35] and its inhibitory effect on NF-κB signal transduction pathway observed in our study. Furthermore, it has recently been demonstrated that SFN is not decreasing the antitumor potential of CIS [46] and in cancer stem cells it in fact can even enhance its cytotoxicity [47].

Another important consideration to address is if the dose of SFN administered in our study bears any human clinical relevance. This is a very difficult question to answer because of the possible differences in the kinetics, distribution and metabolism of the compound in humans and in rats (e.g., human $T_{1/2}$ of SFN has been reported to be more than 10 times longer than in rats [48,49]). However, the SFN plasma levels in rats reach 46 ± 19 ng/ml after administration of 0.5 mg/kg of this isothiocyanate [50], the same dose used in the present study. Similar plasma levels of SFN were reported in humans after broccoli consumption: 12 ± 1.6 ng/ml [51] and 18.2 ± 6.5 ng/ml [52], suggesting that the dose used in the present study may have clinical relevance. In addition, in support of the idea that nutritional amounts of broccoli might exert therapeutic effects, a pilot study has found that consumption of only 68 g of broccoli sprouts was able to inhibit histone deacetylase (a putative target of SFN's anticancer properties) in peripheral blood mononuclear cells [53]. Importantly, the dose of SFN used in our study is also in the range of the amounts reported to exert antitumor effects in animal models of various cancers [36].

Collectively, we demonstrated that the cisplatin-induced nephropathy is associated with activation of various cell death and proinflammatory pathways (p53, JNK, p38α, TNF-α and NF-κB) and impairments of key prosurvival signaling mechanisms (ERK and p38-β); and these pathological alterations can be prevented by a natural, low-cost, antioxidant/antiinflammatory constituent from cruciferous vegetables (e.g., broccoli, Brussels sprouts, etc.), providing a novel approach for preventing this devastating complication of the chemotherapy. These results, coupled with the potent antitumor effect of SFN reported in multiple preclinical studies, and

epidemiological evidence suggesting that dietary intake of cruciferous vegetables may lower cancer risk in humans, are particularly exciting. Well-powered future clinical trials to explore the potential benefit of SFN supplementation and/or whole broccoli consumption in cancer prevention, treatment or against cisplatin-induced nephropathy are warranted.

Acknowledgments

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