



Role of APE1/Ref-1 in hydrogen peroxide-induced apoptosis in human renal HK-2 cells

Ha Yeon Kim^{1,*}, Jung Sun Park^{2,*}, Byeong Hwa Jeon³, Hong Sang Choi², Chang Seong Kim², Seong Kwon Ma²,
Soo Wan Kim^{2,†}, Eun Hui Bae^{2,†}

¹Department of Internal Medicine, Gwangju Veterans Hospital, Gwangju, Republic of Korea

²Department of Internal Medicine, Chonnam National University Medical School, Gwangju, Republic of Korea

³Research Institute of Medical Sciences and Department of Physiology, Chungnam National University College of Medicine, Daejeon, Republic of Korea

Background: Apurinic/aprimidinic endonuclease 1/redox factor-1 (APE1/Ref-1) is a multipotent protein that plays essential roles in cellular responses to oxidative stress.

Methods: To examine the role of APE1/Ref-1 in ischemia-reperfusion (I/R) injuries and hydrogen peroxide (H₂O₂)-induced renal tubular apoptosis, we studied male C57BL/6 mice and human proximal tubular epithelial (HK-2) cells treated with H₂O₂ at different concentrations. The colocalization of APE1/Ref-1 in the proximal tubule, distal tubule, thick ascending limb, and collecting duct was observed with confocal microscopy. The overexpression of APE1/Ref-1 with knockdown cell lines using an APE1/Ref-1-specific DNA or small interfering RNA (siRNA) was used for the apoptosis assay. The promoter activity of nuclear factor kappa B (NF-κB) was assessed and electrophoretic mobility shift assay was conducted.

Results: APE1/Ref-1 was predominantly localized to the renal tubule nucleus. In renal I/R injuries, the levels of APE1/Ref-1 protein were increased compared with those in kidneys subjected to sham operations. The overexpression of APE1/Ref-1 in HK-2 cells enhanced the Bax/Bcl-2 ratio as a marker of apoptosis. Conversely, the suppression of APE1/Ref-1 expression by siRNA in 1-mM H₂O₂-treated HK-2 cells decreased the Bax/Bcl-2 ratio, the phosphorylation of extracellular signal-regulated kinase (ERK) 1/2, p38, c-Jun N-terminal kinase (JNK) 1/2, and NF-κB. In HK-2 cells, the promoter activity of NF-κB increased following H₂O₂ exposure, and this effect was further enhanced by APE1/Ref-1 transfection.

Conclusion: The inhibition of APE1/Ref-1 with siRNA attenuated H₂O₂-induced apoptosis through the modulation of mitogen-activated protein kinase pathways mediated by ERK, JNK, and p38 and the nuclear activation of NF-κB and proapoptotic factors.

Keywords: APE1/Ref-1, APE1/Ref-1 inhibitor, Apoptosis, HK-2 cells, Reperfusion injury

Received: August 4, 2022; **Revised:** November 2, 2022; **Accepted:** November 11, 2022

Correspondence: Soo Wan Kim

Department of Internal Medicine, Chonnam National University Medical School, 42 Jebong-ro, Dong-gu, Gwangju 61469, Republic of Korea.

E-mail: skimw@cnonnam.ac.kr

ORCID: <https://orcid.org/0000-0002-3540-9004>

Eun Hui Bae

Department of Internal Medicine, Chonnam National University Medical School, 42 Jebong-ro, Dong-gu, Gwangju 61469, Republic of Korea.

E-mail: baedak76@gmail.com

ORCID: <https://orcid.org/0000-0003-1727-2822>

*Ha Yeon Kim and Jung Sun Park contributed equally to this study as co-first authors.

†Soo Wan Kim and Eun Hui Bae contributed equally to this study as co-corresponding authors.

© 2024 The Korean Society of Nephrology

© This is an Open Access article distributed under the terms of the Creative Commons Attribution Non-Commercial and No Derivatives License (<http://creativecommons.org/licenses/by-nc-nd/4.0/>) which permits unrestricted non-commercial use, distribution of the material without any modifications, and reproduction in any medium, provided the original works properly cited.

Introduction

Apurinic/apyrimidinic endonuclease 1/redox factor-1 (APE1/Ref-1) is a ubiquitously expressed multipotent protein that plays an essential role in cellular response to oxidative stress. APE1/Ref-1 (also known as HAP1 or APEX) is a protein composed of 318 amino acids including a redox region and a DNA repair region. It is an essential component in maintaining genome stability, serving as an essential master regulator of the stress response [1]. APE1/Ref-1 is a dual-function protein involved both in the base excision repair pathways of DNA lesions, acting as the major apurinic/apyrimidinic endonuclease, as well as in the eukaryotic transcriptional regulation of gene expression [2,3]. APE1/Ref-1 functions to activate many transcription factors including p53, nuclear factor kappa B (NF- κ B), hypoxia-inducible factor (HIF) 1- α , and paired box 5 [2,4]. Moreover, APE1/Ref-1 may be important in the cellular response to oxidative changes triggered during apoptosis. APE1/Ref-1 is localized predominantly in the nucleus and has also been detected in the mitochondria and cytoplasm [5]. The movement of APE1/Ref-1 from the nucleus to the cytoplasm is mediated by an N-terminal nuclear export signal and a C-terminal nuclear localization signal (NLS) [6,7]. Importantly, this subcellular localization is dynamically regulated. APE1/Ref-1 rapidly translocates from the nucleus to the cytoplasm in response to reactive oxygen species (ROS) [8–10]. These two biological activities are located in two functionally distinct domains. The N-terminus, containing the NLS region, is principally devoted to redox activity through Cys65, while the C-terminus exerts enzymatic activity towards the basic sites of DNA [11].

Accumulating evidence has demonstrated that the heterogeneity of APE1/Ref-1 expression patterns is linked to different pathological conditions ranging from metabolic to differentiative disorders, including cancer and neurodegenerative diseases. Different kinds of human tumors are characterized by alterations in the subcellular distribution of APE1/Ref-1 with respect non-tumoral tissue [4,12]. The expression status of APE1/Ref-1 is altered in numerous cancers including prostate, lung, colon, and ovarian tumors. In addition, alterations in APE1/Ref-1 expression and mutations in the *APE1/Ref-1* gene have been detected in patients with a variety of neurodegenerative diseases [2,13–18]. However, the pathophysiological roles of APE1/

Ref-1 in kidney disease remain unclear.

In the present study, we evaluated whether APE1/Ref-1 was involved in ischemia-reperfusion (I/R)-induced kidney injury and whether APE1/Ref-1 played roles in H₂O₂-treated human proximal epithelial tubule (HK-2) cells. We also investigated whether APE1/Ref-1 overexpression exhibited cytoprotective or cytotoxic effects against H₂O₂-mediated apoptosis.

Methods

Animal experiments

The animal experiments were approved by the Animal Care Regulations Committee of the Chonnam National University Medical School (No. CNUH IACUC-18010), and the protocols conformed to the institutional guidelines for experimental animal care and use. Eight-week-old male C57BL6 mice were purchased from Samtako. Kidney I/R injuries were established by clamping both renal arteries for 20 minutes followed by 2 days of reperfusion. During the period of ischemia, body temperature was maintained by placing the rats on a 37 °C heating pad. Following removal of the clamps, the kidneys were inspected for 1 minute for the restoration of blood flow, as noted by a return to their original color, prior to closure of the abdomen. Sham-operated mice received identical surgical procedures with the exception of the microaneurysm clamps. The mice were sacrificed 2 days after reperfusion, blood was collected, and kidney tissue was divided for subsequent protein extraction.

Cell culture and reagents

HK-2 cells were passaged every 3–4 days in 100-mm dishes containing Dulbecco's modified Eagle's medium/F-12 medium supplemented with 10% fetal bovine serum (FBS), 100-U/mL penicillin, and 100- μ g/mL streptomycin (Sigma). When the cells reached 70% to 80% confluence, they were detached with TrypLE Express (Invitrogen), centrifuged at 250 \times g for 3 minutes, replated, and maintained at 37 °C in an incubator containing 5% CO₂ atmosphere. For experimental use, the HK-2 cells were plated in 60-mm dishes in medium containing 10% FBS for 24 hours and then treated with H₂O₂.

Histologic analysis

Kidney tissues were fixed with 4% paraformaldehyde, embedded in paraffin, and cut into 4 μm -thick sections. To assess histological morphology, immunohistochemical staining was performed using indicated antibodies and horseradish peroxidase-conjugated anti-mouse or anti-rabbit immunoglobulin G secondary antibodies (Dako). The stained sections were imaged with a Nikon Eclipse Ni-U microscope. The quantitative analysis of the stained sections was performed using imageJ software (National Institutes of Health).

Confocal laser microscopy

Left kidneys of I/R and control mice were collected for immunofluorescence analyses. Tissue samples were prepared through deparaffination and hydration, and the cells fixed in 4% paraformaldehyde were prepared and blocked at room temperature for 2 hours. Rabbit or mouse monoclonal antibodies against Lotus tetragonolobus lectin (Vector Laboratories), calbindin-D28K (Invitrogen), Tamm-Horsfall protein (AbD serotec), and aquaporin-2 (Santa Cruz Biotechnology) were diluted 1:100 in blocking buffer and applied at 4 °C for 24 hours. After washing, the secondary antibody was diluted 1:200 in blocking buffer and applied at room temperature for 2 hours. After washing, coverslips were mounted onto microslides using a ProLong Gold Antifade Reagent with DAPI (Life Technologies).

Protein extraction

The kidneys were homogenized in ice-cold isolation solution containing 0.3-M sucrose, 25-mM imidazole, 1-mM ethylenediaminetetraacetic acid (EDTA), 8.5- μM leupeptin, and 1-mM phenylmethylsulfonyl fluoride (PMSF; pH 7.2). The homogenates were centrifuged and the total protein concentration was measured using a Pierce BCA protein assay kit (Thermo Fisher Scientific). All the samples were adjusted with isolation solution to normalize protein concentration and stored at -20 °C. HK-2 cells were harvested, washed twice with ice-cold phosphate-buffered saline (PBS), resuspended 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, and 1-mM Na_3VO_4), and briefly sonicated. After

centrifugation, the supernatants were prepared as protein extracts and the protein concentrations were measured (Pierce BCA protein assay reagent kit).

