

Small heterodimer partner attenuates hydrogen peroxide-induced expression of cyclooxygenase-2 and inducible nitric oxide synthase by suppression of activator protein-1 and nuclear factor- κ B in renal proximal tubule epithelial cells

JUNG SUN PARK, HOON IN CHOI, EUN HUI BAE, SEONG KWON MA and SOO WAN KIM

Department of Internal Medicine, Chonnam National University Medical School, Gwangju 501-757, Republic of Korea

Received October 12, 2016; Accepted February 6, 2017

DOI: 10.3892/ijmm.2017.2883

Abstract. The orphan nuclear receptor, small heterodimer partner (SHP), plays a negative regulatory role in innate immune responses and is involved in various inflammatory signaling pathways. In the present study, we aimed to ascertain whether SHP is effective in preventing hydrogen peroxide (H_2O_2)-induced kidney tubular inflammation and explored the molecular mechanisms underlying the protective effects of SHP. Renal ischemia/reperfusion (I/R) injury was induced in mice by clamping both renal pedicles for 30 min. The effects of H_2O_2 on cell viability in human renal proximal tubule (HK-2) cells were determined using MTT assays. 2',7'-DCF-DA was used to determine intracellular reactive oxygen species (ROS). SHP, cyclooxygenase-2 (COX-2) levels, and inducible nitric oxide synthase (iNOS) expression levels were determined by semi-quantitative immunoblotting and real-time polymerase chain reaction. In addition, SHP, nuclear factor- κ B (NF- κ B), and activator protein-1 (AP-1) promoter activities were determined by luciferase assays. SHP mRNA and protein expression levels were reduced, whereas COX-2 and iNOS levels were increased in mice subjected to renal I/R. H_2O_2 treatment in HK-2 cells decreased cell viability, increased ROS production, and induced COX-2 and iNOS expression. These changes were counteracted by transient transfection with SHP. H_2O_2 treatment decreased SHP luciferase activity, which was recovered by treatment with the NF- κ B inhibitor Bay11-7082, transfection with dominant-negative c-Jun or treatment with *N*-acetyl cysteine (NAC). AP-1 and NF- κ B promoter activities were increased by H_2O_2 and this increase was blocked by SHP transfection. To conclude, SHP protected

HK-2 cells from H_2O_2 -induced tubular injury by inhibition of COX-2 and iNOS through suppression of AP-1 and NF- κ B promoter activities.

Introduction

Acute kidney injury (AKI) is related to the toxic effects of various chemical agents and reactive oxygen species (ROS) and results in increased risk for progression to chronic kidney disease. The incidence of AKI in hospital patients has generally been reported to range from 2 to 7%, and AKI has been shown to be associated with mortality (1). Ischemia/reperfusion (I/R) injury is one of the major causes of AKI, resulting from a generalized impairment of oxygen and nutrient delivery along with a mismatch of local tissue oxygen supply (2). I/R kidney injury is characterized by inflammation, peroxidation, DNA damage, apoptosis, vascular leakage, immune activation, endothelial cell activation, leukocyte adhesion and compromised microvascular blood flow (3,4). Moreover, I/R kidney injury increases ROS levels (5).

ROS, including hydrogen peroxide (H_2O_2), enhance tubular stress and epithelial cell injury, interfere with normal regenerative processes and lead to fibrosis (6,7). Furthermore, ROS induced by oxidative stress are implicated in the pathogenesis of many renal diseases, such as acute glomerulonephritis, acute interstitial nephritis and tubular cell injury (8). Additionally, ROS induce pro-inflammatory and chemotactic cytokines, such as cyclooxygenase (COX)-2, inducible nitric oxide synthase (iNOS), tumor necrosis factor- α (TNF- α), transforming growth factor- β (TGF- β), interleukin-1 β (IL-1 β), IL-6, IL-8 and activated inflammatory cells in the kidneys (9,10). In response to oxidative stress, tubular cells also express Toll-like receptor, complement and complement receptors, and costimulatory molecules, which regulate T-lymphocyte activity (11). COX-2 and iNOS are important components in a network of inflammatory cytokines activated by ROS in the kidney (12,13). The expression of COX-2 and iNOS is controlled through the transcription factors, nuclear factor- κ B (NF- κ B) and activator protein-1 (AP-1) (14-16). NF- κ B and AP-1 have been shown to be crucial for the induction of genes involved in inflammation (17). Moreover, NF- κ B and AP-1 are important ROS-sensitive transcription factors

Correspondence to: Professor Soo Wan Kim, Department of Internal Medicine, Chonnam National University Medical School, 42 Jebongro, Gwangju 501-757, Republic of Korea
E-mail: skimw@chonnam.ac.kr

Key words: cyclooxygenase-2, hydrogen peroxide, inducible nitric oxide synthase, inflammation, promoter activity, small heterodimer partner

that regulate the transcription of genes encoding inflammatory cytokines and chemokines (18).

The small heterodimer partner (SHP, also known as NR0B2) is an atypical orphan nuclear receptor that is structurally related to nuclear hormone receptors but lacks both a known physiological ligand and a DNA binding domain (19). The human *SHP* gene is expressed in various tissues, including the heart, pancreas, lung, spleen, smooth muscle and kidney (20-23). SHP functions as a transcriptional co-regulator by directly interacting with other nuclear receptors and transcription factors (24-27). Moreover, SHP plays a crucial role in negatively regulating the transactivation of various transcription factors involved in diverse physiological and metabolic pathways (26). Recent studies have demonstrated that the NF- κ B p65 protein complex requires interaction with SHP, which is an intrinsic negative regulator of Toll-like receptor-triggered inflammation (28). These findings suggest that SHP may exert anti-inflammatory effects.

In the present study, we aimed to ascertain whether SHP is effective in preventing H₂O₂-induced oxidative stress, which can trigger inflammation in tubular epithelial cells, and to explore the molecular mechanisms underlying the protective effects of SHP. We examined whether SHP attenuates H₂O₂-induced COX-2 and iNOS expression through suppression of the transcription factors NF- κ B and AP-1 in human renal proximal tubule epithelial (HK-2) cells.

Materials and methods

Cell culture and reagents. Human renal proximal tubule epithelial HK-2 cells (ATCC, Manassas, VA, USA), were cultured. Cells were passaged every 3-4 days in 100-mm dishes containing combined Dulbecco's modified Eagle's medium (DMEM)-F-12 medium supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 μ g/ml streptomycin (all from Sigma, St. Louis, MO, USA). The cells were incubated in a humidified atmosphere of 5% CO₂ and 95% air at 37°C for 24 h and sub-cultured at 70-80% confluence. For experimental use, the HK-2 cells were plated onto 60-mm dishes in medium containing 10% FBS for 24 h and cells were then switched to DMEM-F12 with 1% FBS for 16 h. The cells were then treated with H₂O₂ (0, 100, 300, 500 and 1,000 μ M). The cells were harvested at the end of treatment for further analysis. SP600125 (a specific JNK inhibitor) was obtained from Calbiochem (San Diego, CA, USA). *N*-acetyl-L-cysteine (NAC) was obtained from Sigma-Aldrich (Steinheim, Germany). Bay11-7082 was obtained from BioMol (Plymouth Meeting, PA, USA).

Animals. The animal experiments were approved by the Animal Care Regulations (ACR) Committee of Chonnam National University Medical School and our protocols conformed to the institution guidelines for experimental animal care and use. Male 8-week-old C57BL6 mice were purchased from Samtako (Osan, Korea). Mice were divided into two groups. The control group (n=8) underwent a sham operation without clamping of the renal pedicle. In the experimental group, in order to induce I/R kidney injury, both renal pedicles of the mice (n=8) were clamped for 30 min. Twenty-four hours later, the mice were anesthetized with 2% isoflurane and 100% oxygen. Blood

samples were collected from the left ventricle and analyzed for creatinine. Plasma creatinine was measured using the Jaffe method (Olympus 5431; Olympus Optical, Tokyo, Japan). The kidney was rapidly removed, and then processed for semi-quantitative immunoblotting. Another series of experiment was carried out for the assay of real-time polymerase chain reaction (PCR). The mice were decapitated and their kidneys were excised and maintained at -70°C until assayed for the mRNA expression by real-time PCR.

