Sulforaphane protects kidneys against ischemia-reperfusion injury through induction of the Nrf2-dependent phase 2 enzyme

Ha-Yong Yoon [a,1], *Nam-In Kang* [b,1], *Hern-Ku Lee* [b], *Kyu Yun Jang* [c], *Jin-Woo Park* [a], *Byung-Hyun Park* [a,*]

[a] *Department of Biochemistry, Medical School and Institute for Medical Sciences, Chonbuk National University, Jeonju, Jeonbuk 561-756, Republic of Korea*
[b] *Department of Immunology, Medical School and Institute for Medical Sciences, Chonbuk National University, Jeonju, Jeonbuk 561-756, Republic of Korea*
[c] *Department of Pathology, Medical School and Institute for Medical Sciences, Chonbuk National University, Jeonju, Jeonbuk 561-756, Republic of Korea*

ARTICLE INFO

ABSTRACT

Reactive oxygen species are important mediators that exert a toxic effect during ischemia-reperfusion injury of various organs. Sulforaphane, which is a naturally occurring isothiocyanate that is present in cruciferous vegetables such as broccoli, is known to be an indirect antioxidant that acts by inducing Nrf2-dependent phase 2 enzymes. Phase 2 enzymes such as heme oxygenase-1, NAD(P)H: quinone oxidoreductase 1, glutathione reductase, and glutathione peroxidase participate in adaptive and protective responses to oxidative stress and various inflammatory stimuli. Therefore, we evaluated the preactivation of Nrf2 by sulforaphane to determine if it could inhibit ischemia-reperfusion-induced kidney damage. Treatment of HK2 renal tubular epithelial cells with sulforaphane effectively protected cells against cytotoxicity induced by hypoxia-reoxygenation, and sulforaphane dramatically induced phase 2 enzymes by decreasing the Keap1 protein levels and increasing Nrf2 nuclear translocation. Additionally, a second set of experiments using a renal ischemia-reperfusion model produced results that were essentially the same as those observed when HK2 cells were used; namely, that sulforaphane induced Nrf2-dependent phase 2 enzymes and thereby improved ischemia-reperfusion-induced changes in the lipid hydroperoxides, glutathione, creatinine clearance, kidney weight, and histologic abnormalities. Collectively, these results suggest that sulforaphane can be used as an effective adjunct for the prevention of renal oxidative insults during ischemia-reperfusion injury.

© 2008 Elsevier Inc. All rights reserved.

1. Introduction

Renal ischemia-reperfusion injury, which occurs in many clinical settings including renal transplantation, shock, and vascular surgery [1], is a major cause of acute renal failure. Clinical and experimental studies have shown that the tissue damage that occurs following ischemia-reperfusion, especially during reperfusion, is due in part to reactive oxygen species (ROS) [2], and the role of ROS in the pathophysiology of ischemia-reperfusion injury is supported by the increased formation of lipid hydroperoxides and other toxic products that occurs following such an injury [3]. Additionally, the administration of antioxidants has been shown to have beneficial effects on both *in vitro* and *in vivo* systems following ischemia-reperfusion injury [4].

Sulforaphane is a naturally occurring isothiocyanate that is present in cruciferous vegetables such as broccoli that has gained attention as a chemopreventive compound [5]. The cytoprotective effect exerted by this compound is mediated by transcription factor NF-E2-related factor-2 (Nrf2), which, under basal conditions, is bound to the Kelch-like ECH-associated protein 1 (Keap1) in the cytoplasm. However, sulforaphane can disrupt the Nrf2/Keap1 complex, thereby permitting Nrf2 to translocate into the nucleus [6–8]. Regulation of both the basal and inducible expressions of cytoprotective genes by Nrf2 is mediated by the antioxidant response element, which is a *cis*-acting sequence found in the 5′-flanking region of genes encoding phase 2 enzymes, including heme oxygenase-1 (HO-1), NAD(P)H: quinone oxidoreductase 1 (NQO1), glutathione reductase (GR), and glutathione peroxidase (GPx). Phase 2 enzymes, in turn, play a major role in the detoxification of ROS produced by xenobiotics [9] or during ischemia-reperfusion [10–13].

Studies conducted to date suggest that Nrf2 activation by sulforaphane results in effective protection from cancers by upregulating antioxidant response element-related detoxification enzymes [14,15]. However, these studies have focused on the chemoprevention by sulforaphane and to our knowledge, no one has evaluated sulforaphane to determine if it can protect renal tissue from ischemia-reperfusion injury. Therefore, in this study, we activated the cellular and renal Nrf2-phase 2 enzyme system by sulforaphane using hypoxia-reoxygenation and renal ischemia-reperfusion models, and then examined the effects on renal function, pathology, and biochemical response. By measuring the amount of lipid hydroperoxides and glutathione, we also demonstrated that the activation of the Nrf2 contributed to the attenuation of ROS generation. Taken together, the results of this study provide insights into the application of the Nrf2-phase 2 enzyme system in acute renal ischemia-reperfusion injury.

2. Materials and methods

2.1. Cell culture and reagents

HK2 cells, the human proximal tubule cell line, were obtained from the American type culture collection and grown at 37 °C under a humidified, 5% CO_2 atmosphere in Dulbecco's Modified Eagle Medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum, 100 U/ml of penicillin, 100 μg/ml of streptomycin, and 2.5 μg/ml of amphotericin B. Sulforaphane was purchased from LKT Laboratories (St. Paul, MN) and dissolved at 20 mM in dimethyl sulfoxide. All reagents were purchased from Sigma (St. Louis, MO) unless otherwise noted.

2.2. Hypoxia-reoxygenation protocol

HK2 cells in Dulbecco's Modified Eagle Medium without fetal bovine serum were incubated at 37 °C in anaerobic jars (Oxoid Ltd., Basingstoke, Hampshire, England) with oxygen absorbing packs (AnaeroGen, Oxoid Ltd.) for 24 h. According to the description by company, this pack, AnaeroGen, reduces the oxygen level in the jar to below 1% and the resulting carbon dioxide level is between 9% and 13%. Reoxygenation was achieved by removing the cells from anaerobic jar and placing them into a normoxic state. Next the cells were incubated in Dulbecco's modified eagle medium supplemented with 10% fetal bovine serum at 37 °C under a humidified, 5% CO_2 atmosphere. In the sulforaphane-pretreated group, cells were incubated with sulforaphane for 12 h prior to placing them in an anaerobic jar.