Western blot analysis

Equal amounts of protein were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 9% or 12% gels. The proteins were electrophoretically transferred onto nitrocellulose membranes using a Bio-Rad Mini Protean II apparatus (Bio-Rad). 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 [pH 7.5]) for 2 hours. Anti-Bcl-2 (3498), anti-Bax (2772), anti-cleaved caspase 3 (9661), anti-phospho extracellular signal-regulated kinase (p-ERK) (9101), anti-phospho-p38 mitogen-activated protein kinase (p-p38 MAPK) (4631), and anti-phospho JNK (p-JNK) (9251) antibodies (Cell Signaling Technology) were used. Phospho-NF- κB p65 (sc-33020; Santa Cruz Biotechnology), anti-NF- κB p65 (8242; Cell Signaling Technology), anti-I $\kappa\text{B}\alpha$ (SC-1643; Santa Cruz Biotechnology), and anti- β -actin (a5316; Sigma) antibodies were diluted in blocking buffer and incubated with the blots overnight at 4 °C. The blots were then washed and incubated with peroxidase-conjugated secondary antibody (1:3,000). The selected bands were scanned (GS-700 Imaging Densitometry; Bio-Rad) and the density was determined (Molecular Analyst version 1.5; Bio-Rad).

Stable cell lines

To generate APE1/Ref-1 overexpressing cell lines, HK-2 cells were transfected with 2 μg of empty vector (Mock) or APE1/Ref-1 DNA using 6 μL of FuGENE HD reagent (Promega) in antibiotic-free DMEM-F12. Starting 1 day after transfection, transfectants were selected in DMEM-F12 containing 800- $\mu\text{g}/\text{mL}$ zeocin which was refreshed every 3 days for 2 weeks. Colonies surviving in the selection medium were collected and sequentially plated in 48-, 12-, and 6-well plates and then in 60- and 100-mm dishes. Total cellular extracts were analyzed for APE1/Ref-1 expression by immunoblotting, thus revealing the expression of the ectopic flagged forms of wild type and the mutant form of the protein.

Small interfering RNA knockdown of APE1/Ref-1

RNA interference of APE1/Ref-1 was performed using an APE1/Ref-1-specific small interfering RNA (siRNA) from Ambion's siRNA Target Finder Program: Ape1/Ref-1 siRNA (534 bp from the ATG), 5-GUCUGGUACGACUGGAGUAtt-3 (sense), and 5-UACUCC AGUCGUACCAGACt-3 (anti-sense). siRNAs were prepared using a transcription-based method with a Silencer siRNA construction kit (Ambion).

Apoptosis assay

The number of apoptotic cells was quantified using the Ezway Annexin V-FITC apoptosis detection kit (KOMA Biotech) according to the manufacturer's protocol. Cells were stained with Annexin V-FITC dye. Fluorescent intensity was measured by FACSCalibur flow cytometry (BD Biosciences).

Promotor activity of NF- κ B

The transcriptional regulation of NF- κ B was examined by the transient transfection of an NF- κ B promoter-luciferase reporter construct (pGL3-NF- κ B). HK-2 cells (5×10^5) were seeded and grown until they reached 60% to 70% confluence, and pGL3-NF- κ B 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 hours after 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 the HK-2 cells were prepared with the NE-PER nuclear extraction reagent (Pierce Biotechnology). The biotin-labeled NF- κ B oligonucleotide sequence was 5'-biotin-AGTTGAGGGGACTTCCCAGGC-3'. The binding reactions contained 10 μ g of the nuclear extract protein, buffer (10-mM Tris [pH 7.5], 50-mM KCl, 5-mM

MgCl₂, 1-mM dithiothreitol, 0.05% Nonidet P-40, and 2.5% glycerol), 1 μ g of poly (dI-dC), and 2-nM biotin-labeled DNA. The reactions were incubated at 23 °C for 20 minutes. The competition reactions were performed by adding 10-fold excess unlabeled double-stranded NF- κ B consensus oligonucleotides to the reaction mixture. The reactions were electrophoresed on a 6% precasted Tris-borate-EDTA gel (Invitrogen) at 100 V for 1 hour 30 minutes in a 100-mM Tris-borate-EDTA buffer. The reactions were then transferred to a nylon membrane. The biotin-labeled DNA was detected with a LightShift chemiluminescent electrophoretic mobility shift assay kit (Pierce Biotechnology).

Statistical analysis

The results are expressed as the mean \pm standard error of mean. Multiple comparisons among the three groups were performed using one-way analysis of variance and *post hoc* Tukey honestly significant difference tests. Differences with the p-values of <0.05 were considered significant.

Results

APE1/Ref-1 expression in *in vivo* ischemia-reperfusion-induced mouse kidney injuries

Fig. 1 shows the expression pattern of APE1/Ref-1 according to mouse I/R injury. The fluorescence of APE1/Ref-1 expression was highest in the cortex, modest in the outer medulla, and lowest in the inner medulla (Fig. 1A). The expression of APE1/Ref-1 that significantly increased after I/R injury compared to the control was observed by immunofluorescence (Fig. 1B). Fig. 1C shows the observation of the expression of APE1/Ref-1 in each part of the kidney by immunofluorescence. APE1/Ref-1 exhibited high expression in the proximal tubule, but weak expression was observed in the thick ascending limb and collecting duct in the mouse kidney (Fig. 1C).

Serum blood urea nitrogen and creatinine levels were significantly increased in I/R injured mice compared to the sham-operated controls (Fig. 2A). Consistent with this finding, the protein expression of APE1/Ref-1 increased after I/R injury (Fig. 2B). In addition, the protein expression levels of Bax and cleaved caspase 3 were increased, whereas Bcl-2 expression was decreased. We observed kidney

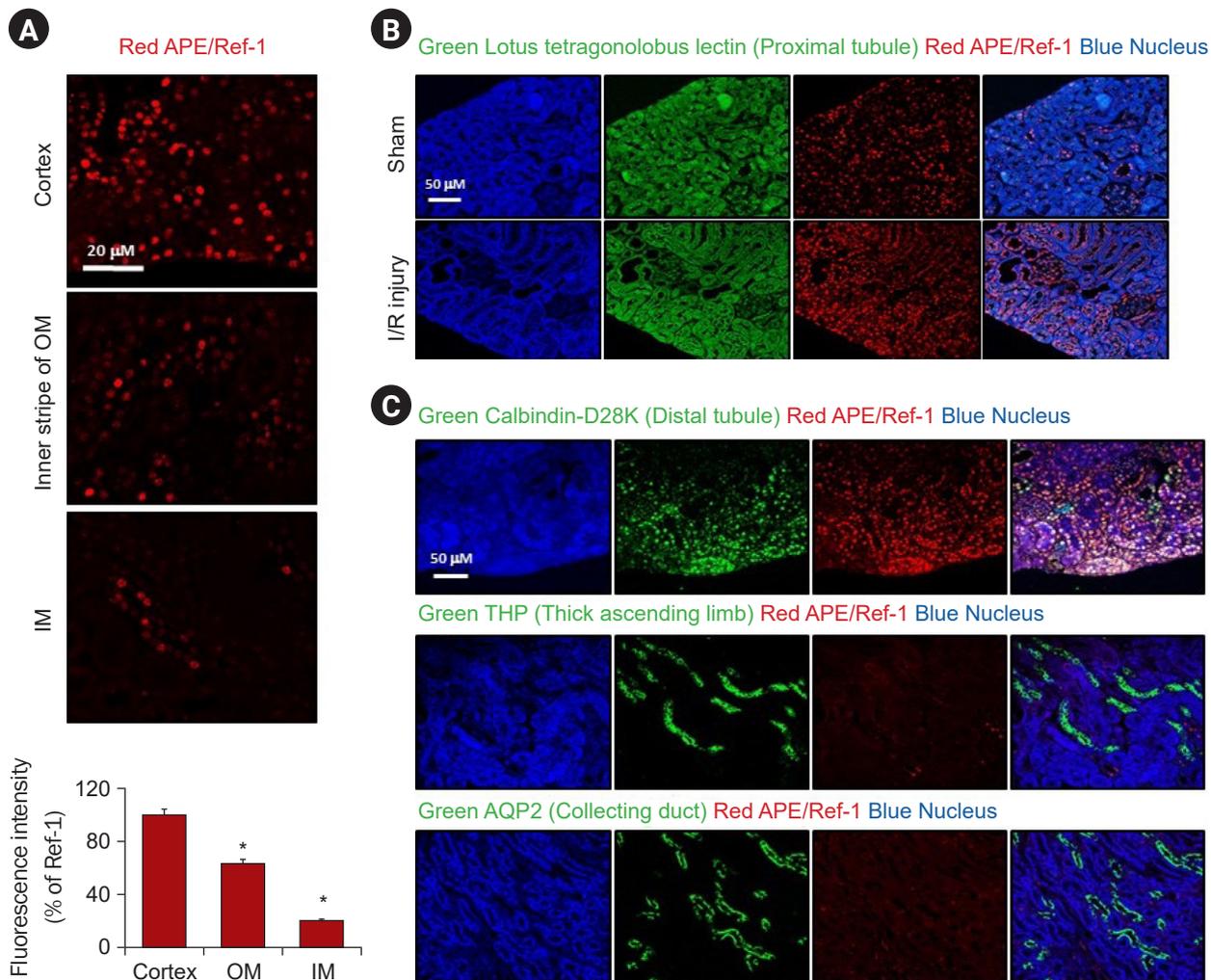


Figure 1. Expression of APE1/Ref-1 in mouse kidneys. (A) The control kidney sections were stained with anti-APE1/Ref-1 antibody (red). The fluorescence indicating APE1/Ref-1 expression was the highest in the cortex, modest in the outer medulla (OM), and lowest in the inner medulla (IM). Scale bar, 20 μm . Original magnification, $\times 200$. (B) Double staining was performed with kidney tubule markers and APE1/Ref-1 to indicate the presence of APE1/Ref-1. The kidney sections were double-stained with anti-APE1/Ref-1 and then with anti-Lotus tetragonolobus lectin antibodies. The fluorescence of APE1/Ref-1 expression is presented as a red color. APE1/Ref-1 was predominantly localized in the nuclei of kidney cortical tubule cells. Scale bar, 50 μm . Original magnification, $\times 200$. (C) The expression of APE1/Ref-1 was observed by the double staining of the distal tubule, thick ascending limb, collecting duct, and APE1/Ref-1 in mouse kidneys. Compared with the distal tubule, the expression of APE1/Ref-1 was weaker in the thick ascending limb and collecting duct. Scale bar, 50 μm . Original magnification, $\times 200$. APE1/Ref-1, apurinic/aprimidinic endonuclease 1/redox factor-1.

tissue damage caused by I/R through H&E and PAS staining. There was a markedly increased kidney tubule-interstitial injury tissue in the I/R model compared to the control (Fig. 2C). Immunohistochemistry was performed to determine whether I/R-induced renal tissue damage was associated with the expression of APE1/Ref-1. Com-

pared with the control, it was observed that the expression of APE1/Ref-1 increased in the I/R injury model (Fig. 2D). These results demonstrate that APE1/Ref-1 expression and apoptosis are induced by I/R kidney tissue damage.