Real-time PCR. Total RNA was isolated with TRIzol reagent (Invitrogen, Carlsbad, CA, USA). cDNA was constructed by reverse transcribing 1 μ g of total RNA using oligo(dT) priming and superscript reverse transcriptase II (Invitrogen). cDNA was quantified using SmartCycler II System (Cepheid, Sunnyvale, CA, USA) and SYBR-Green was used for detection. Each PCR reaction was performed using 10 μ M forward primer, 10 μ M reverse primer, 2X SYBR-Green Premix Ex Taq (Takara Bio, Inc., Shiga, Japan), 0.5 μ l cDNA and H₂O to bring the final volume to 20 μ l. Relative levels of mRNA were determined by real-time PCR, using a Rotor-Gene™ 3000 detector system (Corbett Research, Mortlake, New South Wales, Australia). The specific primers sequences were: hSHP forward, 5'-CAA TGTGGGAGGCGGCT-3' and reverse, 5'-TGAAAGGGA CCATCCTCTTCA-3' (60 bp); hCOX-2 forward, 5'-CGA GGTGTATGTATGAGTGT-3' and reverse, 5'-TCTAGCCAG AGTTTCACCGT-3' (594 bp); hiNOS forward, 5'-ACGTGC GTTACTCCACCAACA-3' and reverse, 5'-CATAGCGGA TGAGCTGAGCATT-3' (114 bp); hIL-1 β forward, 5'-TGATGT TCCATTAGACAGC-3' and reverse, 5'-GAGGTGCTG ATGTACCAGTT-3' (378 bp); hTNF- α forward, 5'-GCATGA TCCGCGACGTGGAA-3' and reverse, 5'-AGATCCATG CCGTTGGCCAG-3' (352 bp); hGAPDH forward, 5'-GCC AAAAGGGTCATCATCTC-3' and reverse, 5'-GGCCATCCA CAGTCTTCT-3' (229 bp). The PCR was performed according to the following steps: i) 95°C for 5 min; ii) 95°C for 20 sec; iii) 58 to 62°C for 20 sec (optimized for each primer pair); iv) 72°C for 30 sec. Steps 2-4 were repeated for an additional 40 cycles, while at the end of the last cycle, the temperature was increased from 60 sec to 95°C to produce a melting curve. Data from the reaction were collected and analyzed with Corbett Research Software. The comparative critical threshold (Ct) values from quadruplicate measurements were used to calculate the gene expression, with normalization to GAPDH as an internal control. Melting curve analysis was performed to enhance specificity of the amplification reaction.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Viability of the HK-2 cells was determined using the MTT assay. HK-2 cells were subcultured in a 96-well plate at an initial density of 5x10³ cells/ml. Cells were incubated with fresh medium containing 0, 300, 500 and 1000 μ M of H₂O₂ for 6 h. After incubation, 50 μ l of 5 mg/ml MTT (Sigma) was added to each well of the 96-well plates and subsequently incubated for 4 h at 37°C. Supernatants were removed by aspiration and then dimethylsulfoxide (DMSO) was added to solubilize the precipitated dyes. Absorbance was measured at a wavelength of 570 nm. The viability of the cells was expressed as the fraction of surviving cells relative to the untreated controls.

Intracellular level of ROS. HK-2 cells were cultured in 96-well plates until they reached confluence. Cells were incubated with fresh medium containing 0, 300, 500 or 1,000 μM of H_2O_2 for 6 h. Cells were washed twice with Hanks' Balanced Salt Solution (HBSS) and incubated with HBSS (without phenol red) containing 10 μM 2',7'-dichlorofluorescein diacetate (DCF-DA; Molecular Probes, Camarillo, CA, USA) for 30 min at 37°C. Fluorescence intensity was analyzed by a fluorescence reader (Fluoroscanner Ascent FL; Labsystems, Helsinki, Finland) using 485 nm excitation and 538 nm emission filter. The images were obtained.

Protein extraction and western blot analysis. The kidney was homogenized in ice-cold isolation solution containing 0.3 M sucrose, 25 mM imidazole, 1 mM EDTA, 8.5 μM leupeptin, and 1 mM phenylmethylsulfonyl fluoride (pH 7.2). The homogenates were centrifuged, and the total protein concentration was measured using the Pierce BCA protein assay kit (Thermo Fisher Scientific, Rockford, IL, USA). All samples were adjusted with isolation solution to normalize the protein concentrations, solubilized at 65°C for 15 min in sodium dodecyl sulfate (SDS)-containing sample buffer, and then stored at -20°C. The HK-2 cells were harvested, washed twice with ice-cold phosphate-buffered saline (PBS) and re-suspended in lysis buffer (20 mM Tris-HCl, pH 7.4, 0.01 mM EDTA, 150 mM NaCl, 1 mM PMSF, 1 $\mu\text{g}/\text{ml}$ leupeptin, 1 mM Na_3VO_4) and sonicated briefly. After centrifugation, the supernatant was prepared as protein extract, and protein concentrations were measured (Pierce BCA protein assay reagent kit; Thermo Fisher Scientific). Equal amounts of protein were separated on 9 or 12% sodium dodecyl sulfate polyacrylamide gels. The proteins were electrophoretically transferred onto nitrocellulose membranes using Bio-Rad Mini Protean II apparatus (Bio-Rad, Hercules, CA, USA). The blots were blocked with 5% milk in PBS-T (80 mM Na_2HPO_4 , 20 mM NaH_2PO_4 , 100 mM NaCl, and 0.1% Tween-20 at pH 7.5) for 2 h. The anti-SHP antibody was provided by Professor Heung-Sik Choi (Chonnam National University, Korea). The NF- κB p65 (8242; Cell Signaling Technology, Beverly, MA, USA), anti-I $\kappa\text{B}\alpha$ (SC-1643; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), anti-iNOS (610600; BD Transduction Laboratories, San Jose, CA, USA), anti-COX-2 (160107; Cayman Chemical, Ann Arbor, MI, USA), and β -actin (a5316; Sigma) antibodies were diluted in a blocking buffer and incubated with the blots overnight at 4°C. The bound antibodies were detected with a 1:2,500 dilution of horseradish peroxidase-conjugated secondary antibody according to the instructions provided with the ECL kit (Amersham, Franklin Lakes, NJ, USA).

Small interfering RNA transfection. For knockdown of SHP expression, siRNAs for SHP were chemically synthesized (Dharmacon Inc., Lafayette, CA, USA) and transfected according to the manufacturer's instructions. HK-2 cells were transfected with siRNA using DharmaFECT 2 reagent (Dharmacon Inc.). Efficiency of knockdown was performed through western blot analysis.

Transient transfection of the plasmid construct, and SHP, AP-1 and NF- κB reporter. pcDNA3-mSHP and the reporter

construct was kindly provided by Professor Heung-Sik Choi (Chonnam National University). The mouse SHP was subcloned into the *NcoI/XhoI* site of the pcDNA3 vector. pcDNA3-mSHP or pcDNA3 was introduced into the HK-2 cells by FuGene HD reagent (Promega, Madison, WI, USA). Two days after transfection, we identified the overexpression of SHP and Flag in the HK-2 cells by western blot analysis. AP-1 and NF- κB reporter construct were purchased from Clontech (Palo Alto, CA, USA). Once the cells had reached 60-70% confluence, they were washed with DMEM-F-12 medium and incubated in the medium without serum and antibiotics for 18 h. The cells were then transfected with SHP, AP-1 and NF- κB reporter containing the pGL3 vector using FuGene HD reagent. Reporter transfected cells were pretreated with NAC and Bay for 1 h and incubated with 500 μM H_2O_2 for 6 h. Also, c-Jun dominant-negative construct and pcDNA3-mSHP were co-transfected with the reporter construct. The luciferase activity was measured using a luminometer.

Promotor activity of SHP, AP-1 and NF- κB . The transcriptional regulation of SHP, AP-1 and NF- κB was examined by transient transfection of an SHP, AP-1 and NF- κB promoter-luciferase reporter construct (pGL3-SHP, pGL3-AP-1 and pGL3-NF- κB). HK-2 cells (5×10^5) were seeded and grown until they reached 60-70% confluence and pGL3-SHP, pGL3-AP-1 and pGL3-NF- κB wild-type and pGL3-empty were transfected into the cells using FuGene HD reagent, according to the manufacturer's protocol. The pRL-null plasmid encoding *Renilla* luciferase was included in all the samples to monitor transfection efficiency. At 24 h post-transfection, the levels of Firefly and *Renilla* luciferase activity were measured sequentially from a single sample using the Dual-Glo Luciferase assay system (Promega). Firefly luciferase activity was normalized to *Renilla* activity and the relative amount of luciferase activity in the untreated cells.