2.3. MTT assay for cell viability

The viability of cultured cells was determined by assaying the reduction of 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) to formazan. After treatment, cells were washed twice with PBS, which was followed by the addition of MTT (100 μg/100 μl of PBS). Next, the cells were incubated at 37 °C for 1 h, after which DMSO (100 μl) was added to dissolve the formazan crystals. The absorbance was then measured at 570 nm using a model Spectra MAX PLUS spectrophotometer (Molecular Devices, Sunnyvale, CA).

2.4. Immunofluorescent staining

For immunofluorescent staining, the control or treated cells were fixed in cold 100% methanol for 2–3 min at room temperature and then washed three times with PBS. Next, the cells were blocked with 0.1% bovine serum albumin in PBS for 1 h at room temperature, after which they were incubated for 18 h at 4 °C with anti-Nrf2 antibody (Santa Cruz Biochemicals, Santa Cruz, CA) in PBS containing 3% bovine serum albumin. The cells were then incubated with FITC-conjugated secondary antibody for 1 h after serial washing with PBS. Counter-staining with propidium iodide (PI, 5 μg/ml) was conducted to verify the location and integrity of the nuclei, and the stained cells were then washed and observed under a confocal microscope (TE2000 & D-eclipse C1, Nikon, Japan).

2.5. Preparation of nuclear extracts

After treatment, cells and tissues were immediately washed twice, scraped into 1.5 ml of ice-cold PBS (pH 7.9), and pelleted at 12,000 × g for 30 s. The cell pellet was then suspended in cold hypotonic lysis buffer (10 mM Hepes, 1.5 mM $MgCl_2$, 0.2 mM KCl, 0.2 mM PMSF, and 0.5 mM dithiothreitol), vortexed for 10 s, and then incubated on ice for 15 min. Next, the packed cells were resuspended in ice-cold hypotonic lysis

buffer in the presence of 50 µl of 10% Nonidet P-40, and then incubated on ice for 25 min. The nuclear fraction was then precipitated by centrifugation at $13,000 \times g$ for 1 min at 4 °C, after which the supernatants (cytoplasmic extracts) were collected and stored at -80 °C. The pelleted nuclei were then resuspended in 50–100 µl of low salt extraction buffer (20 mM Hepes, pH 7.9, 1.5 mM $MgCl_2$, 25% glycerol, 20 mM KCl, 0.2 mM EDTA, 0.2 mM PMSF, and 0.5 mM dithiothreitol), added to an equal volume of high salt extraction buffer (20 mM Hepes, pH 7.9, 1.5 mM $MgCl_2$, 25% glycerol, 80 mM KCl, 0.2 mM EDTA, 0.2 mM PMSF, and 0.5 mM dithiothreitol) in a dropwise fashion, and then incubated under continuous shaking at 4 °C for 45 min. The sample was then centrifuged for 20 min at $12,000 \times g$. Aliquots of the nuclear extracts were stored at -80 °C.

2.6. Western blot analysis

Cells and tissues were homogenized in 100 µl of ice-cold lysis buffer (20 mM Hepes, pH 7.2, 1% Triton X-100, 10% glycerol, 1 mM PMSF, 10 µg/ml leupeptin, 10 µg/ml aprotinin). The homogenates, which contained 20 µg of protein, were then separated by SDS-PAGE with 12% resolving and 3% acrylamide stacking gels, after which they were transferred to nitrocellulose membranes. Next, the nitrocellulose membranes were blocked with 2% bovine serum albumin and then incubated for 4 h with 1 µg/ml of primary antibodies for Nrf2, Keap1, HO-1, NQO1, GR, GPx, PCNA, and actin (Santa Cruz Biochemicals, Santa Cruz, CA). Horseradish peroxidase-conjugated IgG (Zymed, South San Francisco, CA) was then used as a secondary antibody, after which the protein expression levels were determined by analyzing the signals captured on the nitrocellulose membranes using a Chemi-doc image analyzer (Bio-Rad, Hercules, CA).

2.7. RNA isolation and real-time PCR

RNA was isolated from HK2 cells or kidney tissues using Trizol reagent (Invitrogen, Carlsbad, CA). RNA was then precipitated with isopropanol and dissolved in DEPC-treated distilled water, after which, total RNA (2 µg) was treated with RNase-free DNase (Invitrogen) and first-strand cDNA was generated using the random hexamer primer provided

in the first-strand cDNA synthesis kit (Applied Biosystems, Foster City, CA). Specific primers for each gene (Table 1) were designed using primer express software (Applied Biosystems), and the sequence for the control 18S ribosomal RNA was purchased from Applied Biosystems and used as the invariant control. The real-time PCR reaction, which was conducted in a mixture that had a final volume of 10 µl, consisted of 10 ng of reverse transcribed total RNA, 167 nM of forward and reverse primers and $2 \times$ PCR master mixtures. The PCR reaction was conducted in 384-well plates using the ABI Prism 7900HT Sequence Detection System (Applied Biosystems), and all reactions were conducted in triplicate.

2.8. Ischemia-reperfusion protocol

Renal ischemia-reperfusion was conducted using 6-week-old male Sprague–Dawley rats that were anaesthetized with a ketamine–xylazine mixture. In the ischemia-reperfusion group, both renal pedicles were clamped through flank incisions for 20 min using a non-traumatic microvascular clip and then subjected to reperfusion. In addition, another group of rats was only subjected to the flank incision (sham-operated control group). Sulforaphane (500 µg/body weight kg, i.v.) was suspended in phosphate buffered saline (PBS, pH 7.4) and injected intravenously 24 h before the clamping operation (sulforaphane + ischemia-reperfusion group). Three h after reperfusion, kidneys were collected for glutathione assay, Western blot and real-time PCR analyses, and histologic evaluation and lipid hydroperoxides assay were performed 48 h after reperfusion. All experimental procedures were approved by the Institutional Animal Care and Use Committee at Chonbuk National University.