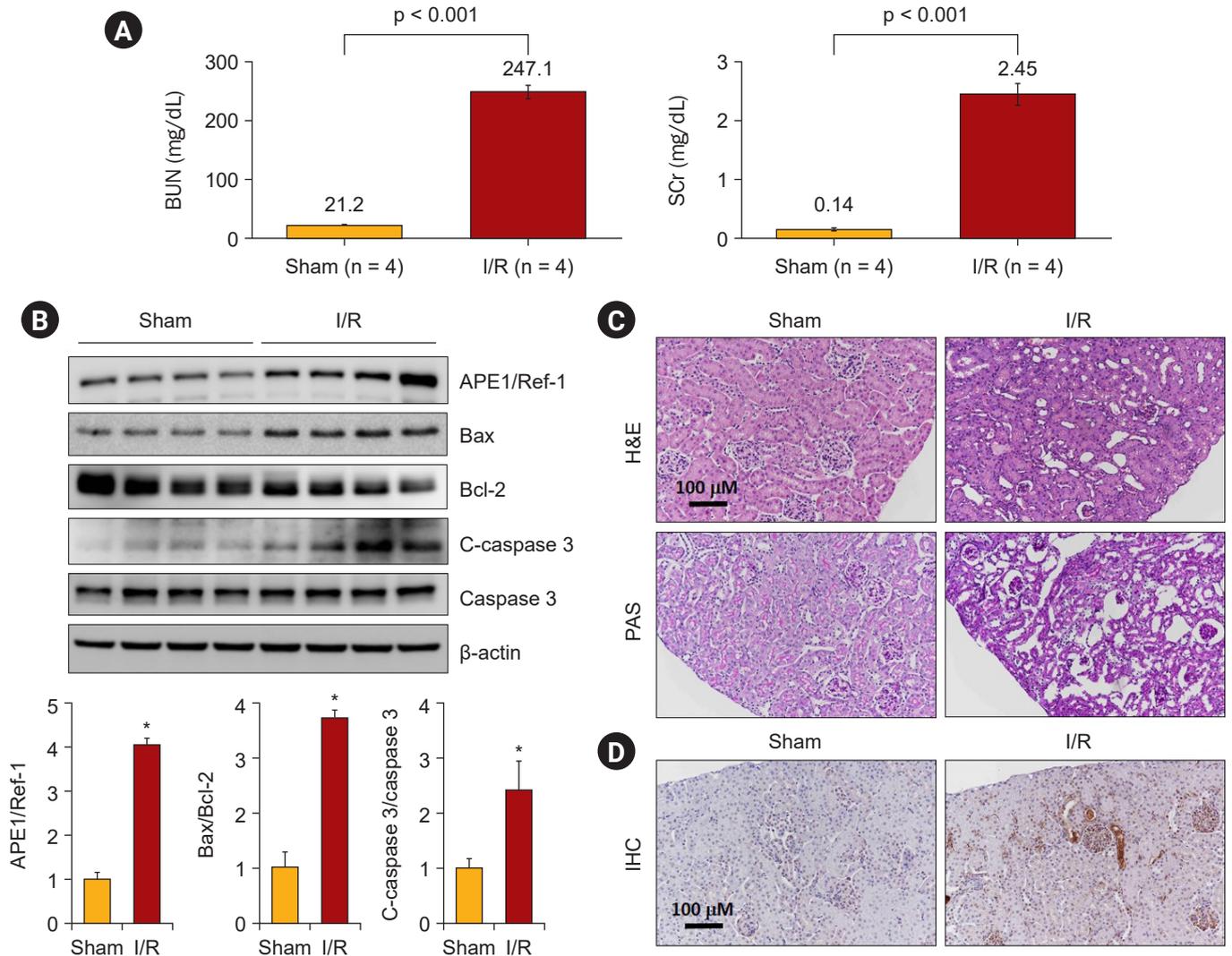


Figure 2. Increased APE1/Ref-1 and apoptotic markers in mice with I/R injuries after 2 days. (A) Mice with renal I/R injury exhibited significantly higher serum creatinine (SCr) and blood urea nitrogen (BUN) levels compared to untreated I/R injury controls. (B) Western blotting analysis showed that the expression levels of apoptotic markers, such as pro-/anti-apoptotic Bax/Bcl-2, and cleaved caspase 3 (C-caspase 3) was altered and APE1/Ref-1 were increased in the I/R injury mouse model compared with those in the sham. (C) Immunohistochemical (IHC) staining of hematoxylin and eosin (H&E) and periodic acid-Schiff (PAS) in the kidneys of the control and I/R mice. Scale bar, 100 μ m. Original magnification, $\times 200$. (D) IHC staining of APE1/Ref-1 in the kidneys of the control and I/R mice. Scale bar, 100 μ m. Original magnification, $\times 200$.

APE1/Ref-1, apurinic/aprimidinic endonuclease 1/redox factor-1; Bax, Bcl-2-associated protein X; Bcl-2, B-cell lymphoma 2; I/R, ischemia-reperfusion.

Columns, means of three independent cases; bars, standard deviation.

* $p < 0.05$, I/R injury vs. sham.

Aggravation of H_2O_2 -mediated injury by APE1/Ref-1 over-expression in HK-2 cells *in vitro*

Next, we investigated whether H_2O_2 treatment increased the expression of APE1/Ref-1 and apoptosis proteins in

kidney proximal tubular HK-2 cells. Fig. 3A shows that 0-, 0.1-, 0.2-, 0.5-, and 1-mM H_2O_2 were treated in a concentration-dependent manner and harvested after 24 hours. APE1/Ref-1, Bax, and cleaved caspase-3 increased according to the concentration. Fig. 3B shows the time-de-

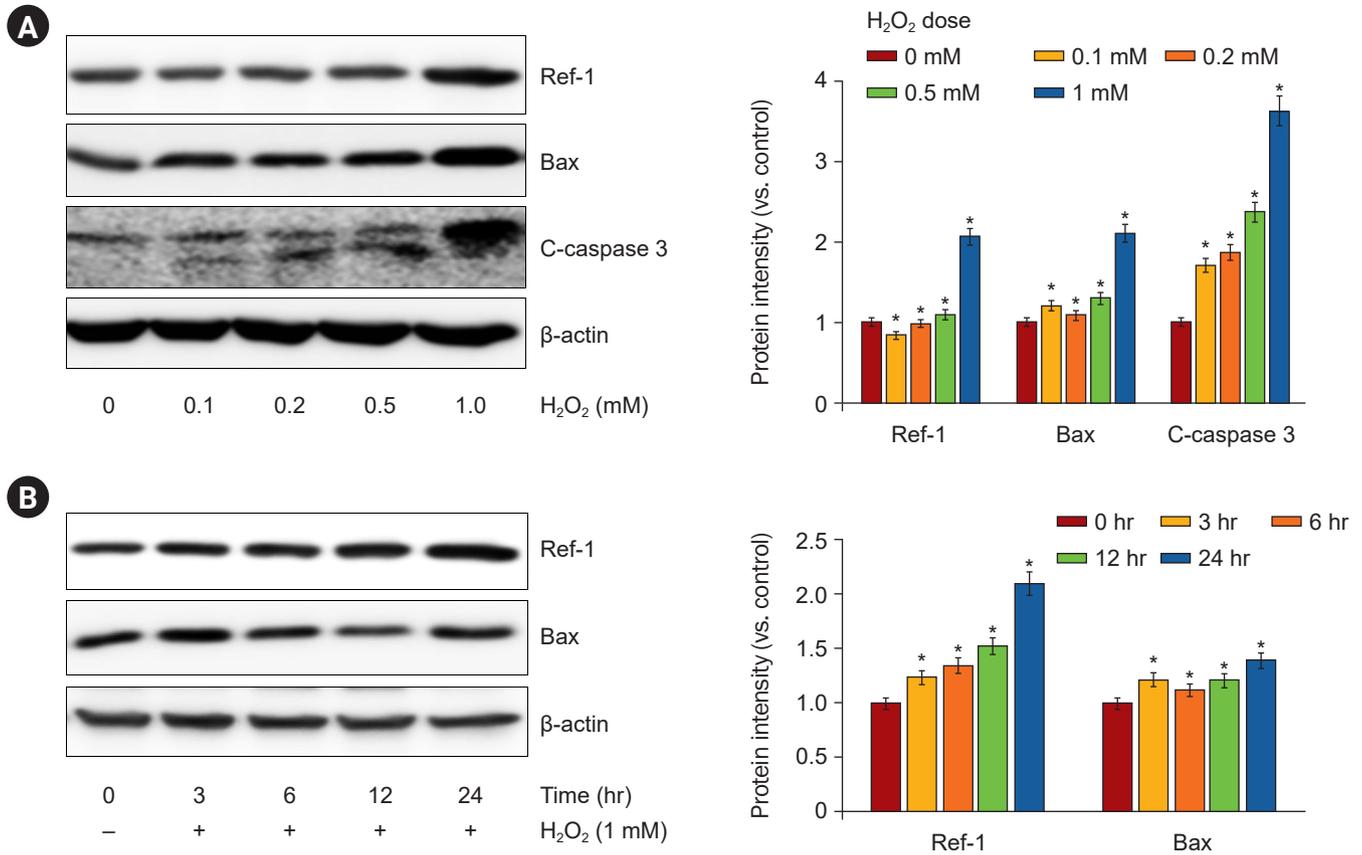


Figure 3. H₂O₂-induced APE1/Ref-1 expression in HK-2 cells with various concentrations and exposure times of H₂O₂. (A) Exposure to H₂O₂ exhibited a dose-dependent increase in the protein expression of APE1/Ref-1, Bax, and cleaved caspase 3 (C-caspase 3). (B) The protein expression levels of APE1/Ref-1, Bax, and C-caspase 3 increased after H₂O₂ treatment in HK-2 cells. APE1/Ref-1, apurinic/aprimidinic endonuclease 1/redox factor-1; Bax, B-cell lymphoma 2-associated protein X. Columns, means of three independent cases; bars, standard deviation. *p < 0.05 vs. H₂O₂-untreated cells.