Electrophoretic mobility shift assay. Nuclear extracts of HK-2 cells were prepared with the NE-PER nuclear extraction reagent (Pierce Biotechnology). Biotin labeled oligonucleotides were 5'-biotin-AGTTGAGGGGACTTTCCCAGGC-3' for NF- κB and 5'-biotin-CGCTTGATGACTCAGCGGAA-3' for AP-1 as well as nonlabeled NF- κB oligonucleotide. The binding reactions contained 10 μg of nuclear extract protein, buffer (10 mM Tris, pH 7.5, 50 mM KCl, 5 mM MgCl_2 , 1 mM dithiothreitol, 0.05% Nonidet P-40, and 2.5% glycerol), 1 μg of poly(dI-dC) and 2 nM of biotin-labeled DNA. The reactions were incubated at 23°C for 20 min. The competition reactions were performed by adding 10-fold excess unlabeled double-stranded NF- κB consensus oligonucleotide to the reaction mixture. The reactions were electrophoresed on a 6% precasted Tris-borate-EDTA gel (Invitrogen) at 100 V for 1 h 30 min in a 100 mM Tris-borate-EDTA buffer. The reactions were transferred to a nylon membrane. The biotin-labeled DNA was detected with LightShift chemiluminescent electrophoretic mobility shift assay kit (Pierce Biotechnology).

Statistical analysis. The results are expressed as mean \pm SEM. Multiple comparisons among 3 groups were performed using one-way ANOVA and the post hoc Tukey's honestly significant

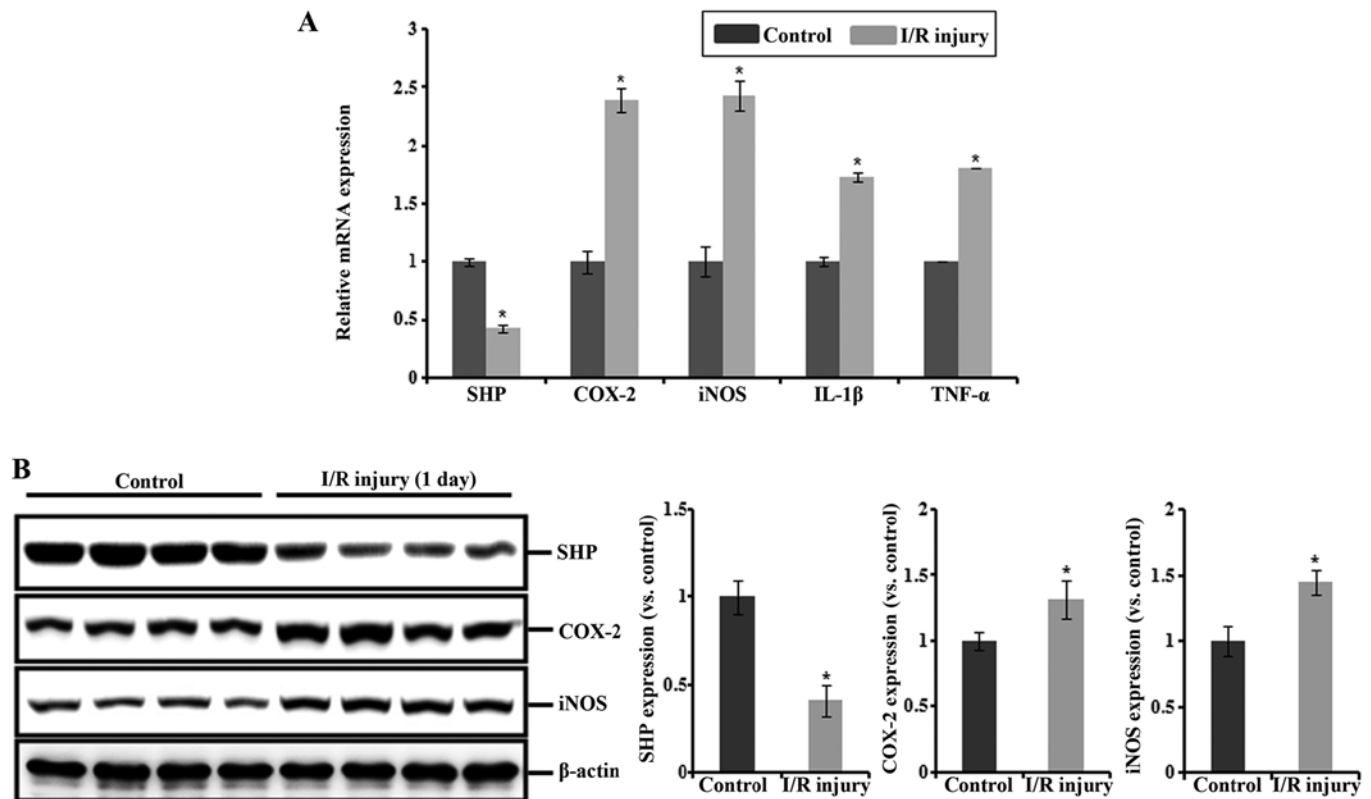


Figure 1. Expression of small heterodimer partner (SHP) and inflammatory cytokines in ischemia/reperfusion (I/R) kidney injury in mice. (A) SHP mRNA and (B) protein expression were reduced in the I/R kidney injury mice, while (A and B) expression levels of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) were increased. Each column represents the mean \pm SEM of 8 mice. β -actin protein levels were analyzed as internal controls. * p <0.05 compared to the control.

difference test. Differences with values of p <0.05 were considered significant.

Results

Expression of SHP and inflammatory proteins in I/R-induced kidney injury. Serum creatinine levels were significantly increased in the I/R injury mice compared with that in the sham-operated controls. The mRNA expression levels of COX-2, iNOS, IL-1 β and TNF- α were increased in the I/R injury mice compared with those in the controls, whereas that of SHP was reduced in these mice (Fig. 1A). Consistent with this, the protein expression of SHP was decreased after I/R injury, whereas the expression levels of COX-2 and iNOS proteins were increased (Fig. 1B).

Expression of SHP, COX-2, and iNOS and the effect of SHP on the production of ROS by H₂O₂ exposure in HK-2 cells. H₂O₂ treatment (300, 500 and 1,000 μ M) for 6 h decreased HK-2 cell viability in a concentration-dependent manner as determined by MTT assays. To examine the physiological effects of SHP on HK-2 cells, we determined the viability of SHP-transfected HK-2 cells treated with H₂O₂. Decreased cell viability induced by H₂O₂ treatment was recovered by overexpression of SHP (Fig. 2A). We next assessed the formation of ROS using the ROS-sensitive fluorescent dye DCF-DA in HK-2 cells. The level of intracellular ROS increased progressively after incubation of cells with 500 μ M

H₂O₂, reaching a peak at 30 min, whereas overexpression of SHP attenuated the increased production of ROS (Fig. 2B). H₂O₂ exposure increased ROS production in a concentration-dependent manner in the HK-2 cells. In contrast, following H₂O₂ exposure at 0, 300, 500 and 1,000 μ M, ROS production was attenuated by 10, 11, 12 and 13% in the SHP-transfected HK-2 cells, respectively compared with levels in the non-SHP-transfected cells (Fig. 2C). We also performed additional experiments to examine whether SHP may play a role in the inhibition of ROS production through mitochondrial uncoupling protein 2 (UCP2). H₂O₂ exposure decreased UCP2 mRNA levels in a concentration-dependent manner in the HK-2 cells (Fig. 2D). SHP transfection induced gene expression of UCP2, suggesting that UCP2 is involved in the SHP-mediated suppression of ROS production (Fig. 2E).

HK-2 cells were incubated with 0, 300, 500 or 1,000 μ M H₂O₂ for 6 h, and the expression levels of SHP, COX-2 and iNOS were determined by real-time PCR and western blotting. As shown in Fig. 3, H₂O₂ treatment increased the mRNA and protein expression of COX-2 and iNOS, whereas levels of SHP mRNA and protein were decreased.