2.9. Histology and histopathologic scoring

Sections of sham-operated, ischemia-reperfusion-induced, and sulforaphane-pretreated and ischemia-reperfusion-induced kidneys were fixed in 10% neutral buffered formalin and then embedded in paraffin. Tissue was sectioned at 4 µm and then stained with hematoxylin and eosin for light microscopic analysis. Histopathologic scoring was performed using a blind method and the score was assessed by grading tubular necrosis, loss of brush border, cast formation, and tubular dilatation in 10 randomly chosen, non-overlapping fields ($\times 200$ magnification) as follows: 0, none; 1, $\leq 10\%$; 2, 11–25%; 3, 26–45%; 4, 46–75%; and 5, $\geq 76\%$ [16].

2.10. Lipid hydroperoxides assay

Lipid hydroperoxides were measured in fresh plasma using a Lipid Hydroperoxide Assay Kit (Calbiochem, La Jolla, CA) according to the manufacturer's instructions.

2.11. Glutathione measurement

Total and reduced glutathione was measured in kidneys using Total Glutathione Detection Kit (Assay Designs, Inc., Ann Arbor, MI, USA) according to the manufacturer's instruction.

Table 1 – Sequences and accession numbers for primers (forward, FOR and reverse, REV) used in real-time RT-PCR		
Gene	Sequences for primers	Accession No.
HO-1	FOR: CAGCCCCACCAAGTTCAAA REV: CAGCCCCACCAAGTTCAAA	NM_012580
NQO1	FOR: GCCCGCATGCAGATCCT REV: GGTCTCCTCCCA-GACGGTTT	NM_017000
GR	FOR: CAAGATCACCCAGAG-CAATGC REV: CATCCAGGTGGTGCTTTCG	NM_053906
GPx	FOR: GCCCGCATGCAGATCCT REV: GGTCTCCTCCCA-GACGGTTT	X07365

2.12. Statistical analysis

Statistical analysis of the data was conducted using ANOVA and Duncan's test. Differences with a $p < 0.05$ were considered statistically significant.

3. Results

3.1. Prevention of hypoxia-reoxygenation-induced HK2 cell death by sulforaphane

HK2 cells were cultured to near confluence, and then cells that were pretreated with or without sulforaphane for 12 h were exposed to hypoxia-reoxygenation injury for 48 h, at which time they were harvested and their viability was assessed using an MTT assay. Hypoxia-reoxygenation significantly decreased the cell viability to $57.0 \pm 8.1\%$ ($p < 0.01$) when compared to that of the control (Fig. 1). However, prior treatment with sulforaphane (1–20 μM) for 12 h before hypoxia-reoxygenation resulted in substantial concentration-dependent protection against hypoxia-reoxygenation-induced cytotoxicity, and the survival of HK2 cells was improved as the concentration of sulforaphane increased, with $92.9 \pm 6.0\%$ of cells pretreated with 20 μM of sulforaphane being viable.

3.2. Inductions of phase 2 enzymes by sulforaphane through Nrf2 activation in HK2 cells

Sulforaphane is known to activate the Nrf2-dependent signaling pathway. Under normal quiescent conditions, the cytoplasmic protein, Keap1, binds directly to Nrf2 and represses the transactivation [6–8]. However, exposure of HK2 cells to sulforaphane for 12 h decreased the cytoplasmic Keap1 protein level and increased the level of nuclear Nrf2
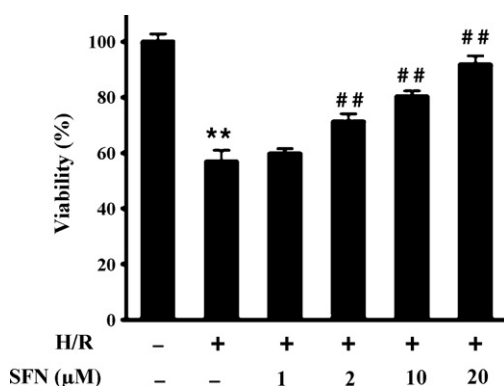

Fig. 1 – Sulforaphane prevents hypoxia-reoxygenation-induced cell death in HK2 cells. HK2 cells (1×10^5) were pretreated with the indicated concentrations of sulforaphane (SFN) for 12 h, and then incubated in anaerobic jars for 24 h. After hypoxic incubation, cells were reoxygenated for 24 h. The cell viability was then determined using a MTT assay. Each value represents the mean ± S.E.M. of four independent experiments. $^{**}p < 0.01$ vs. control; $^{\#\#}p < 0.01$ vs. hypoxia-reoxygenation (H/R).

protein (Fig. 2A). Immunofluorescent staining also confirmed the increased nuclear translocation of Nrf2 after treatment with sulforaphane (Fig. 2B). Next, we evaluated the activation and enhanced nuclear translocation of Nrf2 by sulforaphane to determine if it is involved in the induction of phase 2 enzymes. To accomplish this, the mRNA expression profiles of phase 2 enzymes were examined by real-time PCR. Treatment with hypoxia-reoxygenation alone induced only a slight increase in the expression of HO-1, NQO1, GR, and GPx mRNA, these increases were not significant. However, prior treatment of the HK2 cells with sulforaphane moderately augmented the expressions of all of the aforementioned genes (Fig. 2C). In addition, increased transcription as a result of sulforaphane treatment was accompanied by increased expression of their protein levels in HK2 cells (Fig. 2D).

3.3. Effect of sulforaphane on ischemia-reperfusion-mediated renal injury

To induce ischemia-reperfusion, the renal pedicles were bilaterally clamped for 20 min, after which they were reopened. Sulforaphane was administered via an i.v. injection 24 h prior to inducing ischemia-reperfusion to evaluate its effects. Forty-eight hours after ischemia-reperfusion, kidneys and blood were collected. The serum creatinine concentration of the ischemia-reperfusion group was significantly greater than that of the control group (Fig. 3A), however, when sulforaphane was administered prior to ischemia-reperfusion, the serum creatinine level was significantly depressed ($p < 0.01$). Macroscopically, kidneys subjected to ischemia-reperfusion were clearly enlarged and appeared edematous (data not shown). In addition, the ischemia-reperfusion group had significantly greater kidney weight than that of the control (Fig. 3B), however, the kidney weight of the sulforaphane + ischemia-reperfusion group was significantly reduced ($p < 0.01$). Upon histological examination, the ischemia-reperfusion group showed features typical of severe acute tubular damage, including extensive tubular necrosis, tubular dilatation, and loss of brush border (Fig. 4A). A grading system was introduced to compare the severity of the histologic signs of ischemia-reperfusion, and the histopathologic score of the ischemia-reperfusion-induced kidneys was increased by more than ten-fold when compared with that of the control group (Fig. 4B). Conversely, pretreatment with sulforaphane preserved the normal morphology of the kidney and resulted in slight swelling of the tubular epithelium and a slight loss of brush border. The histopathologic scores supported the histologic findings, with sulforaphane treatment reducing the score from 3.8 ± 0.2 to 1.2 ± 0.3 ($p < 0.01$).