pendent observations of 0, 3, 6, 12, and 24 hours by treatment with 1-mM H₂O₂. The expression of APE1/Ref-1 and Bax was significantly increased after 24 hours. Next, we evaluated the effects of the overexpression of APE1/Ref-1 in H₂O₂-treated HK-2 cells *in vitro*. To determine the physiological effects of APE1/Ref-1, HK-2 cells were stably transfected with an empty vector (Mock) or a plasmid encoding human APE1/Ref-1. Stable cells were selected through the confirmation of the expression of zeocin, which was present in the backbone plasmid pCDNA4. The evaluation of Mock and APE1/Ref-1 stable cells following H₂O₂-mediated injury revealed decreased cell viability and increased the number of Annexin-V-positive cells in cells stably expressing APE1/Ref-1. Apoptotic cells were defined as Annexin V-FITC-positive cells. This result suggested

that APE1/Ref-1 overexpression aggravated H₂O₂-mediated apoptotic and necrotic cell death (65.7% apoptosis in H₂O₂-treated HK-2 cells with APE1/Ref-1 overexpression versus 50.4% apoptosis in H₂O₂-treated HK-2 cells without APE1/Ref-1 overexpression) (Fig. 4).

Proapoptotic effects of APE1/Ref-1

To verify the apoptotic effects of APE1/Ref-1 in injured proximal tubule cells, the expression of proapoptotic proteins was evaluated in H₂O₂-treated Mock cells and cells stably expressing APE1/Ref-1. The expression level of the Bax/Bcl-2 ratio was increased in APE1/Ref-1 overexpressing cells (Fig. 5), and this effect was abolished by APE1/Ref-1 specific siRNA knockdown (Fig. 6).

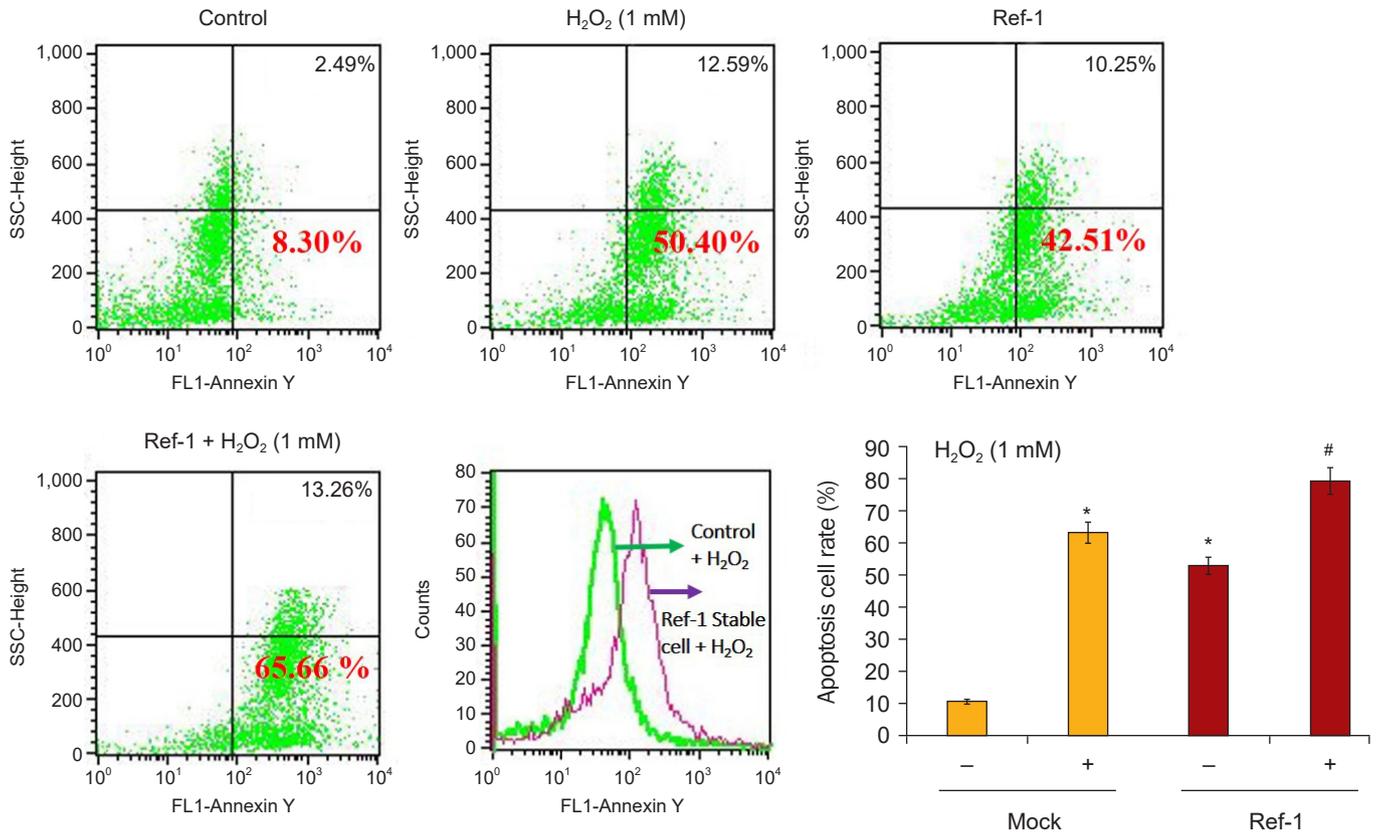


Figure 4. Expression of apoptotic cells following APE1/Ref-1 overexpression. HK-2 cells and APE1/Ref-1 overexpressing HK-2 cells were treated with 1-mM H₂O₂ for 6 hours and stained with Annexin V-FITC for 30 minutes, followed by flow cytometry analysis for the presence of apoptotic cells. APE1/Ref-1 overexpressing cells (65.7%) treated with H₂O₂ exhibited increased apoptotic cells compared to H₂O₂-treated cells without overexpression (50.4%). The bar graph reveals apoptosis according to fluorescence values.

APE1/Ref-1, apurinic/aprimidinic endonuclease 1/redox factor-1; SSC, side scatter.

Columns, means of three independent cases; bars, standard deviation.

*p < 0.05 vs. H₂O₂-untreated Mock cells; #p < 0.05 vs. H₂O₂-treated Mock cells.

Role of APE1/Ref-1 in mitogen-activated protein kinase signaling

Following treatment with 1-mM H₂O₂, the MAPK pathway components (e.g., extracellular signal-regulated kinase [ERK], c-Jun N-terminal kinase [JNK], and p38) were upregulated in APE1/Ref-1-overexpressing cells compared with Mock cells (Fig. 7A). The phosphorylation of p38, which is involved in the stabilization and mitochondrial accumulation of p38, was higher in H₂O₂-treated APE1/Ref-1 cells compared to Mock cells. Furthermore, the levels of activated p-ERK1/2 and phospho-JNK1/2 were enhanced in H₂O₂-treated APE1/Ref-1-overexpressing cells, and these effects were attenuated by transfection with siRNA target-

ing APE1/Ref-1 (Fig. 7B). Therefore, the involvement of the ERK/p38/JNK axis in apoptosis was associated with APE1/Ref-1 overexpression.

Effects of APE1/Ref-1 on NF-κB signaling

To further analyze the mechanisms through which APE1/Ref-1 was associated with the enhancement of apoptosis, we studied the expression of NF-κB, which is involved in the coordinated induction of genes that encode many stress-responsive and cytotoxic enzymes and related proteins. After treatment with 1-mM H₂O₂, NF-κB protein expression was observed in a 0, 1-, 2-, 4-, and 6-hour dependent manner. The expression of NF-κB protein was