Effects of SHP on the expression of COX-2 and iNOS. COX-2 and iNOS mRNAs were increased in cells exposed to 500 μ M H₂O₂, and transfection with SHP blocked this increase (Fig. 4A and B). SHP mRNA expression was significantly increased in the SHP-transfected cells (data not shown). In addition, SHP-Flag-tag construct transfection

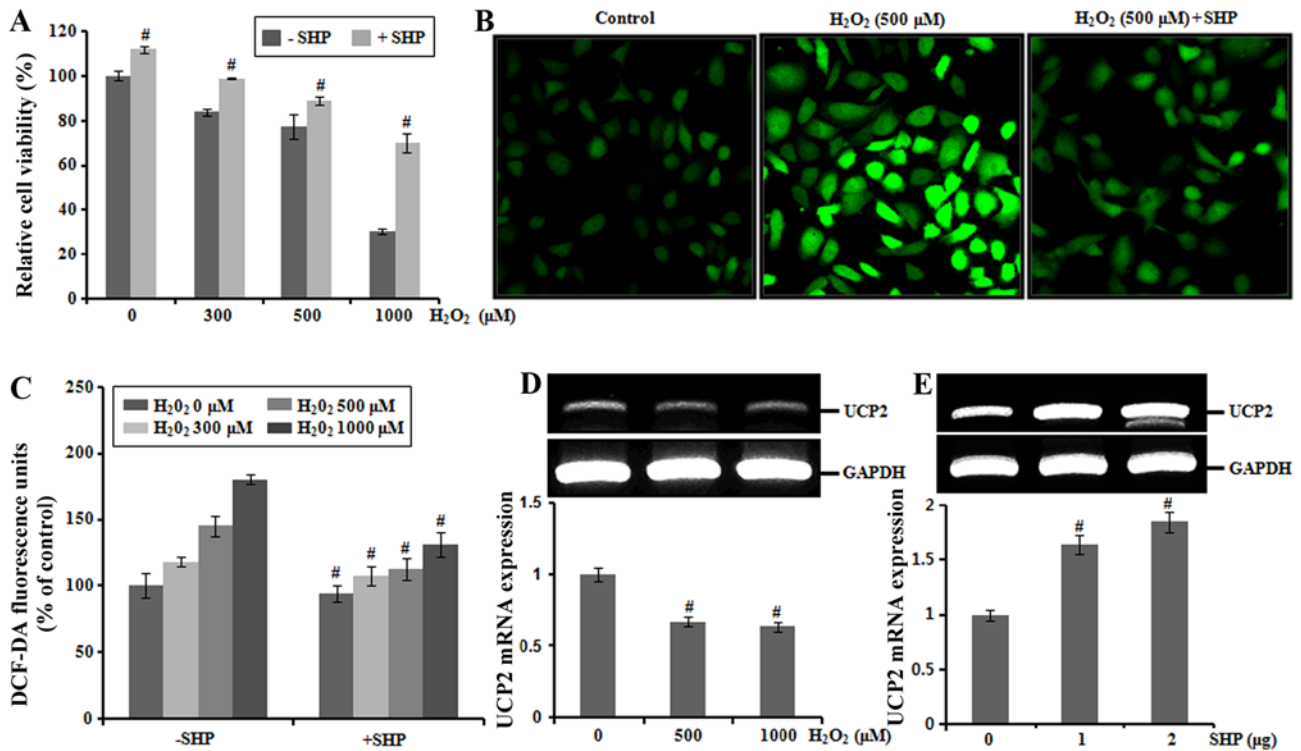


Figure 2. Cell viability and reactive oxygen species (ROS) production in H_2O_2 -treated human renal proximal tubular (HK-2) cells. (A) Cell viability in the H_2O_2 -treated HK-2 cells. H_2O_2 exposure dose-dependently decreased the viability of the HK-2 cells, which was attenuated by small heterodimer partner (SHP) transfection. Cell viability was determined by MTT assay and data are presented as percentage of viability. (B and C) Dichlorofluorescein diacetate (DCF-DA)-sensitive ROS production following exposure of HK-2 cells to H_2O_2 . The level of intracellular ROS was increased after incubation of cells with H_2O_2 , whereas overexpression of SHP attenuated the increased ROS production. (D and E) Uncoupling protein 2 (UCP2) mRNA expression was reduced in H_2O_2 -treated HK-2 cells, which was counteracted by SHP transfection. $^{\#}p < 0.05$ compared to the untransfected HK-2 cells. Data are representative of at least 3 independent experiments.

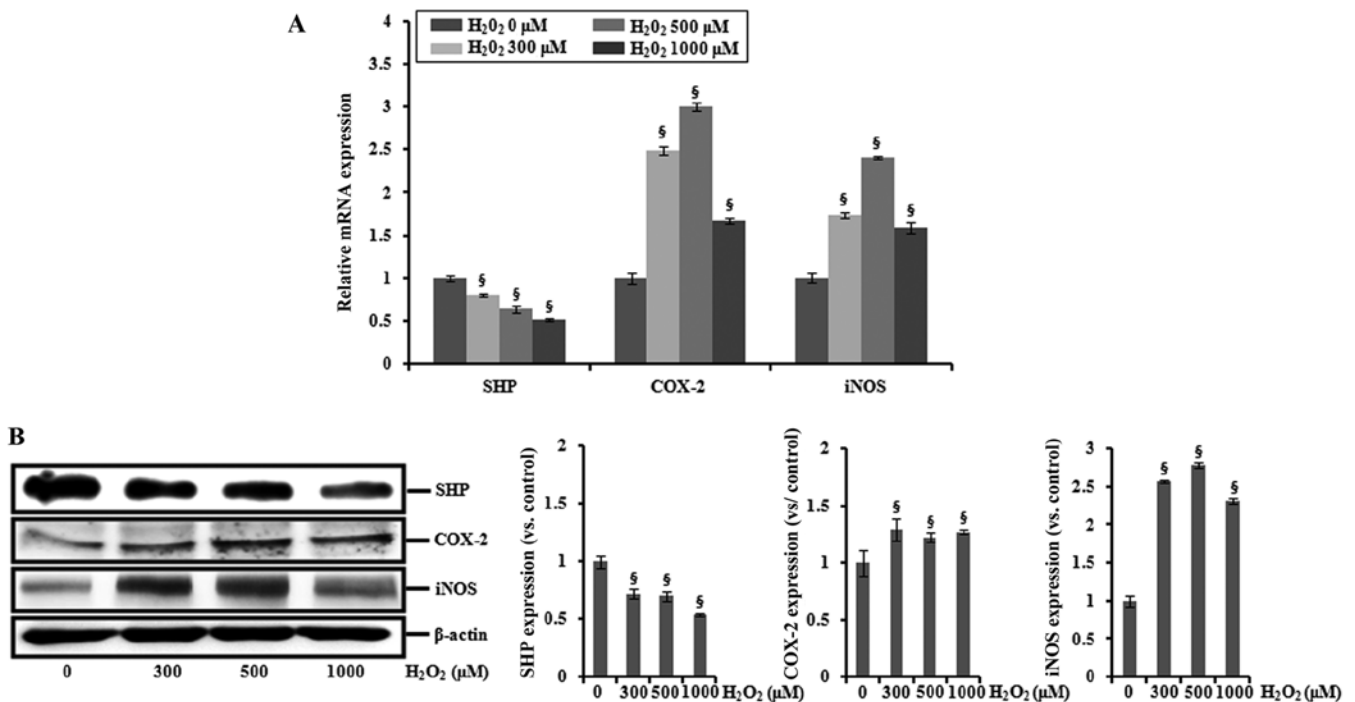


Figure 3. Expression of small heterodimer partner (SHP), cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) in H_2O_2 -treated human renal proximal tubular (HK-2) cells. H_2O_2 exposure increased the (A) mRNA and (B) protein expression of COX-2 and iNOS, while (A) SHP mRNA and (B) SHP protein levels were decreased. Each column represents mean \pm SEM. β -actin protein levels were analyzed as internal controls. $^{\$}p < 0.05$ compared to the H_2O_2 untreated HK-2 cells. Data are representative of at least 3 independent experiments.