3.4. Induction of phase 2 enzymes by sulforaphane through Nrf2 activation in rat renal tissues

Because Nrf2 activation by sulforaphane protected the HK2 cells from hypoxia-reoxygenation by inducing Nrf2-dependent phase 2 enzymes, we evaluated sulforaphane to determine if it could activate Nrf2 and induce phase 2 enzymes in renal tissues as well. Consistent with the results of the in vitro studies, Nrf2 was activated by sulforaphane in renal tissues, which was demonstrated by the presence of

decreased cytoplasmic levels of Keap1 protein and a resultant increase in nuclear Nrf2 translocation (Fig. 5A). In addition, real-time PCR data showed that sulforaphane caused dramatic increases in phase 2 enzymes including HO-1, NQO1, GR, and GPx (Fig. 5B). Furthermore, the change in protein levels was consistent with that of the mRNA levels (Fig. 5C). Eschwege et al. [17] demonstrated that renal ischemia-reperfusion is associated with lipid peroxidation, which is an autocatalytic mechanism that leads to oxidative destruction of cellular membranes. We therefore determined the lipid hydroperoxides levels in the plasma to compare the effects of systemic exposure to oxidative insult during ischemia-reperfusion. We found that renal ischemia-reperfusion increased the plasma lipid hydroperoxides levels from 17.9 ± 1.1 to 30.2 ± 2.5 nmol/ml (Fig. 6A), however, pretreatment of the rats with sulforaphane abolished these effects. The endogenous antioxidant, reduced form of glutathione (GSH), level in the renal tissue was decreased significantly after ischemia-reperfusion (8.2 ± 0.9 μmol/g). On the other hand sulforaphane treatment

significantly reversed this ischemia-reperfusion-induced GSH reduction (18.2 ± 0.8 μmol/g) (Fig. 6B).

4.   Discussion

Evidences suggest the critical role of ROS in ischemia-reperfusion-mediated renal injury [2–4]. Therefore, development of simple long-term strategies for reducing oxidative stress by inducing endogenous phase 2 enzymes with dietary means is attractive. In the present study, we demonstrated that Nrf2 activation by sulforaphane treatment protected kidney against renal damage induced by ischemia-reperfusion. Sulforaphane induced Nrf2-activated phase 2 enzymes, which consequently resulted in a significant increase in reduced form of glutathione and decrease in lipid hydroperoxides level. In accordance with these biochemical changes, the histologic evaluation of the tissues revealed that sulforaphane was also effective at protecting the kidneys against
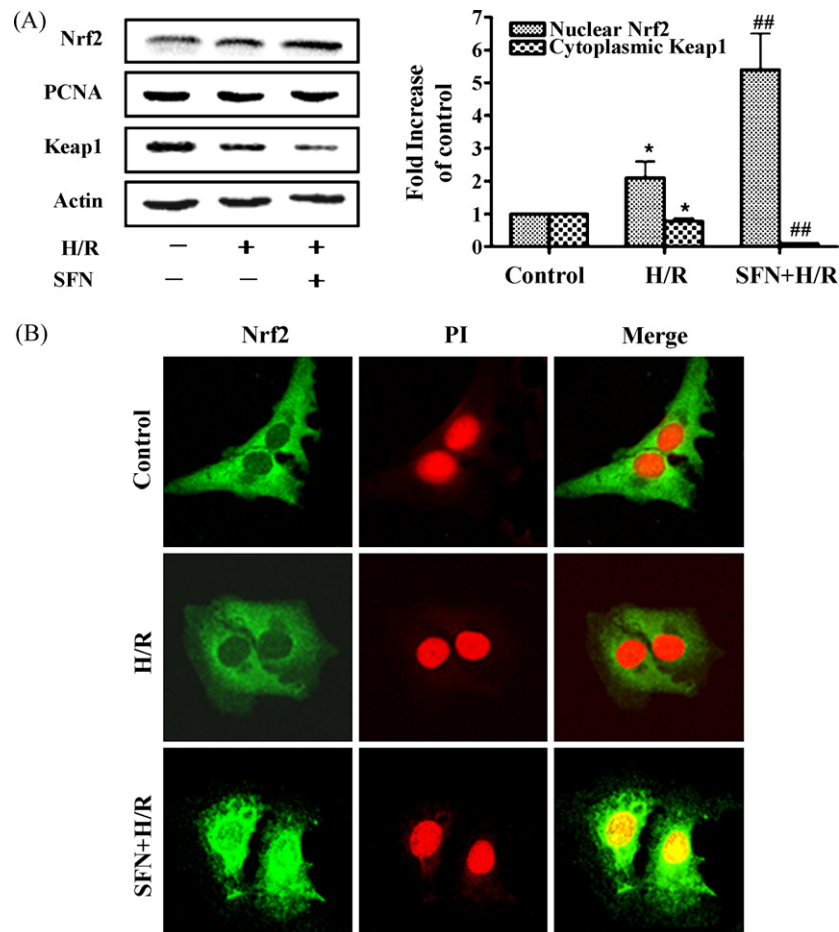