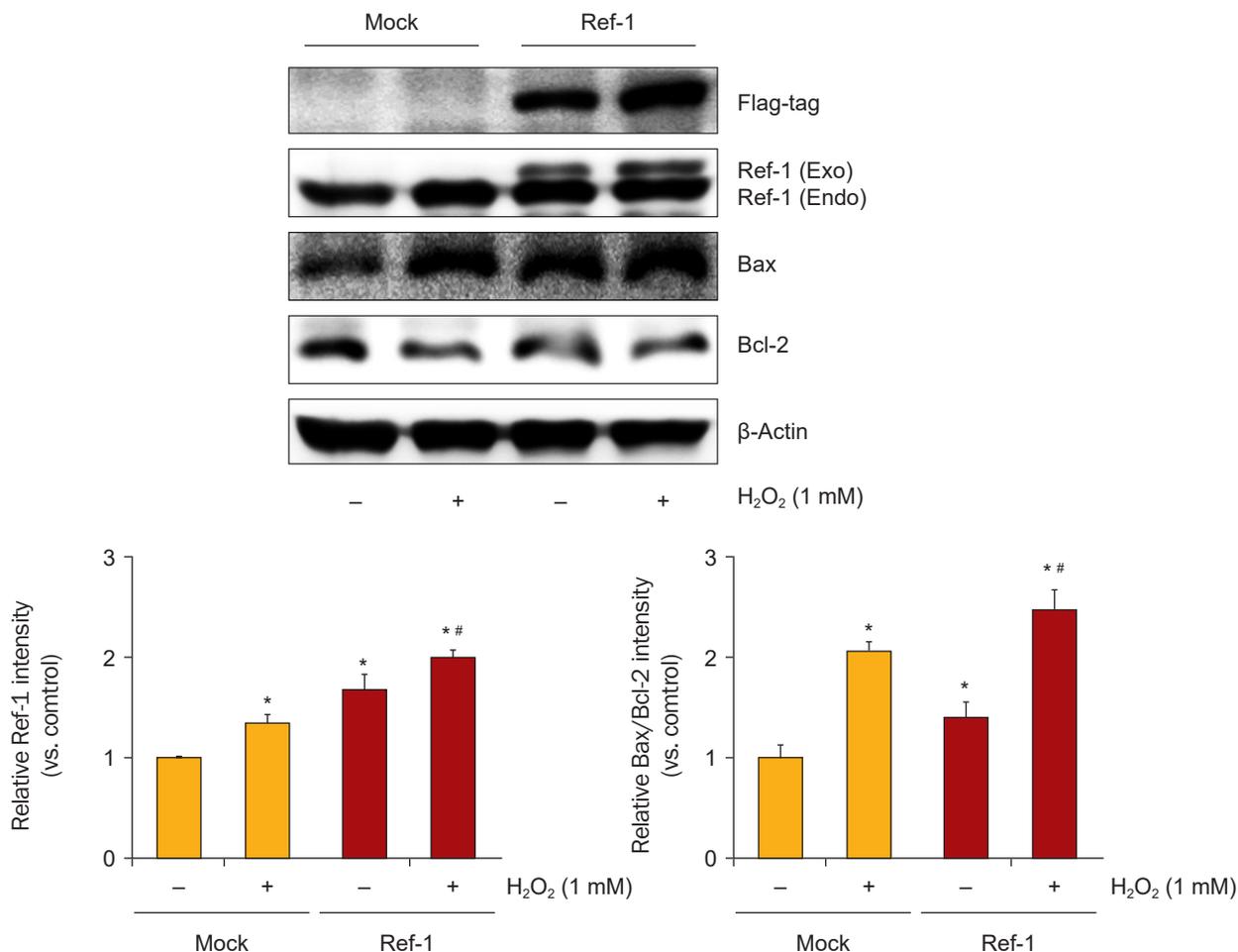


Figure 5. Enhancement of apoptotic protein expression induced by H₂O₂ in an APE1/Ref-1-overexpressing cell line. HK-2 cells transfected with APE1/Ref-1 exhibited increased apoptotic protein expression levels (Bax/Bcl-2 ratio) compared with Mock cells. APE1/Ref-1, apurinic/aprimidinic endonuclease 1/redox factor-1; Bax, Bcl-2-associated protein X; Bcl-2, B-cell lymphoma 2. *p < 0.05 vs. H₂O₂-untreated Mock cells; #p < 0.05 vs. H₂O₂-treated Mock cells. Data are representative of at least three independent experiments.

significantly increased in APE1/Ref-1 overexpressing cells compared with Mock cells (Fig. 8A). Next, NF-κB expression by APE1/Ref-1 specific siRNA knockdown was observed. Cells were treated with 30 nM of scrambled siRNA and APE1/Ref-1 siRNA for 24 hours, and then treated with 1-mM H₂O₂ for 6 hours to obtain nuclear and cytoplasmic proteins, respectively, and NF-κB expression was observed. We observed the decreased expression of NF-κB nucleoproteins transfected with siRNA targeting APE1/Ref-1 (Fig. 8B). These results suggest that the expression of APE/Ref-1 and NF-κB is related to apoptosis.

Effects of APE1/Ref-1 on the transcriptional activation of NF-κB

NF-κB is an important transcription factor activating the expression of cyclooxygenase-2 and inducible nitric oxide synthase. Moreover, NF-κB is known to be activated by ROS. Therefore, we examined the role of APE1/Ref-1 in the H₂O₂-induced transcriptional activation of NF-κB. First, it was observed whether NF-κB phosphorylation increased in nucleoproteins after treatment with 1-mM H₂O₂. The expression of NF-κB proteins was observed to significantly increase the phosphorylation of NF-κB in APE1/Ref-1 overexpressing cells compared to Mock cells (Fig. 9A).

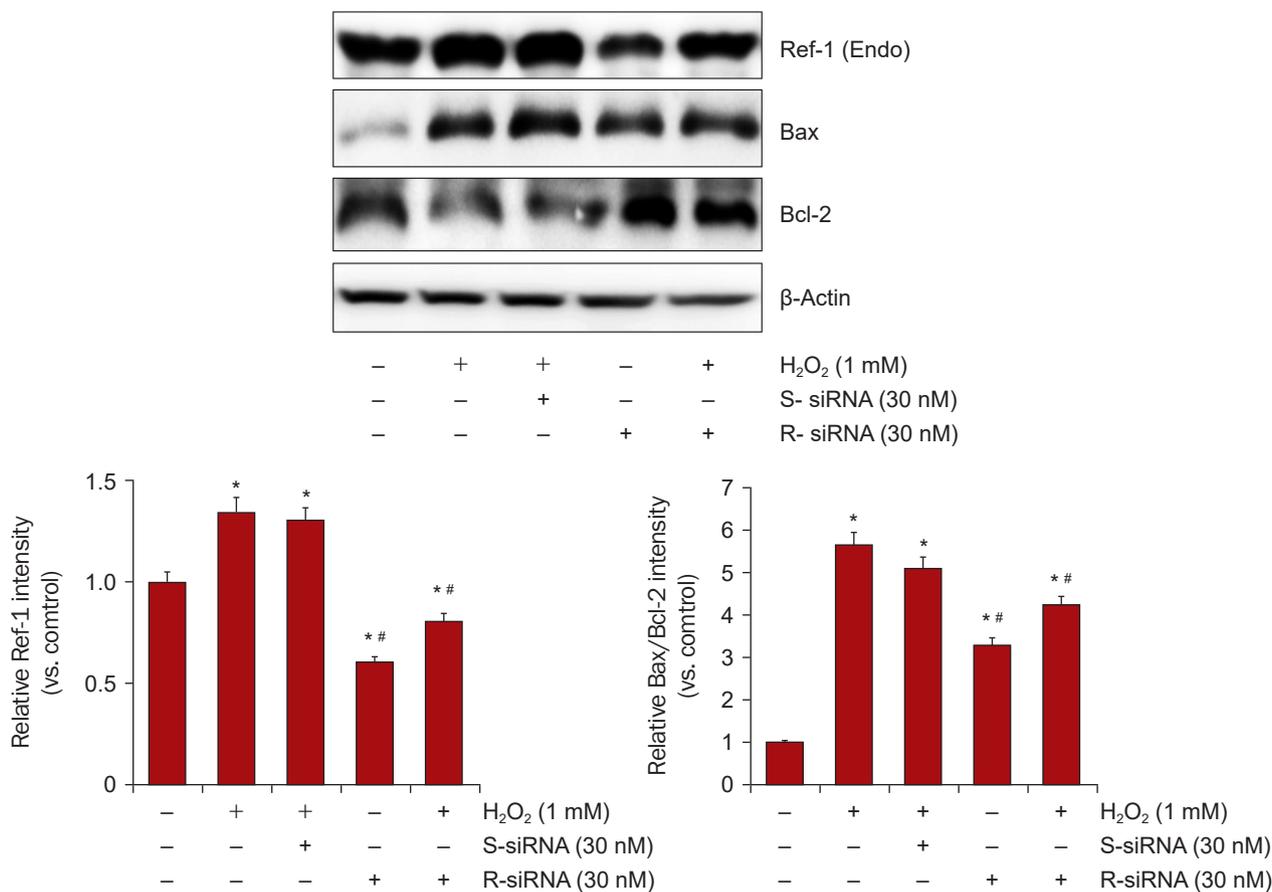


Figure 6. Downregulation of APE1/Ref-1 in H₂O₂-treated HK-2 cells. HK-2 cells were harvested and equivalent amounts of protein were immunoblotted with anti-Bax, Bcl-2, and β -actin. RNA interference of APE1/Ref-1 was performed using an APE1/Ref-1-specific small interfering RNA (siRNA). The bar graph shows the relative protein expression of APE1/Ref-1 measured by densitometry. β -actin levels were analyzed as internal controls.

APE1/Ref-1, apurinic/aprimidinic endonuclease 1/redox factor-1; Bax, Bcl-2-associated protein X; Bcl-2, B-cell lymphoma 2; siRNA, small interfering RNA; S-siRNA, scrambled siRNA; R-siRNA, Ref-1 siRNA.

Columns, means of three independent cases; bars, standard deviation.

* $p < 0.05$ vs. H₂O₂-untreated HK-2 cells; # $p < 0.05$ vs. H₂O₂-treated HK-2 cells in the absence of siRNA of APE1/Ref-1.

NF- κ B is activated by a Cys-65 redox activation reaction that regulates several transcription factors present in the N-terminal region of APE1/Ref-1. We treated cells with an inhibitor of APE1/Ref-1 (E3330) and observed whether NF- κ B activity was inhibited. NF- κ B was significantly reduced by treatment with 100 μ M E3330 (Fig. 9B). The promoter activity of NF- κ B was increased following H₂O₂ exposure in HK-2 cells, and this increase was enhanced by APE1/Ref-1 transfection (Fig. 9C). Nuclear extracts from cells analyzed by electrophoretic mobility shift assays for activated NF- κ B confirmed these findings (Fig. 9D). These results suggest that APE1/Ref-1 increased NF- κ B pathway activity direct-

ly by activating the transcription factor NF- κ B. Based on these results, a schematic diagram showing that APE1/Ref-1 induces apoptosis in association with MAPK and NF- κ B signaling is presented in Fig. 10.