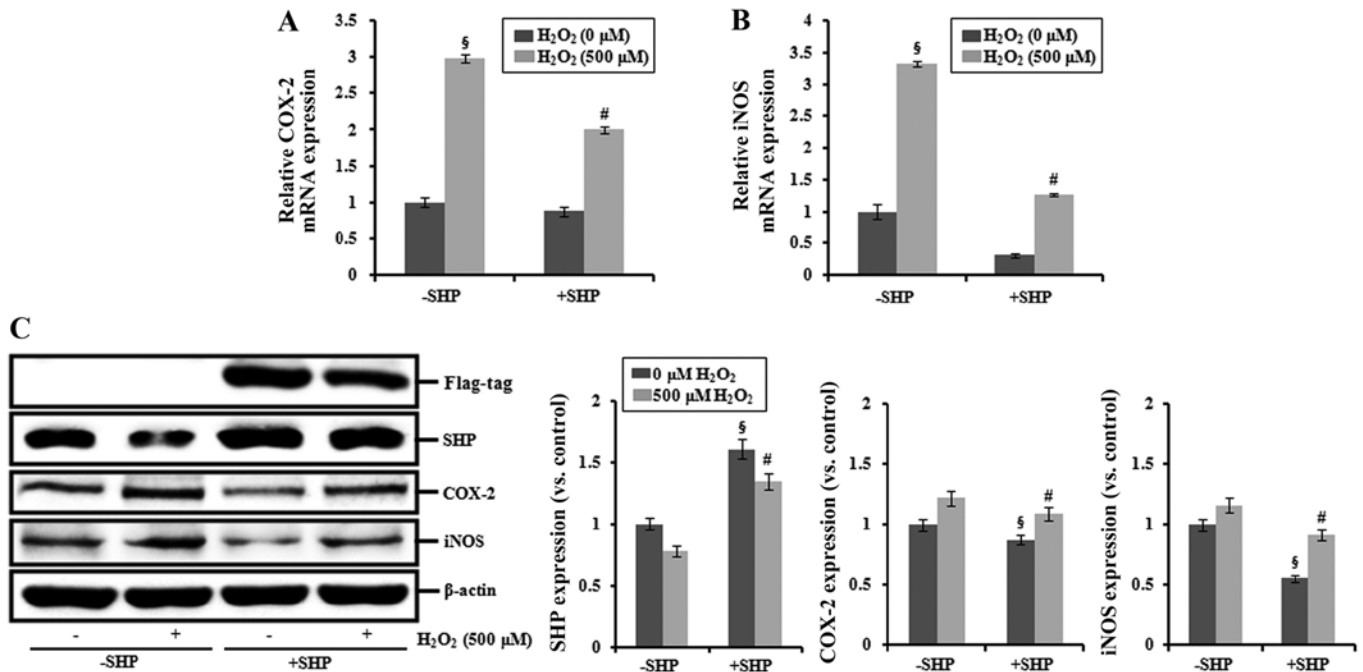


Figure 4. Expression of cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) following H₂O₂ exposure in small heterodimer partner (SHP)-transfected human renal proximal tubular (HK-2) cells. mRNA expression of (A) COX-2 and (B) iNOS was increased in the 500 μM H₂O₂-treated cells, which was attenuated to a certain degree in the SHP-transfected cells. (C) Accordingly, the protein expression of COX-2 and iNOS was increased in the 500 μM H₂O₂-treated cells compared to these levels in the untreated controls, which was counteracted by the transfection of SHP. [§]p<0.05 compared to the H₂O₂-untreated HK-2 cells; [#]p<0.05 compared to the untransfected HK-2 cells. Data are representative of at least 3 independent experiments.

induced increased protein expression of SHP compared with that noted in the non-SHP-transfected cells. Additionally, the expression levels of COX-2 and iNOS proteins were increased in cells exposed to 500 μM H₂O₂ compared with those in the untreated controls, and transfection with SHP suppressed this effect (Fig. 4C).

Effects of an NF-κB inhibitor, dominant-negative c-Jun, and NAC on the transcriptional activation of SHP. Next, we investigated whether NAC could modulate the expression of SHP in HK-2 cells exposed to H₂O₂. H₂O₂ exposure increased the expression of COX-2 and iNOS, but decreased SHP expression. These changes were counteracted by pretreatment with NAC for 1 h. In addition, transfection with SHP siRNA attenuated the inhibitory effects of NAC on the expression of COX-2 and iNOS in the H₂O₂-treated HK-2 cells (Fig. 5A).

To further investigate the transcriptional regulation of SHP, HK-2 cells were transiently transfected with a mouse SHP promoter luciferase reporter construct (pGL3-SHP). HK-2 cells were pretreated with 10 μM Bay11-7082 (an NF-κB inhibitor) and 20 mM NAC and cotransfected with dominant-negative c-Jun before H₂O₂ exposure. H₂O₂ exposure decreased SHP promoter activity, and this decrease was blocked by treatment with Bay11-7082 or NAC and by cotransfection with dominant-negative c-Jun owing to competitive inhibition of AP-1 activation (Fig. 5B).

Effect of SHP on the transcriptional activation of AP-1 and NF-κB. AP-1 and NF-κB are important transcription factors activating the expression of COX-2 and iNOS (14-16), and

AP-1 and NF-κB are activated by ROS (17,18). We examined the role of SHP in the H₂O₂-induced activation of AP-1 and NF-κB. The promoter activity of AP-1 and NF-κB was increased following H₂O₂ exposure in the HK-2 cells, and this increase was attenuated by SHP transfection and NAC treatment (Figs. 6A and B and 7A and B). Cotransfection with dominant-negative c-Jun inhibited the H₂O₂-induced increase in AP-1 promoter activity (Fig. 6B). Furthermore, pretreatment with 10 μM Bay11-7082 reduced H₂O₂-induced NF-κB promoter activity (Fig. 7B). The nuclear extracts from cells analyzed by EMSA for activated AP-1 and NF-κB confirmed these findings (Figs. 6C and 7C).

Discussion

I/R kidney injury is widely utilized as an experimental model of AKI. Increased generation of ROS, endothelial dysfunction, tubular necrosis and inflammation are major players in the pathogenesis of I/R kidney injury (29). Post-ischemic tissues generate inflammatory mediators that can stimulate circulating neutrophils (30). Inflammation involves a complex cascade of intercellular cytokine signals. Activated monocytes and macrophages release a variety of inflammatory mediators, such as TNF-α, IL-1β, nitric oxide and ROS. Nitric oxide has various effects in renal physiology and pathophysiology (31). Moreover, nitric oxide produced by constitutive NOS (eNOS and nNOS) is essential and plays a role in maintaining cellular function, whereas nitric oxide produced by iNOS is an important mediator of inflammation (32). In addition, COX-2 is also an inducible enzyme involved in the pathogenesis of