Fig. 2 – Sulforaphane induces phase 2 enzymes through nuclear translocation of Nrf2 and Keap1 degradation in HK2 cells. HK2 cells (5×10^6) were treated with 20 μM sulforaphane for 12 h, and then incubated in anaerobic jars. Following 24 h of incubation, cells were removed from the anaerobic jar and reoxygenated for 3 h. The cytoplasmic and nuclear fractions were separated and then the Nrf2 protein levels in the nucleus and Keap1 degradation in the cytoplasm were determined by Western blotting (A). Nuclear translocation of Nrf2 by sulforaphane treatment was determined by immunofluorescent staining (B). Real-time PCR (C) and Western blotting (D) analyses for phase 2 enzymes were conducted. Each value represents the mean ± S.E.M. of four independent experiments. $^*p < 0.05$ vs. control; $^{##}p < 0.01$ vs. hypoxia-reoxygenation.
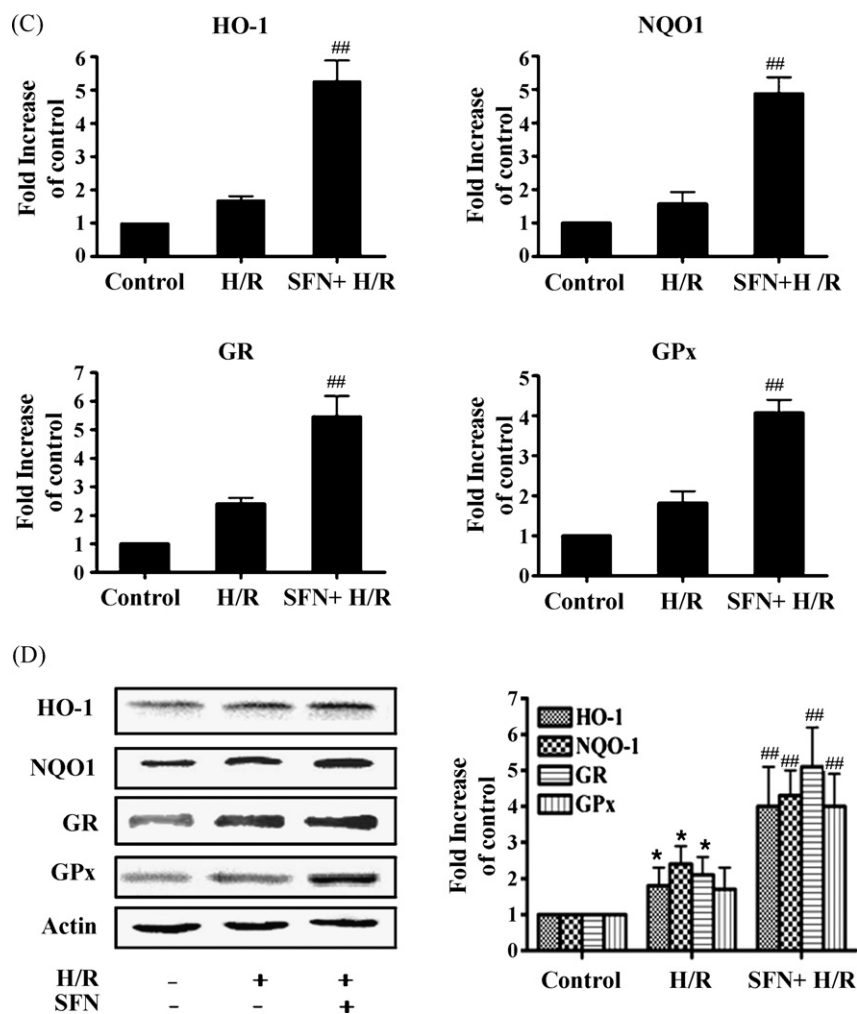
Fig. 2. (Continued).

ischemia-reperfusion, and that this protective effect led to the restoration of renal function.

The findings of this study revealed that sulforaphane activated Nrf2 and induced phase 2 enzymes in HK2 cells and protected HK2 cells from hypoxia-reoxygenation injury. Hypoxia-reoxygenation injury is known to mirror the response obtained in animal models of ischemia-reperfusion [18,19], and HK2 cells originate from human renal tubular epithelium, which is the component of the kidney that is most susceptible to ischemia-reperfusion. In addition, damage to the proximal epithelium is a major cause of renal dysfunction [20]. We showed that sulforaphane exerted a cytoprotective effect against hypoxia-reoxygenation toxicity in HK2 cells by up-regulating the expressions of Nrf2-dependent phase 2 enzymes. In addition, we found that sulforaphane strongly decreased the level of Keap1. Because Keap1 retains Nrf2 in the cytoplasm, it appears that suppression of Keap1 by sulforaphane may result in an overall increase in Nrf2 accumulation in the nucleus and subsequent inductions of phase 2 enzymes, which then participate in adaptive and protective responses to oxidative stress [10–13].

We also studied the effects of sulforaphane in vivo using a renal ischemia-reperfusion model. Kidneys from rats that

underwent ischemia-reperfusion showed characteristic morphological changes, such as extensive tubular necrosis, tubular dilatation, and loss of brush border, however, pretreatment with sulforaphane reduced the amount of renal dysfunction that was induced by ischemia-reperfusion, which was demonstrated by the decreased serum creatinine concentration. These favorable effects of sulforaphane resulted in the maintenance of normal kidney weight following ischemia-reperfusion injury. Ischemia-reperfusion is known to promote the formation of a variety of active mediators such as thrombospondin 1 [21] and tumor necrosis factor-α [22], which affect renal function directly by causing tubular or glomerular damage. Therefore, we cannot exclude the possibility that sulforaphane directly or indirectly affects the formation of those mediators.