Discussion

Ischemic stress contributed to the upregulation of APE1/Ref-1 expression in the kidney. In HK-2 cells, oxidative stress induced increased APE1/Ref-1 expression, and APE1/Ref-1 aggravated H₂O₂-mediated apoptosis in APE1/Ref-1-overexpressing HK-2 cells. Moreover, the Bax/Bcl-

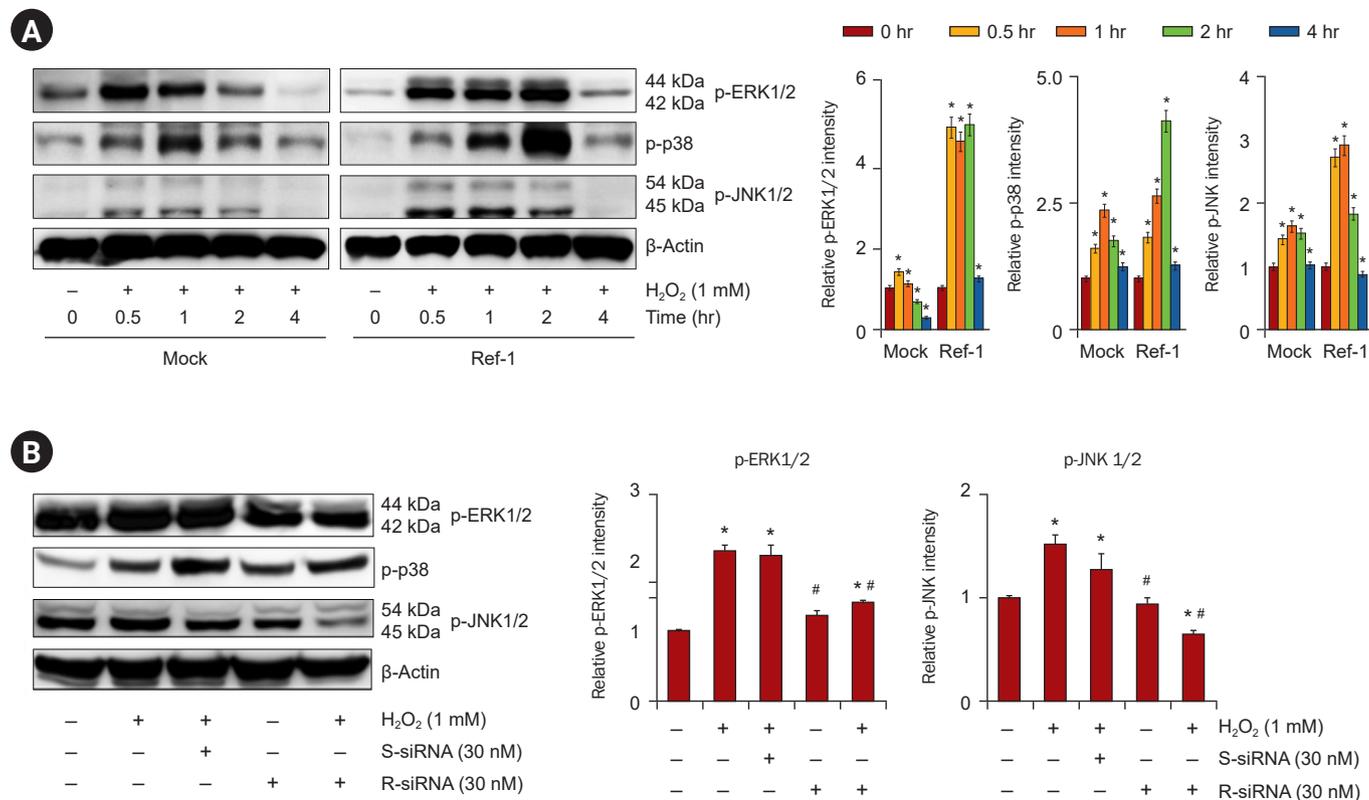


Figure 7. Effects of APE1/Ref-1 siRNA on the p-ERK1/2, p-p38, and p-JNK in HK-2 cells treated with H₂O₂. (A) Compared with Mock, APE1/Ref-1-overexpressing HK-2 cells exhibited increased protein expressions of p-ERK, p38, and p-JNK after pretreatment with H₂O₂ (1 mM). (B) Increased expression of p-ERK-1/2 and p-JNK was attenuated by siRNA of APE1/Ref-1, while p-p38 was not affected by APE1/Ref-1 overexpression.

APE1/Ref-1, apurinic/aprimidinic endonuclease 1/redox factor-1; p-ERK, phosphorylation of extracellular signal-regulated kinase; p-JNK, phosphorylation of c-Jun N-terminal kinase; p-p38, phosphorylation of p38; siRNA, small interfering RNA; S-siRNA, scrambled siRNA; R-siRNA, Ref-1 siRNA.

*p < 0.05 vs. H₂O₂-untreated HK-2 cells, #p < 0.05 vs. H₂O₂-treated HK-2 cells in the absence of siRNA of APE1/Ref-1. Data are representative of at least three independent experiments.

2 ratio was increased in APE1/Ref-1-overexpressing cells but was decreased in HK-2 cells transfected with siRNA targeting APE1/Ref-1. We also demonstrated that APE1/Ref-1 was associated with the MAPK pathway and NF-κB signaling. These findings suggest that the upregulation of APE1/Ref-1 is related to apoptosis after oxidative stress and that the inhibition of APE1/Ref-1 could have therapeutic potential in the management of acute kidney injuries.

APE1/Ref-1 is involved in the repair of DNA damage caused by oxidative stress [19] and has been shown to possess roles in the redox regulation of various transcription factors such as activator protein-1 [20], NF-κB [21], early growth response-1 [22], NF-Y [23], HIF-1 [24], Myb [25], and p53 [26]. Moreover, this protein is involved in controlling

cell-cycle arrest and apoptotic programs, with different functions in various contexts. Notably, the overexpression of APE1/Ref-1 confers resistance to apoptosis induced by chemotherapeutic drugs, radiation, hypoxia, and tumor necrosis factor (TNF) [27]. However, studies have also shown that APE1/Ref-1 is a potent signaling molecule in hyperacetylated breast cancer cells and promotes apoptosis-induced cell death [28]. In addition, H₂O₂ and/or hydroxyl radicals efficiently and rapidly promote a transient increase in APE1/Ref-1 protein levels, and various transcription factors are involved in the inducible expression of APE1/Ref-1 [18].

The regulatory functions modulating the activity of APE1/Ref-1 are complex and controlled through three

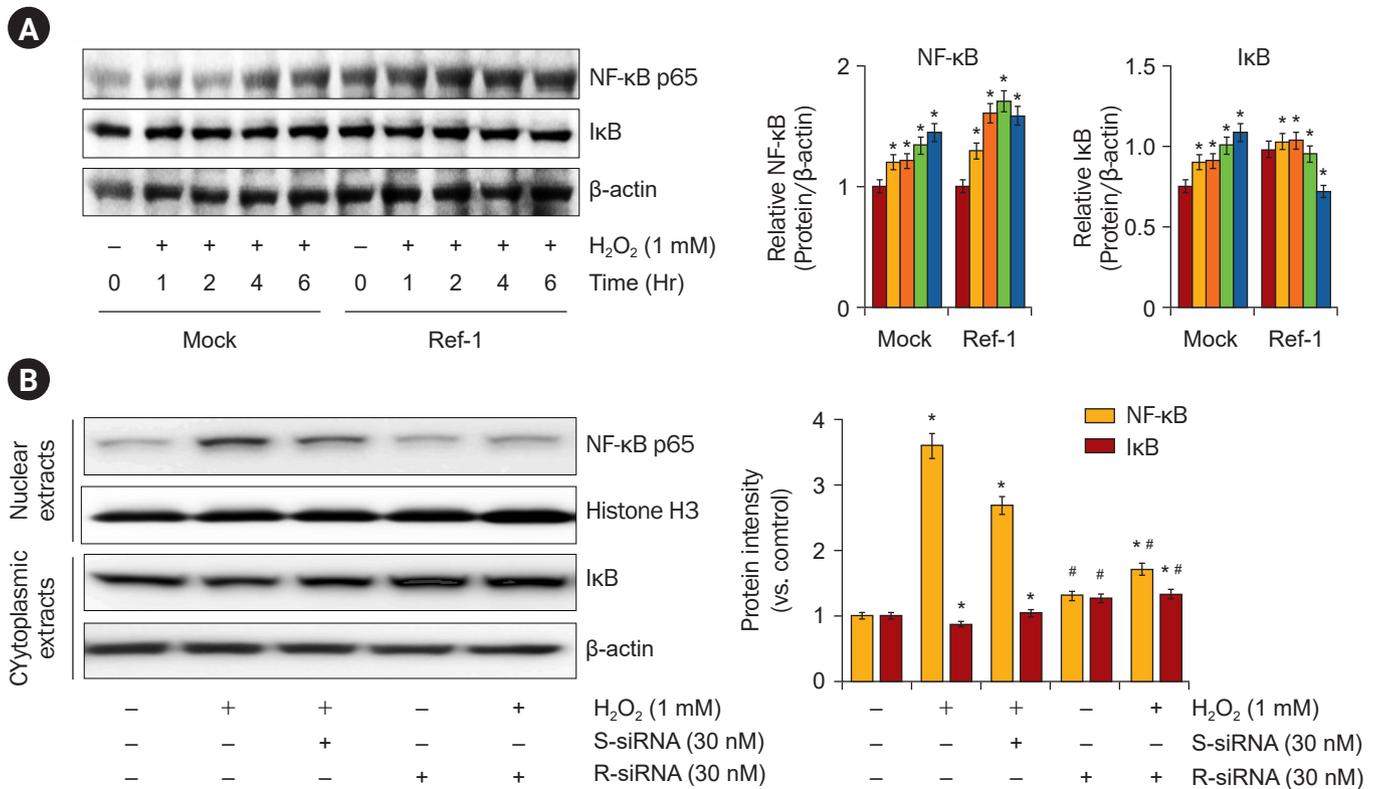


Figure 8. Effects of siRNA of APE1/Ref-1 on the expression of nuclear NF- κ B p65 subunit proteins in HK-2 cells treated with H₂O₂. (A) Time-dependent NF- κ B p65 expression levels were observed in Mock and APE/Ref-1 overexpressing cells after treatment with 1 mM H₂O₂. In the Mock cells, NF- κ B p65 expression increased from 4 hours after H₂O₂ treatment, but it was observed that NF- κ B p65 expression was highly activated in APE/Ref-1 overexpressing cells. (B) The H₂O₂-induced increase in the expression of the NF- κ B p65 subunit in nuclear extracts of HK-2 cells was attenuated by siRNA of APE1/Ref-1.

APE1/Ref-1, apurinic/aprimidinic endonuclease 1/redox factor-1; I κ B, I kappa B; NF- κ B, nuclear factor kappa B; siRNA, small interfering RNA; S-siRNA, scrambled siRNA; R-siRNA, Ref-1 siRNA.

Bars, standard deviation. Data are representative of at least three independent experiments.