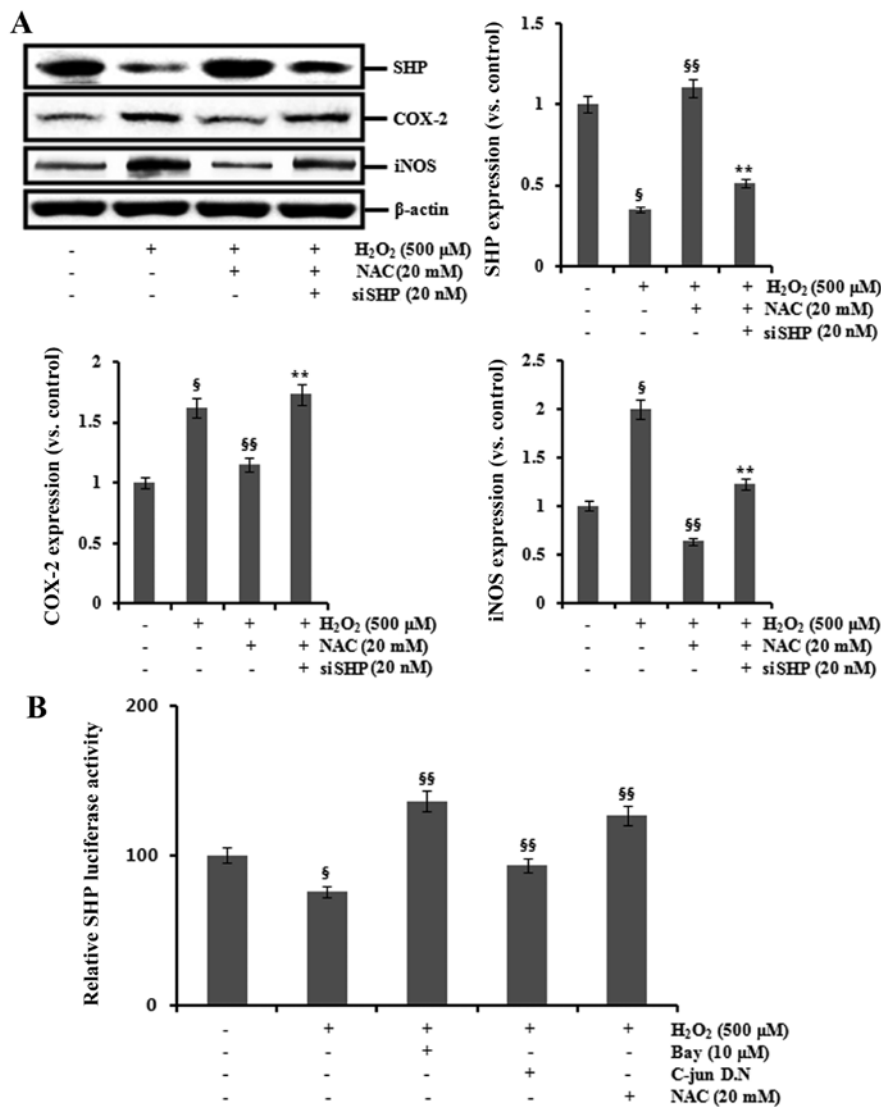


Figure 5. Effects of small heterodimer partner (SHP) siRNA, *N*-acetyl cysteine (NAC), Bay11-7082, and dominant-negative c-Jun in human renal proximal tubular (HK-2) cells exposed to H₂O₂. (A) H₂O₂ treatment increased expression of cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS), but decreased SHP expression. These changes were counteracted by a 1-h pretreatment with NAC. In addition, transfection of SHP siRNA (siSHP) attenuated the inhibitory effects of NAC on the expression of COX-2 and iNOS in the H₂O₂-treated HK-2 cells. (B) HK-2 cells were pretreated with 10 μM Bay11-7082 [nuclear factor-κB (NF-κB) inhibitor], 20 mM NAC and co-transfection of dominant-negative c-Jun (DN c-Jun) before H₂O₂ exposure. H₂O₂ exposure decreased the SHP promoter activity, which was counteracted by Bay11-7082, NAC and co-transfection of dominant-negative c-Jun. Transient transfection of SHP was used for the positive control. [§]p<0.05 compared to the H₂O₂-untreated HK-2 cells; ^{§§}p<0.05 compared to the H₂O₂ treated HK-2 cells; ^{**}p<0.05 compared to the H₂O₂ and NAC-treated HK-2 cells. Data are representative of at least 3 independent experiments.

inflammation. Many studies have reported that iNOS-derived nitrogen reactive species and COX-2-derived oxidative stress play roles in inflammatory kidney injury (33,34). In the present study, we examined changes in the expression levels of inflammatory mediators in response to induction of AKI. Consistent with previous studies, the expression of COX-2 and iNOS was increased, and associated with upregulation of IL-1β and TNF-α (33,34). Moreover, renal dysfunction caused by I/R-induced kidney injury resulted in marked reduction in SHP expression. These results suggest that SHP is associated with the pathogenesis of renal inflammation in I/R kidney injury.

Recently, we demonstrated the effect of SHP on cisplatin-induced kidney injury using a farnesoid X receptor ligand (35). Farnesoid X receptor ligand prevented cisplatin-induced kidney injury by inhibiting renal inflammation, fibrosis and

apoptosis through induction of SHP. In the present study, we examined the hypothesis that SHP is involved in the inflammatory signaling pathway in HK-2 cells. An imbalance between cell survival and death, a key process in many degenerative and inflammatory diseases, may be caused by aberrant turnover of ROS, which regulates the crosstalk between mitogen-activated protein kinases (MAPKs) and NF-κB (36). Because H₂O₂ is a strong inducer of ROS production, we examined the effects of SHP on H₂O₂-mediated ROS production using the fluorescent dye H₂DCF-DA. H₂O₂ exposure strongly induced ROS production, which was ameliorated by the overexpression of SHP. Accordingly, cell viability was decreased by H₂O₂ exposure, which was again attenuated by SHP transfection. Thus, SHP increased the cell viability following H₂O₂-mediated kidney tubule cell injury in HK-2 cells through inhibition

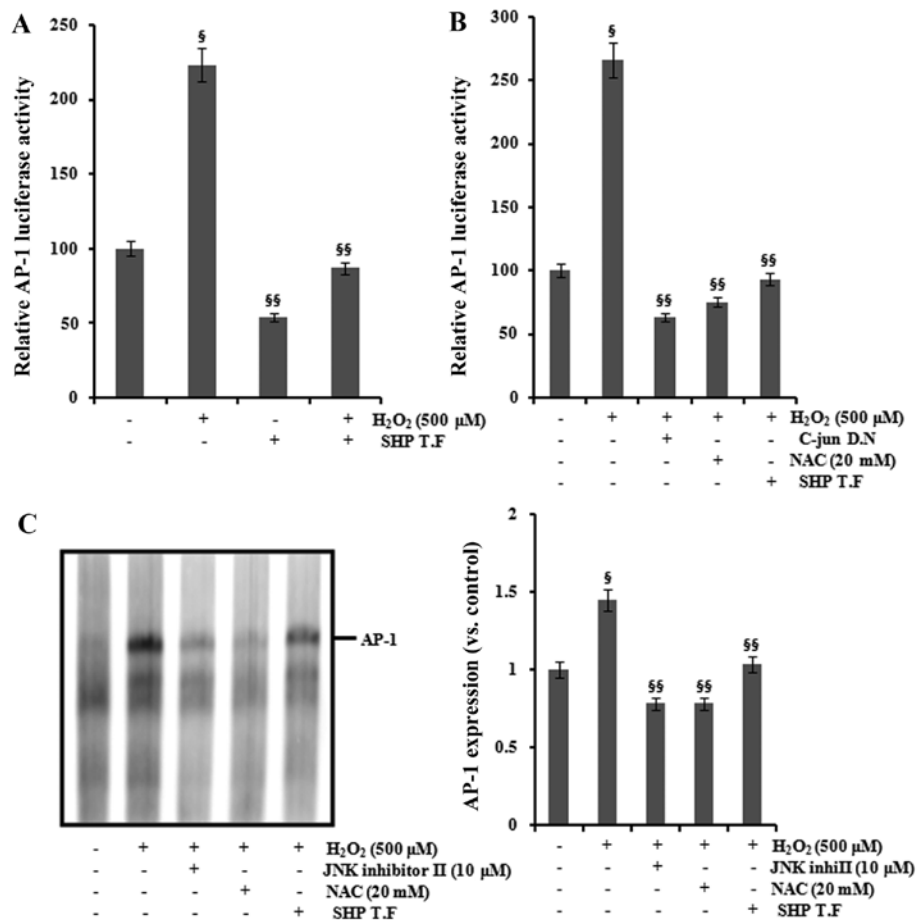


Figure 6. Effects of small heterodimer partner (SHP) on H₂O₂-induced activator protein-1 (AP-1) activation in human renal proximal tubular (HK-2) cells. (A and B) HK-2 cells were cotransfected with an SHP construct and dominant-negative mutant of c-Jun with pGL3-AP-1. The transfected cells were pretreated with 20 mM *N*-acetyl cysteine (NAC) for 1 h, followed by incubation with 500 μM H₂O₂ for 6 h. Luciferase activity was then determined using a luminometer. Pretreatment of 20 mM NAC, co-transfection of c-Jun dominant-negative and overexpression of SHP construct inhibited H₂O₂-induced increase in AP-1 promoter activity. (C) Nuclear extracts from HK-2 cells were analyzed by EMSA for activated AP-1 using biotin-labeled oligonucleotide probes. HK-2 cells overexpressing SHP were pretreated with 10 μM JNK inhibitor II and 20 mM NAC for 1 h and then incubated with 500 μM H₂O₂ for 6 h. After incubation, the lysates were analyzed by EMSA. [§]p<0.05 compared to the H₂O₂-untreated HK-2 cells; ^{§§}p<0.05 compared to the H₂O₂ exposed HK-2 cells. Data are representative of at least 3 independent experiments.

of ROS production. Mitochondrial uncoupling proteins may play a role in minimizing mitochondrial ROS production and function in the protection against oxidative stress (37). We examined whether SHP may play a role in the inhibition of ROS production through mitochondrial uncoupling protein 2 (UCP2). SHP transfection induced gene expression of UCP2, suggesting that UCP2 is involved in SHP-mediated suppression of ROS production. In addition, H₂O₂ exposure increased the expression of COX-2 and iNOS, which was ameliorated by NAC pretreatment. These findings suggest that the protective activity of SHP on ROS-mediated inflammation is through suppression of ROS production.

We then aimed to ascertain whether SHP prevents H₂O₂-induced inflammation in HK-2 cells. Both iNOS and COX-2 exhibited increased expression after H₂O₂ treatment, and SHP transfection prevented this H₂O₂-mediated increase in iNOS and COX-2 expression. Therefore, SHP expression may be essential for suppression of inflammatory markers such as COX-2 and iNOS in H₂O₂-induced injury of proximal tubular cells.