Because ROS acts as a key mediator in both hypoxia-reoxygenation and ischemia-reperfusion injury models, cellular defense mechanisms against oxidative stress are necessary to maintain normal cellular function. The Nrf2-phase 2 enzyme system functions as one of the most important anti-oxidant defense mechanisms by upregulating antioxidant response element-related detoxification. Among the tested phase 2 enzymes, the expression of GPx and GR
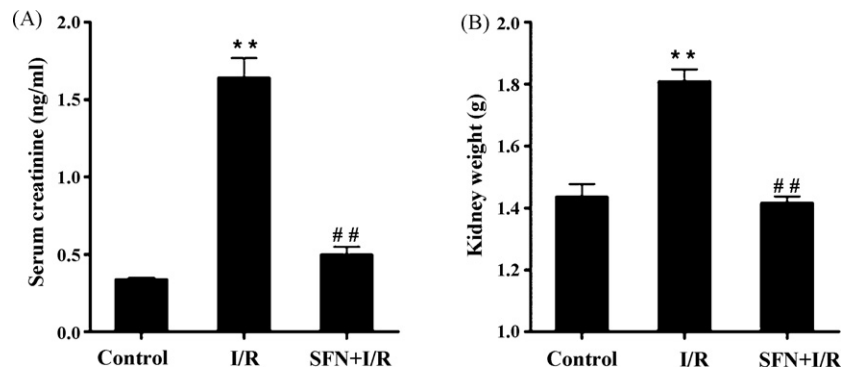
Fig. 3 – Sulforaphane conserves renal function. Sprague–Dawley rats were injected with 500 μg/kg sulforaphane via an i.v. 24 h before the clamping operation. The bilateral renal pedicles were then clamped for 20 min using microclamps while the rats were under anesthesia, after which the kidneys were reperfused. Two days after surgery, blood was collected for the measurement of serum creatinine (A) and the kidneys were then collected and weighed (B). Each value represents the mean ± S.E.M. of five independent experiments. [**]$p < 0.01$ vs. control; [##]$p < 0.01$ vs. ischemia-reperfusion (I/R).
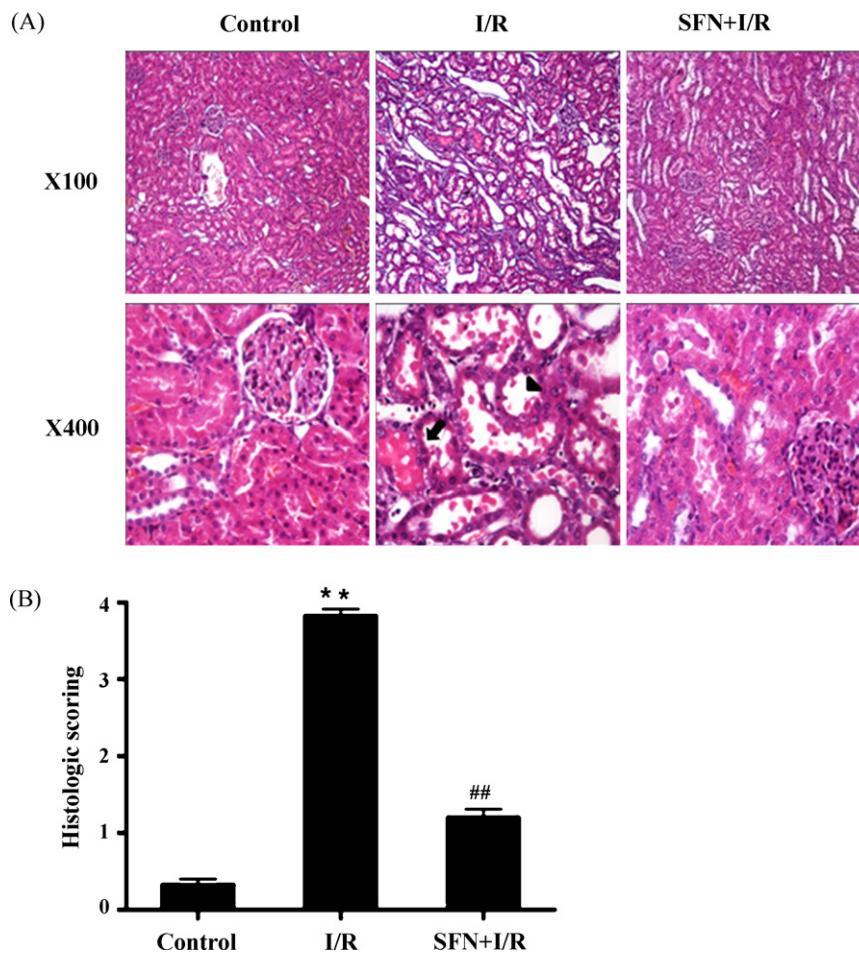

Fig. 4 – Sulforaphane inhibits ischemia-reperfusion-induced renal injury. All experimental procedures for inducing ischemia-reperfusion were the same as those used to generate Fig. 3. Two days after ischemia-reperfusion, kidneys were collected for histologic examination. Tissues were sectioned at 4 μm and then stained with hematoxylin and eosin for light microscopic analysis (A). Histopathologic scoring (B) was performed in a blind method. Each value represents the mean ± S.E.M. of four independent experiments. [**]$p < 0.01$ vs. control; [##]$p < 0.01$ vs. ischemia-reperfusion. Arrow indicates the loss of brush border, and arrowhead indicates the dilatation of tubular lumen.
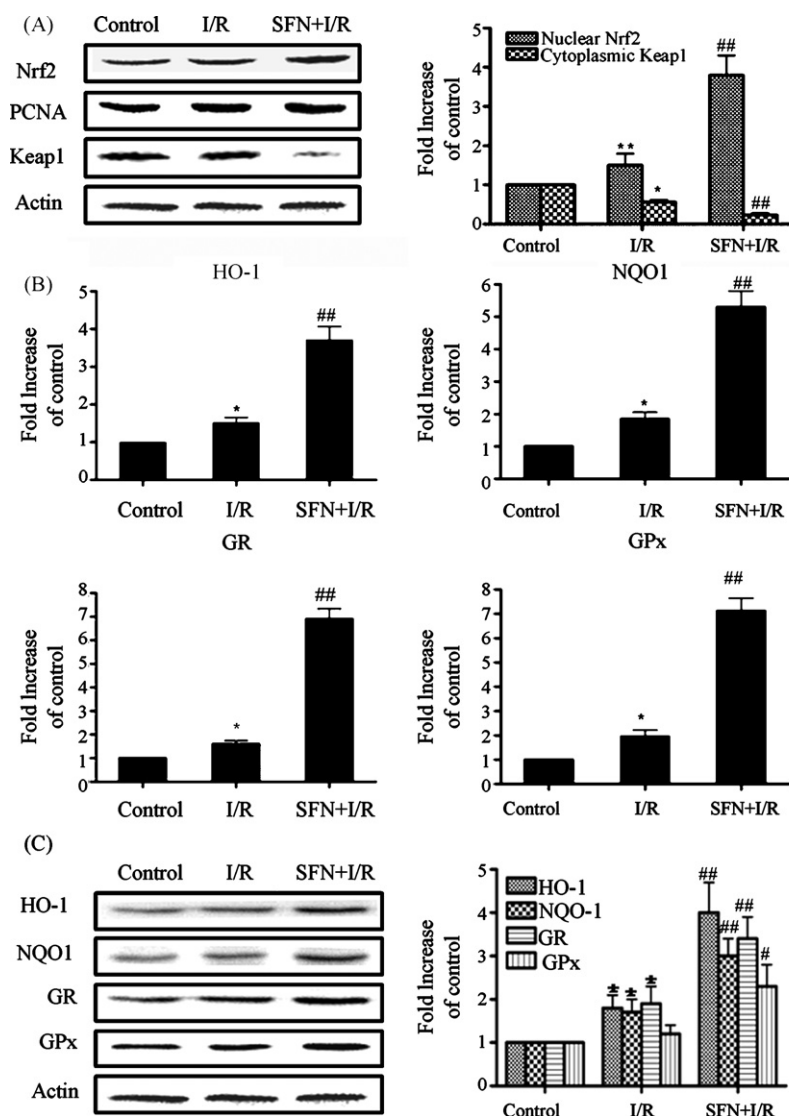
Fig. 5 – Sulforaphane induces phase 2 enzymes through nuclear translocation of Nrf2 and Keap1 repression in kidney tissues. All experimental procedures for inducing ischemia-reperfusion were the same as those described for Fig. 3. Three hours after reperfusion, kidneys were collected and the cytoplasmic and nuclear fractions were separated, after which, the Nrf2 protein levels in the nucleus and the Keap1 degradation in the cytoplasm were determined by Western blotting (A). Real-time PCR (B) and Western blotting (C) for phase 2 enzymes were conducted. Each value represents the mean ± S.E.M. of four independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$ vs. control; $^{#}p < 0.05$, $^{##}p < 0.01$ vs. ischemia-reperfusion.