* $p < 0.05$ vs. H₂O₂-untreated HK-2 cells, # $p < 0.05$ vs. H₂O₂-treated HK-2 cells in the absence of siRNA of APE1/Ref-1.

different mechanisms [29,30]: 1) increase in APE1/Ref-1 expression after transcriptional activation; 2) re-localization of APE1/Ref-1 from the cytoplasm to the nucleus; and 3) modulation of APE1/Ref-1 posttranslational modifications such as acetylation and phosphorylation. Selenomethionine, a posttranslational modification, is another mechanism through which p53 is stimulated by an APE1/Ref-1-dependent redox mechanism [26].

Oxidative DNA damage induces apoptosis and is involved in the activation of stress response pathways, including JNK and p38 MAPK pathways [31–33]. Previous studies examining the associations of APE1/Ref-1 and MAPKs have demonstrated that these proteins have protective effects against ROS owing to the redox function of

APE1/Ref-1 [34,35]. Therefore, we also hypothesized that increases in APE1/Ref-1 by H₂O₂-induced ROS may be a stress response to modulate the adaptation to oxidative stress. However, when APE1/Ref-1 was knocked down using siRNA, the activation of JNK and p38 decreased. The current data also revealed the transcriptional activation of NF- κ B in APE1/Ref-1-overexpressing cells using promoter assays. These conclusions somewhat differed from those of most studies on APE1/Ref-1, which have evaluated cancer cells or other organs with abnormal cell signaling and survival. Moreover, these discrepancies may also be related to the use of artificial overexpression rather than the natural internal expression by ROS. Alternatively, these results may also be related to the complexity of APE1/Ref-1 signaling,

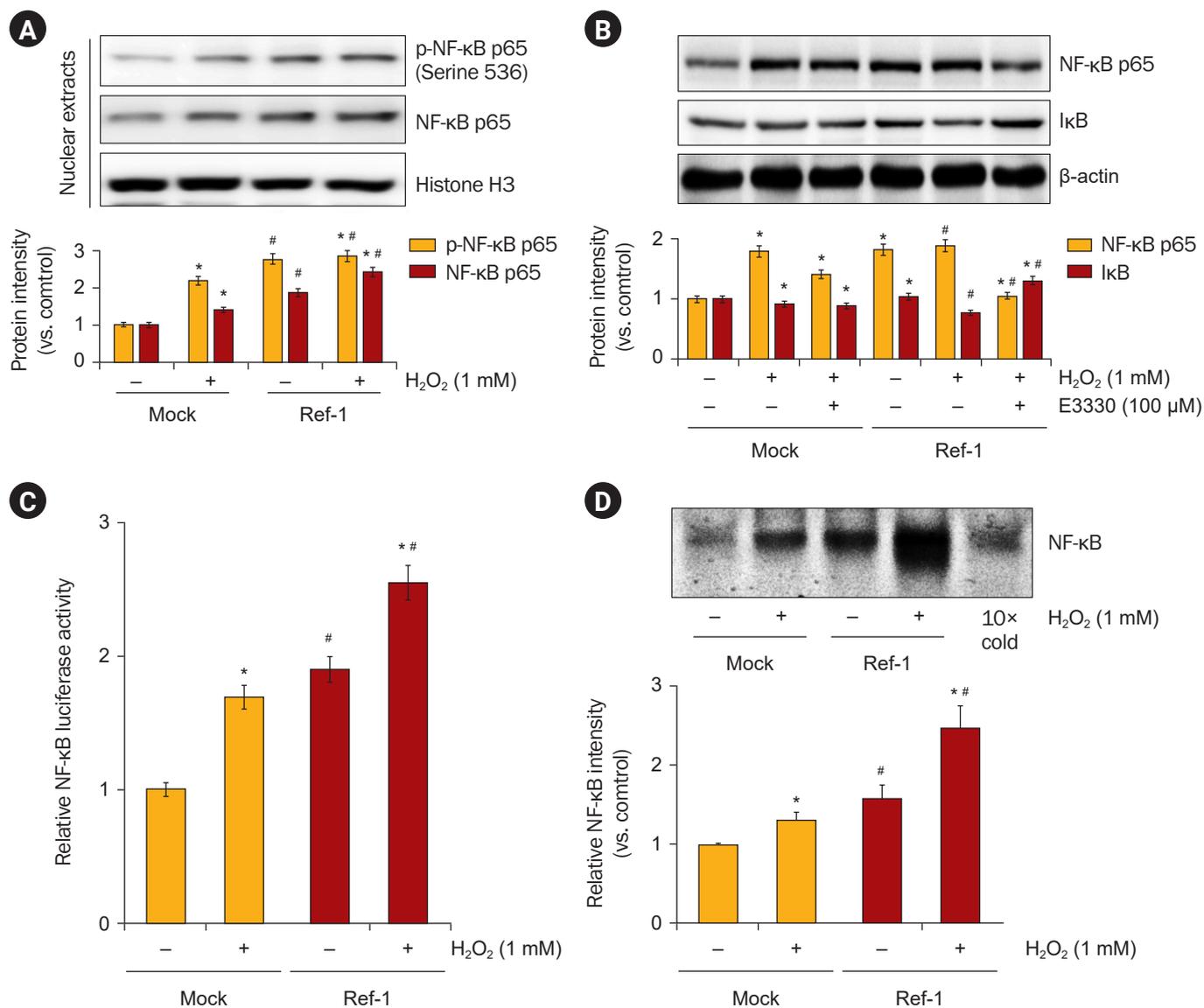


Figure 9. Effects of APE1/Ref-1 on H₂O₂-induced NF-κB activation in HK-2 cells. (A) To observe the activity of NF-κB, the p-NF-κB was observed by extracting nucleoproteins from H₂O₂ treatment and APE/Ref-1 overexpression cells. The p-NF-κB was significantly increased in cells overexpressing APE/Ref-1 treated with 1-mM H₂O₂. (B) The inhibition of NF-κB activity was observed by treatment with E3330, an inhibitor of APE/Ref-1. NF-κB was significantly reduced by treatment with 100 μM E3330. (C) The transcriptional regulation of NF-κB was examined by the transient transfection of an NF-κB promoter-luciferase reporter construct (pGL3-NF-κB). Firefly luciferase activity was normalized to Renilla activity and the relative amount of luciferase activity in the untreated cells. The promoter activity of NF-κB was increased following H₂O₂ exposure in HK-2 cells, and this increase was enhanced by APE1/Ref-1 transfection. (D) Electrophoresis mobility gel shift assay was used to observe whether the overexpression of H₂O₂-treated APE1/Ref-1 increased the binding of nucleoproteins to NF-κB. The expression of NF-κB was also increased in cells treated with 1 mM H₂O₂ and cells overexpressing APE1/Ref-1, but NF-κB transcription factor binding was further increased in cells overexpressing APE1/Ref-1 treated with H₂O₂. APE1/Ref-1, apurinic/apurimidinic endonuclease 1/redox factor-1; NF-κB, nuclear factor kappa B; p-NF-κB, phosphorylation of NF-κB. Data are representative of at least three independent experiments.

*p < 0.05 vs. H₂O₂-untreated Mock cells; #p < 0.05 vs. H₂O₂-treated Mock cells.

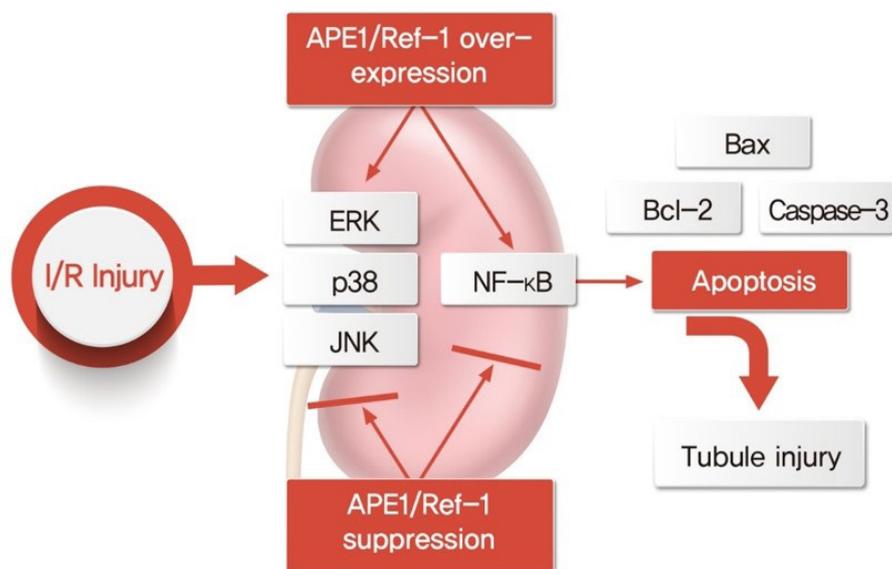


Figure 10. Schematic diagram of tubular injury by APE1/Ref-1 regulation. Overexpression and knockdown of APE1/Ref-1 in the I/R mouse model and in H₂O₂-treated HK-2 cells are associated with apoptosis regulation, suggesting that they act on renal tubular injuries.

APE1/Ref-1, apurinic/aprimidinic endonuclease 1/redox factor-1; Bax, Bcl-2-associated protein X; Bcl-2, B-cell lymphoma 2; ERK, extracellular signal-regulated kinase; I/R, ischemia-reperfusion; JNK, c-Jun N-terminal kinase; NF-κB, nuclear factor kappa B.

which includes various mechanisms and interactions with other proteins and effector genes.

NF-κB is a major transcription factor involved in the synthesis of critical cell survival proteins in response to cellular stress [36,37]. Controversy regarding the dual roles of NF-κB in enhancing or inhibiting apoptosis has been difficult to reconcile [38]. In apoptosis, NF-κB is activated following TNF-α treatment in several cell lines [39]. Moreover, the presence of NF-κB binding sites in genes encoding interleukin-1β converting enzyme protease, c-myc, and TNF-α, which are all involved in apoptosis and cell death, has been demonstrated [1,40]. The current study revealed that H₂O₂ increased APE1/Ref-1 expression and apoptosis and was associated with the activation of transcription factor NF-κB.