Next, we investigated whether the antioxidant, NAC modulates the expression of SHP, COX-2 and iNOS in HK-2 cells following exposure to H₂O₂. H₂O₂ exposure increased the

expression of COX-2 and iNOS, but decreased SHP expression. These changes were ameliorated by NAC pretreatment. In addition, transfection with SHP siRNA attenuated the inhibitory effects of NAC on the expression of COX-2 and iNOS in the H₂O₂-treated HK-2 cells. These findings indicated that the inhibitory effects of NAC on the expression of COX-2 and iNOS in the H₂O₂-treated HK-2 cells may be attributed in part to SHP.

Our results also showed that H₂O₂ exposure decreased SHP promoter activity and that this effect was blocked by treatment with an NF-κB inhibitor or cotransfection with dominant-negative c-Jun. These findings indicate that the promoter activity of SHP is regulated by AP-1 and NF-κB in kidney-related inflammatory signaling. In addition, SHP promoter activation was increased by elimination of ROS using NAC treatment. Because AP-1 and NF-κB are activated by ROS (17,18), these data suggest that SHP promoter activation may be inhibited by ROS through the activation of NF-κB and AP-1. However, further studies are needed to elucidate the exact interactive mechanisms that couple NF-κB and AP-1 to oxidative stress and the role of these mechanisms in the regulation of SHP in kidney injury.

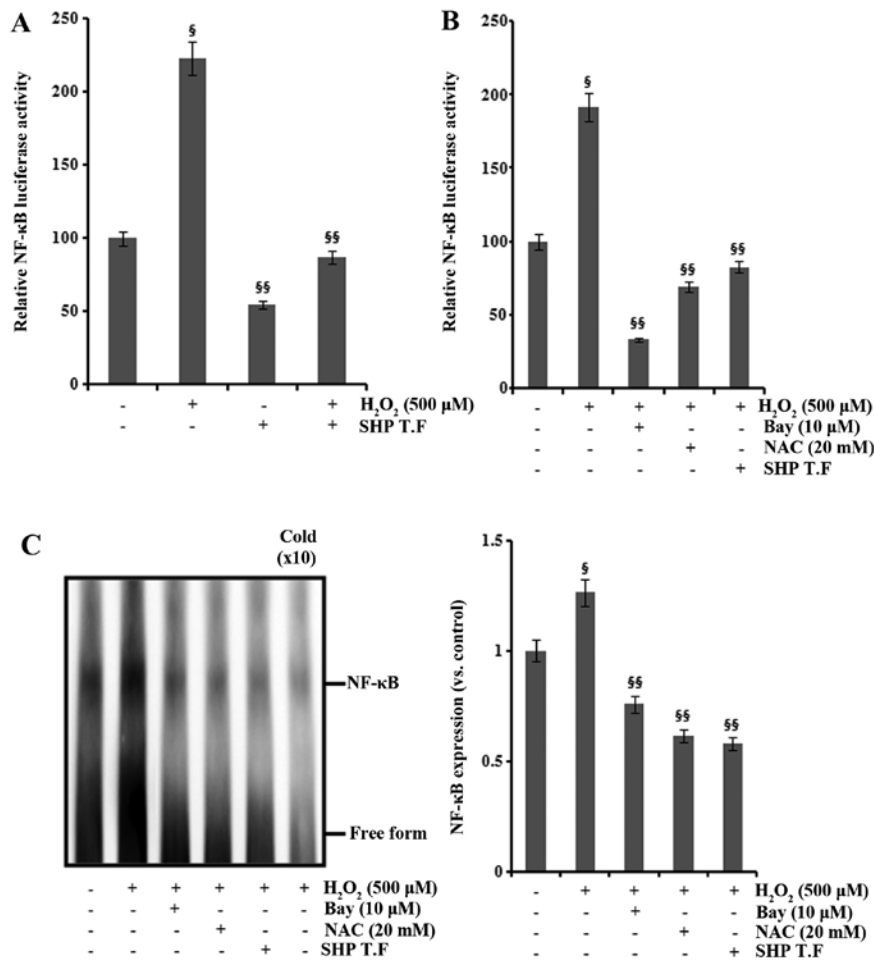


Figure 7. Effects of small heterodimer partner (SHP) on H₂O₂-induced NF-κB activation in human renal proximal tubular (HK-2) cells. (A and B) HK-2 cells were cotransfected with SHP constructs and pGL3-NF-κB. The transfected cells were pretreated with 10 μM Bay11-7082 and 20 mM *N*-acetyl cysteine (NAC) for 1 h and then incubated with 500 μM H₂O₂ for 6 h. Luciferase activity was then determined using a luminometer. Pretreatment of 10 μM Bay (NF-κB inhibitor), 20 mM NAC, and overexpression of the SHP construct reduced H₂O₂-induced NF-κB promoter activity. (C) The nuclear extracts from the cells were analyzed by EMSA for activation of NF-κB using biotin-labeled oligonucleotide probes. HK-2 cells overexpressing SHP were pretreated with 10 μM Bay11-7082 and 20 mM NAC for 1 h, followed by incubation with 500 μM H₂O₂ for 6 h. After incubation, the lysates were analyzed by EMSA. [§]p<0.05 compared to the H₂O₂-untreated HK-2 cells; ^{§§}p<0.05 compared to the H₂O₂-treated HK-2 cells. Data are representative of at least 3 independent experiments.

In the present study, H₂O₂ exposure increased the promoter activity of AP-1 and NF-κB. COX-2 may induce stimulation of pro-inflammatory cytokines and growth factors (38), and the COX-2 promoter has transcription binding sites for AP-1, GATA BOX, C/EBP, CRE and NF-κB (39). Additionally, the iNOS promoter has binding sites for NF-κB, AP-1, STAT1, C/EBP and IRF-1 (40). Importantly, in the present study, the promoter activity of AP-1 and NF-κB was increased by H₂O₂ exposure in the HK-2 cells, and this effect was blocked by SHP transfection. Taken together with the observation that SHP transfection prevented the H₂O₂-mediated increases in iNOS and COX-2 expression, these findings suggest that SHP decreased the expression of COX-2 and iNOS through inhibition of NF-κB and AP-1 promoter activities. Pretreatment with NAC and cotransfection with dominant-negative c-Jun ameliorated the H₂O₂-induced increase in AP-1 promoter activity. Moreover, pretreatment with NAC and an NF-κB inhibitor reduced the H₂O₂-induced increase in NF-κB promoter activity. Furthermore, EMSA results indicated that H₂O₂ exposure markedly increased the amount of AP-1 and NF-κB that could form complexes with the biotin-labeled oligonucleotide

probe. In contrast, the promoter activities of AP-1 and NF-κB were decreased by pretreatment with JNK inhibitor II and Bay11-7082, respectively. The present study demonstrated that SHP protected HK-2 cells from H₂O₂-induced tubular injury by inhibition of COX-2 and iNOS through inhibition of AP-1 and NF-κB promoter activities. This knowledge may lead to an important new therapeutic target for the treatment of AKI such as I/R kidney injury.

Acknowledgements

This study was supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education (no. 2014R1A1A2008333), by the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Science, ICT and future Planning (no. 2016R1A2B4007870), by the Pioneer Research Center Program through the National Research Foundation of Korea funded by the Ministry of Science, ICT and Future Planning (no. 2014M3C1A3053036), and by a grant of the

Korea Health Technology R&D Project through the Korea Health Industry Development Institute (KHIDI), funded by the Ministry of Health and Welfare, Republic of Korea (grant no. HI14C2084).