were increased by sulforaphane treatment. Both of these enzymes are involved in the reduction of oxidized glutathione (GSSG), thereby inducing an increase in the reduced form of glutathione (GSH), which contains a sulfhydryl (SH) group that is known to be sensitive to oxidative stress and depleted following ischemic insult [23]. Therefore, it is possible that the increased expression of GPx and GR by sulforaphane increases the reduced form of intracellular glutathione concentrations that provide resistance to oxidative stress. Another Nrf2-target protein, HO-1, also represents a protective system against various oxidative and inflammatory stresses by generating the vasoactive molecule, carbon monoxide, and the potent antioxidant, bilirubin [10,24]. In addition, Nrf2 activation has been found to protect other organs from ischemia-reperfusion in various experimental animal models [12,25,26], and Leonard et al. [13] recently reported an increase in nuclear translocation of Nrf2 and up-regulation of phase 2 enzymes following renal ischemia-reperfusion injury.

We also assessed the effect of sulforaphane by studying its impact on lipid peroxidation of cellular membrane, an index for the ROS production. Plasma lipid peroxide level has been reported to show a strong transient increase after revascularization operations such as kidney transplantation [27]. In this study, lipid peroxidation levels increased significantly following renal ischemia-reperfusion, however, pretreatment with sulforaphane reversed the increase in lipid hydroperoxides levels to a considerable extent. Reversal effect of sulforaphane for plasma lipid hydroperoxide level confirms its role as an antioxidant in ischemia-reperfusion injury.
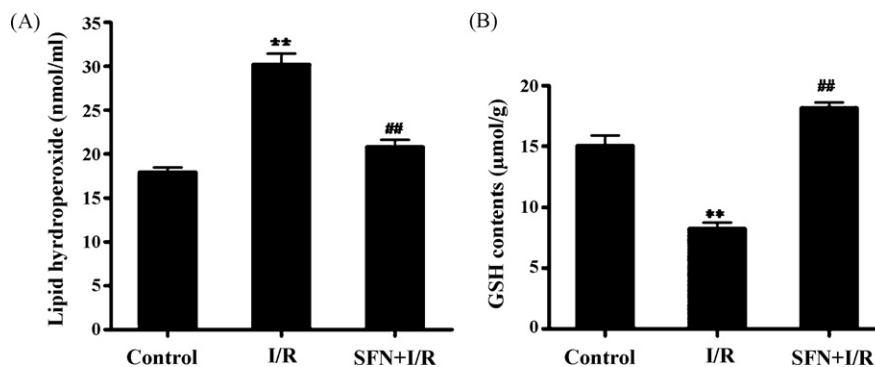
Fig. 6 – Sulforaphane inhibits lipid peroxidation and GSH reduction. All experimental procedures for inducing ischemia-reperfusion were the same as those described for Fig. 3. Two days after operation, plasma samples were collected and the level of lipid hydroperoxides (A) was determined. Three h after operation, kidneys were collected and the reduced form of glutathione contents (B) was determined. Each value represents the mean ± S.E.M. of four (A) or three (B) independent experiments. $^{**}p < 0.01$ vs. control; $^{##}p < 0.01$ vs. ischemia-reperfusion.

Ischemia-reperfusion injury remains of great importance in kidney transplantation and can be a major determinant in early graft dysfunction, as well as in chronic renal dysfunction. Although several strategies may be used to prevent ischemia-reperfusion injury in kidney transplantation, one of the major advantages of sulforaphane is related to the fact that it is already a component of the human diet and is therefore likely to be relatively safe for chronic administration. The beneficial effect of sulforaphane possibly relates to its ability of reducing lipid peroxidation and free radical generation, which contributes to the preservation of tubular cell structure and function.

In summary, sulforaphane effectively reduced the renal dysfunction and injury caused by ischemia-reperfusion of the rat kidney, and the protective mechanism of sulforaphane was mediated by preconditioning via activation of Nrf2 and the resultant induction of phase 2 enzymes. Further studies, however, are required to get more insights on the clinical modality which sulforaphane exerts its beneficial effect on renal ischemia-reperfusion injury. Nevertheless, dietary consumption of sulforaphane-containing cruciferous vegetables such as broccoli could be useful in patients who are at risk of oxidative insult during reperfusion, which may improve patient's outcome after kidney transplantation.