In summary, the findings of this study demonstrated that the promoter activity of NF-κB was increased by H₂O₂ exposure in HK-2 cells and that this effect was blocked by siRNA targeting APE1/Ref-1. Furthermore, APE1/Ref-1 sequentially activated the ERK/p38/JNK axis in the apoptotic pathway. These findings demonstrated that APE1/Ref-1 suppression protected HK-2 cells from H₂O₂-induced tubular injury through the inhibition of the promoter activity of the NF-κB promoter. Therefore, APE1/Ref-1 inhibitors

may represent novel therapeutic agents for the treatment of acute kidney injuries, such as I/R-induced kidney injury.

Conflicts of interest

All authors have no conflicts of interest to declare.

Funding

This research was supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF) grant funded by the Korean government, MSIT (NRF-2019R1A2C1003971 and 2020R1A2C1003310) and by a grant (BCRI22040) from the Chonnam National University Hospital Biomedical Research Institute.

Data sharing statement

The data presented in this study are available on request from the corresponding author.

Authors' contributions

Conceptualization: HYK, SWK, EHB

Data curation: HYK, JSP, HSC

Methodology: HYK, JSP

Formal analysis: CSK, SKM

Funding acquisition: HYK

Resources: BHJ, SKM

Writing-original draft: HYK, EHB

Writing-review & editing: All authors

All authors read and approved the final manuscript.

ORCID

Ha Yeon Kim, <https://orcid.org/0000-0002-6661-641X>

Byeong Hwa Jeon, <https://orcid.org/0000-0002-3347-2739>

Hong Sang Choi, <https://orcid.org/0000-0001-8191-4071>

Chang Seong Kim, <https://orcid.org/0000-0001-8753-7641>

Seong Kwon Ma, <https://orcid.org/0000-0002-5758-8189>

Soo Wan Kim, <https://orcid.org/0000-0002-3540-9004>

Eun Hui Bae, <https://orcid.org/0000-0003-1727-2822>

References

- Collins T, Read MA, Neish AS, Whitley MZ, Thanos D, Maniatis T. Transcriptional regulation of endothelial cell adhesion molecules: NF-kappa B and cytokine-inducible enhancers. *FASEB J* 1995;9:899-909.
- Thakur S, Sarkar B, Cholia RP, Gautam N, Dhiman M, Mantha AK. APE1/Ref-1 as an emerging therapeutic target for various human diseases: phytochemical modulation of its functions. *Exp Mol Med* 2014;46:e106.
- Bennett RA, Wilson DM 3rd, Wong D, Demple B. Interaction of human apurinic endonuclease and DNA polymerase beta in the base excision repair pathway. *Proc Natl Acad Sci U S A* 1997;94:7166-7169.
- Tell G, Damante G, Caldwell D, Kelley MR. The intracellular localization of APE1/Ref-1: more than a passive phenomenon? *Antioxid Redox Signal* 2005;7:367-384.
- Chattopadhyay R, Wiederhold L, Szczesny B, et al. Identification and characterization of mitochondrial abasic (AP)-endonuclease in mammalian cells. *Nucleic Acids Res* 2006;34:2067-2076.
- Jackson EB, Theriot CA, Chattopadhyay R, Mitra S, Izumi T. Analysis of nuclear transport signals in the human apurinic/aprimidinic endonuclease (APE1/Ref1). *Nucleic Acids Res* 2005;33:3303-3312.
- Qu J, Liu GH, Huang B, Chen C. Nitric oxide controls nuclear export of APE1/Ref-1 through S-nitrosation of cysteines 93 and 310. *Nucleic Acids Res* 2007;35:2522-2532.
- Choi S, Joo HK, Jeon BH. Dynamic regulation of APE1/Ref-1 as a therapeutic target protein. *Chonnam Med J* 2016;52:75-80.
- Chattopadhyay R, Bhattacharyya A, Crowe SE. Dual regulation by apurinic/aprimidinic endonuclease-1 inhibits gastric epithelial cell apoptosis during Helicobacter pylori infection. *Cancer Res* 2010;70:2799-2808.
- Sengupta S, Mitra S, Bhakat KK. Dual regulatory roles of human AP-endonuclease (APE1/Ref-1) in CDKN1A/p21 expression. *PLoS One* 2013;8:e68467.
- Xanthoudakis S, Miao GG, Curran T. The redox and DNA-repair activities of Ref-1 are encoded by nonoverlapping domains. *Proc Natl Acad Sci U S A* 1994;91:23-27.
- Kakolyris S, Kaklamanis L, Giatromanolaki A, et al. Expression and subcellular localization of human AP endonuclease 1 (HAP1/Ref-1) protein: a basis for its role in human disease. *Histopathology* 1998;33:561-569.
- Barnham KJ, Masters CL, Bush AI. Neurodegenerative diseases and oxidative stress. *Nat Rev Drug Discov* 2004;3:205-214.
- Lee YR, Lim JS, Shin JH, Choi S, Joo HK, Jeon BH. Altered secretory activity of APE1/Ref-1 D148E variants identified in human patients with bladder cancer. *Int Neurourol J* 2016;20:S30-S37.
- Thomson BG, Tritt R, Davis M, Perlman EJ, Kelley MR. Apurinic/aprimidinic endonuclease expression in pediatric yolk sac tumors. *Anticancer Res* 2000;20(6B):4153-4157.
- Stetler RA, Gao Y, Leak RK, et al. APE1/Ref-1 facilitates recovery of gray and white matter and neurological function after mild stroke injury. *Proc Natl Acad Sci U S A* 2016;113:E3558-E3567.
- Evans AR, Limp-Foster M, Kelley MR. Going APE over ref-1. *Mutat Res* 2000;461:83-108.
- Yoo DG, Song YJ, Cho EJ, et al. Alteration of APE1/ref-1 expression in non-small cell lung cancer: the implications of impaired extracellular superoxide dismutase and catalase antioxidant systems. *Lung Cancer* 2008;60:277-284.
- Grösch S, Fritz G, Kaina B. Apurinic endonuclease (Ref-1) is induced in mammalian cells by oxidative stress and involved in clastogenic adaptation. *Cancer Res* 1998;58:4410-4416.
- Xanthoudakis S, Curran T. Identification and characterization of Ref-1, a nuclear protein that facilitates AP-1 DNA-binding activity. *EMBO J* 1992;11:653-665.
- Nishi T, Shimizu N, Hiramoto M, et al. Spatial redox regulation of a critical cysteine residue of NF-kappa B in vivo. *J Biol Chem*

- 2002;277:44548–44556.
22. Cesaratto L, Calligaris SD, Vascotto C, et al. Bilirubin-induced cell toxicity involves PTEN activation through an APE1/Ref-1-dependent pathway. *J Mol Med (Berl)* 2007;85:1099–1112.
 23. Nakshatri H, Bhat-Nakshatri P, Currie RA. Subunit association and DNA binding activity of the heterotrimeric transcription factor NF-Y is regulated by cellular redox. *J Biol Chem* 1996;271:28784–28791.
 24. Huang LE, Arany Z, Livingston DM, Bunn HF. Activation of hypoxia-inducible transcription factor depends primarily upon redox-sensitive stabilization of its alpha subunit. *J Biol Chem* 1996;271:32253–32259.
 25. Xanthoudakis S, Miao G, Wang F, Pan YC, Curran T. Redox activation of Fos-Jun DNA binding activity is mediated by a DNA repair enzyme. *EMBO J* 1992;11:3323–3335.
 26. Gaidon C, Moorthy NC, Prives C. Ref-1 regulates the transactivation and pro-apoptotic functions of p53 in vivo. *EMBO J* 1999;18:5609–5621.
 27. Fan Z, Beresford PJ, Zhang D, et al. Cleaving the oxidative repair protein Ape1 enhances cell death mediated by granzyme A. *Nat Immunol* 2003;4:145–153.
 28. Lee YR, Kim KM, Jeon BH, Choi S. Extracellularly secreted APE1/Ref-1 triggers apoptosis in triple-negative breast cancer cells via RAGE binding, which is mediated through acetylation. *Oncotarget* 2015;6:23383–23398.
 29. Pines A, Bivi N, Romanello M, et al. Cross-regulation between Egr-1 and APE/Ref-1 during early response to oxidative stress in the human osteoblastic HOBIT cell line: evidence for an autoregulatory loop. *Free Radic Res* 2005;39:269–281.
 30. Pines A, Perrone L, Bivi N, et al. Activation of APE1/Ref-1 is dependent on reactive oxygen species generated after purinergic receptor stimulation by ATP. *Nucleic Acids Res* 2005;33:4379–4394.
 31. Chang L, Karin M. Mammalian MAP kinase signalling cascades. *Nature* 2001;410:37–40.
 32. Davis RJ. Signal transduction by the JNK group of MAP kinases. *Cell* 2000;103:239–252.
 33. Johnson GL, Lapadat R. Mitogen-activated protein kinase pathways mediated by ERK, JNK, and p38 protein kinases. *Science* 2002;298:1911–1912.
 34. Montaldi AP, Godoy PR, Sakamoto-Hojo ET. APE1/REF-1 down-regulation enhances the cytotoxic effects of temozolomide in a resistant glioblastoma cell line. *Mutat Res Genet Toxicol Environ Mutagen* 2015;793:19–29.
 35. Hong J, Chen Z, Peng D, et al. APE1-mediated DNA damage repair provides survival advantage for esophageal adenocarcinoma cells in response to acidic bile salts. *Oncotarget* 2016;7:16688–16702.
 36. Lin A, Karin M. NF-kappaB in cancer: a marked target. *Semin Cancer Biol* 2003;13:107–114.
 37. Yamamoto Y, Gaynor RB. Role of the NF-kappaB pathway in the pathogenesis of human disease states. *Curr Mol Med* 2001;1:287–296.
 38. Lipton SA. Janus faces of NF-kappa B: neurodestruction versus neuroprotection. *Nat Med* 1997;3:20–22.
 39. Manna SK, Mukhopadhyay A, Aggarwal BB. Resveratrol suppresses TNF-induced activation of nuclear transcription factors NF-kappa B, activator protein-1, and apoptosis: potential role of reactive oxygen intermediates and lipid peroxidation. *J Immunol* 2000;164:6509–6519.
 40. Beg AA, Baldwin AS Jr. The I kappa B proteins: multifunctional regulators of Rel/NF-kappa B transcription factors. *Genes Dev* 1993;7:2064–2070.