References

- Lameire N, Van Biesen W and Vanholder R: The changing epidemiology of acute renal failure. *Nat Clin Pract Nephrol* 2: 364-377, 2006.
- Le Dorze M, Legrand M, Payen D and Ince C: The role of the microcirculation in acute kidney injury. *Curr Opin Crit Care* 15: 503-508, 2009.
- Bonventre JV and Zuk A: Ischemic acute renal failure: An inflammatory disease? *Kidney Int* 66: 480-485, 2004.
- Thurman JM: Triggers of inflammation after renal ischemia/reperfusion. *Clin Immunol* 123: 7-13, 2007.
- Sasaki M and Joh T: Oxidative stress and ischemia-reperfusion injury in gastrointestinal tract and antioxidant, protective agents. *J Clin Biochem Nutr* 40: 1-12, 2007.
- Basile DP: The endothelial cell in ischemic acute kidney injury: Implications for acute and chronic function. *Kidney Int* 72: 151-156, 2007.
- Kwon O, Hong SM, Sutton TA and Temm CJ: Preservation of peritubular capillary endothelial integrity and increasing pericytes may be critical to recovery from postischemic acute kidney injury. *Am J Physiol Renal Physiol* 295: F351-F359, 2008.
- Rubattu S, Mennuni S, Testa M, Mennuni M, Pierelli G, Pagliaro B, Gabriele E, Coluccia R, Autore C and Volpe M: Pathogenesis of chronic cardiorenal syndrome: Is there a role for oxidative stress? *Int J Mol Sci* 14: 23011-23032, 2013.
- Ruiz S, Pergola PE, Zager RA and Vaziri ND: Targeting the transcription factor Nrf2 to ameliorate oxidative stress and inflammation in chronic kidney disease. *Kidney Int* 83: 1029-1041, 2013.
- Nath KA and Norby SM: Reactive oxygen species and acute renal failure. *Am J Med* 109: 665-678, 2000.
- Gentle ME, Shi S, Daehn I, Zhang T, Qi H, Yu L, D'Agati VD, Schlondorff DO and Bottinger EP: Epithelial cell TGF β signaling induces acute tubular injury and interstitial inflammation. *J Am Soc Nephrol* 24: 787-799, 2013.
- Lessio C, de Assunção Silva F, Glória MA, Di Tommaso AB, Gori Mouro M, Di Marco GS, Schor N and Higa EM: Cyclosporine A and NAC on the inducible nitric oxide synthase expression and nitric oxide synthesis in rat renal artery cultured cells. *Kidney Int* 68: 2508-2516, 2005.
- Ostergaard M, Christensen M, Nilsson L, Carlsen I, Frøkiær J and Nørregaard R: ROS dependence of cyclooxygenase-2 induction in rats subjected to unilateral ureteral obstruction. *Am J Physiol Renal Physiol* 306: F259-F270, 2014.
- Jedinak A, Dudhgaonkar S, Wu QL, Simon J and Sliva D: Anti-inflammatory activity of edible oyster mushroom is mediated through the inhibition of NF- κ B and AP-1 signaling. *Nutr J* 10: 52, 2011.
- Liu DY, Li XW, Li H, Li XM and Ye WL: Expression of cyclooxygenase-2 in a mouse macula densa cell lines and signal transduction of NF- κ B and AP-1. *Zhongguo Yi Xue Ke Xue Yuan Xue Bao* 29: 78-82, 2007 (In Chinese).
- Jung KJ, Lee EK, Kim JY, Zou Y, Sung B, Heo HS, Kim MK, Lee J, Kim ND, Yu BP, *et al*: Effect of short term calorie restriction on pro-inflammatory NF- κ B and AP-1 in aged rat kidney. *Inflamm Res* 58: 143-150, 2009.
- Ahn KS and Aggarwal BB: Transcription factor NF- κ B: A sensor for smoke and stress signals. *Ann NY Acad Sci* 1056: 218-233, 2005.
- Roebuck KA: Oxidant stress regulation of IL-8 and ICAM-1 gene expression: Differential activation and binding of the transcription factors AP-1 and NF- κ B (Review). *Int J Mol Med* 4: 223-230, 1999.
- Seol W, Choi HS and Moore DD: An orphan nuclear hormone receptor that lacks a DNA binding domain and heterodimerizes with other receptors. *Science* 272: 1336-1339, 1996.
- Lee HK, Lee YK, Park SH, Kim YS, Park SH, Lee JW, Kwon HB, Soh J, Moore DD and Choi HS: Structure and expression of the orphan nuclear receptor SHP gene. *J Biol Chem* 273: 14398-14402, 1998.
- Sanyal S, Kim JY, Kim HJ, Takeda J, Lee YK, Moore DD and Choi HS: Differential regulation of the orphan nuclear receptor small heterodimer partner (SHP) gene promoter by orphan nuclear receptor ERR isoforms. *J Biol Chem* 277: 1739-1748, 2002.
- Nishizawa H, Yamagata K, Shimomura I, Takahashi M, Kuriyama H, Kishida K, Hotta K, Nagaretani H, Maeda N, Matsuda M, *et al*: Small heterodimer partner, an orphan nuclear receptor, augments peroxisome proliferator-activated receptor gamma transactivation. *J Biol Chem* 277: 1586-1592, 2002.
- Masuda N, Yasuno H, Tamura T, Hashiguchi N, Furusawa T, Tsukamoto T, Sadano H and Osumi T: An orphan nuclear receptor lacking a zinc-finger DNA-binding domain: Interaction with several nuclear receptors. *Biochim Biophys Acta* 1350: 27-32, 1997.
- Kim YD, Park KG, Lee YS, Park YY, Kim DK, Nedumaran B, Jang WG, Cho WJ, Ha J, Lee IK, *et al*: Metformin inhibits hepatic gluconeogenesis through AMP-activated protein kinase-dependent regulation of the orphan nuclear receptor SHP. *Diabetes* 57: 306-314, 2008.
- Lee YS, Chanda D, Sim J, Park YY and Choi HS: Structure and function of the atypical orphan nuclear receptor small heterodimer partner. *Int Rev Cytol* 261: 117-158, 2007.
- Chanda D, Park JH and Choi HS: Molecular basis of endocrine regulation by orphan nuclear receptor Small Heterodimer Partner. *Endocr J* 55: 253-268, 2008.
- Bälvner A, Sanyal S, Gustafsson JA and Treuter E: Transcriptional corepression by SHP: Molecular mechanisms and physiological consequences. *Trends Endocrinol Metab* 16: 478-488, 2005.
- Yuk JM, Shin DM, Lee HM, Kim JJ, Kim SW, Jin HS, Yang CS, Park KA, Chanda D, Kim DK, *et al*: The orphan nuclear receptor SHP acts as a negative regulator in inflammatory signaling triggered by Toll-like receptors. *Nat Immunol* 12: 742-751, 2011.
- Carden DL and Granger DN: Pathophysiology of ischaemia-reperfusion injury. *J Pathol* 190: 255-266, 2000.
- Molitoris BA, Sandoval R and Sutton TA: Endothelial injury and dysfunction in ischemic acute renal failure. *Crit Care Med* 30 (Suppl 5): S235-S240, 2002.
- Mount PF and Power DA: Nitric oxide in the kidney: Functions and regulation of synthesis. *Acta Physiol (Oxf)* 187: 433-446, 2006.
- Chatterjee PK, Patel NS, Sivarajah A, Kvale EO, Dugo L, Cuzzocrea S, Brown PA, Stewart KN, Mota-Filipe H, Britti D, *et al*: GW274150, a potent and highly selective inhibitor of iNOS, reduces experimental renal ischemia/reperfusion injury. *Kidney Int* 63: 853-865, 2003.
- Choi YJ, Kim HS, Lee J, Chung J, Lee JS, Choi JS, Yoon TR, Kim HK and Chung HY: Down-regulation of oxidative stress and COX-2 and iNOS expressions by dimethyl lithospermate in aged rat kidney. *Arch Pharm Res* 37: 1032-1038, 2014.
- Villanueva S, Céspedes C, González AA, Vio CP and Velarde V: Effect of ischemic acute renal damage on the expression of COX-2 and oxidative stress-related elements in rat kidney. *Am J Physiol Renal Physiol* 292: F1364-F1371, 2007.
- Bae EH, Choi HS, Joo SY, Kim IJ, Kim CS, Choi JS, Ma SK, Lee J and Kim SW: Farnesoid X receptor ligand prevents cisplatin-induced kidney injury by enhancing small heterodimer partner. *PLoS One* 9: e86553, 2014.
- Nakano H, Nakajima A, Sakon-Komazawa S, Piao JH, Xue X and Okumura K: Reactive oxygen species mediate crosstalk between NF- κ B and JNK. *Cell Death Differ* 13: 730-737, 2006.
- Mailloux RJ and Harper ME: Uncoupling proteins and the control of mitochondrial reactive oxygen species production. *Free Radic Biol Med* 51: 1106-1115, 2011.
- Murakami M and Kudo I: Prostaglandin E synthase: A novel drug target for inflammation and cancer. *Curr Pharm Des* 12: 943-954, 2006.
- Corral RS, Iñiguez MA, Duque J, López-Pérez R and Fresno M: Bombesin induces cyclooxygenase-2 expression through the activation of the nuclear factor of activated T cells and enhances cell migration in Caco-2 colon carcinoma cells. *Oncogene* 26: 958-969, 2007.
- Guo Z, Shao L, Du Q, Park KS and Geller DA: Identification of a classic cytokine-induced enhancer upstream in the human iNOS promoter. *FASEB J* 21: 535-542, 2007.