Acknowledgements

REFERENCES

[1] Gueler F, Gwinner W, Schwarz A, Haller H. Long-term effects of acute ischemia and reperfusion injury. Kidney Int 2004;66:523–7.

[2] McCord JM. Oxygen-derived free radicals in postischemic tissue injury. N Engl J Med 1985;312:159–63.

[3] Beckman JK, Yoshioka T, Knobel SM, Greene HL. Biphasic changes in phospholipid hydroperoxide levels during renal ischemia/reperfusion. Free Radic Biol Med 1991;11:335–40.

[4] Nitescu N, Ricksten SE, Marcussen N, Haraldsson B, Nilsson U, Basu S, et al. N-Acetylcysteine attenuates kidney injury in rats subjected to renal ischaemia-reperfusion. Nephrol Dial Transplant 2006;21:1240–7.

[5] Zhang Y, Talalay P, Cho CG, Posner GH. A major inducer of anticarcinogenic protective enzymes from broccoli: isolation and elucidation of structure. Proc Natl Acad Sci USA 1992;89:2399–403.

[6] Farombi EO, Surh YJ. Heme oxygenase-1 as a potential therapeutic target for hepatoprotection. J Biochem Mol Biol 2006;39:479–91.

[7] Juge N, Mithen RF, Traka M. Molecular basis for chemoprevention by sulforaphane: a comprehensive review. Cell Mol Life Sci 2007;64:1105–27.

[8] Zhang DD. Mechanistic studies of the Nrf2-Keap1 signaling pathway. Drug Metab Rev 2006;38:769–89.

[9] Rushmore TH, Kong AN. Pharmacogenomics, regulation and signaling pathways of phase I and II drug metabolizing enzymes. Curr Drug Metab 2002;3:481–90.

[10] Blydt-Hansen TD, Katori M, Lassman C, Ke B, Coito AJ, Iyer S, et al. Gene transfer-induced local heme oxygenase-1 overexpression protects rat kidney transplants from ischemia/reperfusion injury. J Am Soc Nephrol 2003;14:745–54.

[11] Shah ZA, Li RC, Thimmulappa RK, Kensler TW, Yamamoto M, Biswal S, et al. Role of reactive oxygen species in modulation of Nrf2 following ischemic reperfusion injury. Neuroscience 2007;147:53–9.

[12] Tanaka Y, Maher JM, Chen C, Klaassen CD. Hepatic ischemia-reperfusion induces renal heme oxygenase-1 via NF-E2-related factor 2 in rats and mice. Mol Pharmacol 2007;71:817–25.

[13] Leonard MO, Kieran NE, Howell K, Burne MJ, Varadarajan R, Dhakshinamoorthy S, et al. Reoxygenation-specific activation of the antioxidant transcription factor Nrf2 mediates cytoprotective gene expression in ischemia-reperfusion injury. FASEB J 2006;20:2624–6.

[14] Hu R, Khor TO, Shen G, Jeong WS, Hebbar V, Chen C, et al. Cancer chemoprevention of intestinal polyposis in ApcMin/+ mice by sulforaphane, a natural product derived from cruciferous vegetable. Carcinogenesis 2006;27:2038–46.

[15] Shen G, Khor TO, Hu R, Yu S, Nair S, Ho CT, et al. Chemoprevention of familial adenomatous polyposis by natural dietary compounds sulforaphane and dibenzoylmethane alone and in combination in ApcMin/+ mouse. Cancer Res 2007;67:9937–44.

[16] Melnikov VY, Faubel S, Siegmund B, Lucia MS, Ljubanovic D, Edelstein CL. Neutrophil-independent mechanisms of caspase-1- and IL-18-mediated ischemic acute tubular necrosis in mice. J Clin Invest 2002;110:1083–91.

[17] Eschwege P, Paradis V, Conti M, Holstege A, Richet F, Deteve J, et al. In situ detection of lipid peroxidation by-products as markers of renal ischemia injuries in rat kidneys. J Urol 1999;162:553–7.

[18] Carmago S, Shah SV, Walker PD, Meprin. a brush-border enzyme, plays an important role in hypoxic/ischemic acute renal tubular injury in rats. Kidney Int 2002;61:959–66.

[19] Zager RA, Johnson AC, Hanson SY, Lund S. Ischemic proximal tubular injury primes mice to endotoxin-induced TNF-α generation and systemic release. Am J Physiol Renal Physiol 2005;289:F289–97.

[20] Lieberthal W, Levine JS. Mechanisms of apoptosis and its potential role in renal tubular epithelial cell injury. Am J Physiol 1996;271:F477–88.

[21] Thakar CV, Zahedi K, Revelo MP, Wang Z, Burnham CE, Barone S, et al. Identification of thrombospondin 1 (TSP-1) as a novel mediator of cell injury in kidney ischemia. J Clin Invest 2005;115:3451–9.

[22] Meldrum KK, Meldrum DR, Meng X, Ao L, Harken AH. TNF-α-dependent bilateral renal injury is induced by unilateral renal ischemia-reperfusion. Am J Physiol Heart Circ Physiol 2002;282:H540–6.

[23] Soszynski M, Bartosz G. Decrease in accessible thiols as an index of oxidative damage to membrane proteins. Free Radical Biol Med 1997;23:463–9.

[24] Otterbein LE, Soares MP, Yamashita K, Bach FH. Heme oxygenase-1: unleashing the protective properties of heme. Trends Immunol 2003;24:449–55.

[25] Cao Z, Zhu H, Zhang L, Zhao X, Zweier JL, Li Y. Antioxidants and phase 2 enzymes in cardiomyocytes: chemical inducibility and chemoprotection against oxidant and simulated ischemia-reperfusion injury. Exp Biol Med (Maywood) 2006;231:1353–64.

[26] Shih AY, Li P, Murphy TH. A small-molecule-inducible Nrf2-mediated antioxidant response provides effective prophylaxis against cerebral ischemia in vivo. J Neurosci 2005;25:10321–35.

[27] Rabl H, Khoschsorur G, Colombo T, Tatzber F, Esterbauer H. Human plasma lipid peroxide levels show a strong transient increase after successful revascularization operations. Free Radical Bio Med 1992;13:281–8.