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Synergistic inhibition of mesothelioma cell growth by the combination of clofarabine and resveratrol involves Nrf2 downregulation

Yoon-Jin Lee[1,2], Jae-Hyuk Im[3], David M. Lee[4], Ji-Sung Park[3], Seong Youn Won[2], Moon-Kyun Cho[2], Hae-Seon Nam[2], Yong-Jin Lee[1] & Sang-Han Lee[1,3,]*

[1]Soonchunhyung Environmental Health Center for Asbestos, [2]Division of Molecular Cancer Research, Soonchunhyang Medical Research Institute and [3]Department of Biochemistry, College of Medicine, Soonchunhyang University, Cheonan 330-090, Korea, [4]Cell Biology and Genetics in Biological Sciences, College of Computer, Mathematical, and Natural Sciences, University of Maryland, College Park, MD 20742, USA

We previously reported that MSTO-211H cells have a higher capacity to regulate Nrf2 activation in response to changes in the cellular redox environment. To further characterize its biological significance, the response of Nrf2, a transcription factor that regulates ARE-containing genes, on the synergistic cytotoxic effect of clofarabine and resveratrol was investigated in mesothelioma cells. The combination treatment showed a marked growth-inhibitory effect, which was accompanied by suppression of Nrf2 activation and decreased expression of heme oxygenase-1 (HO-1). While transient overexpression of Nrf2 conferred protection against the cytotoxicity caused by their combination, knockdown of Nrf2 expression using siRNA enhanced their cytotoxic effect. Pretreatment with Ly294002, a PI3K inhibitor, augmented the decrease in HO-1 level by their combination, whereas no obvious changes were observed in Nrf2 levels. Altogether, these results suggest that the synergistic cytotoxic effect of clofarabine and resveratrol was mediated, at least in part, through suppression of Nrf2 signaling. [BMB Reports 2012; 45(11): 647-652]

INTRODUCTION

Malignant pleural mesothelioma (MPM) is an asbestos-related tumor arising from the mesothelial surface of the pleural cavity. The highly aggressive behavior of this tumor results in a poor prognosis, and the median survival is 9-12 months after diagnosis. At this time, there are only a few effective chemotherapeutic options for treatment of MPM, including cisplatin, vinorelbin, and gemcitabine; however, a majority of patients experience a relapse and ultimately die of the disease (1). Although the precise molecular mechanism(s) underlying chemoresistance in MPM remains controversial, dysregulation of cell death signaling has been implicated as a significant contributor to the chemoresistance (2). MPM is highly resistant to apoptosis. It is likely for this reason that chemotherapy has had very little success in improving survival in patients who develop MPM. Hence, new strategies to enhance the apoptotic signal and overcome resistance to therapeutic drugs are necessary to improve MPM therapy.

The nuclear factor E2-related factor 2 (Nrf2) plays a vital role in the prevention of cell dysfunction in response to oxidative stress and in protection against toxic and carcinogenic exposure, through antioxidant response element (ARE)-mediated expression of phase II detoxifying and antioxidant enzymes (3). The Nrf2 protein is sequestrated by its cytoplasmic partner, Kelch-like ECH-associated protein 1 (Keap1). Regulation of Nrf2 nuclear shuttling is mainly based on the interaction of reactive oxygen species (ROS) or electrophiles with one or more of the multiple cysteines on Keap1, which results in a conformational change that liberates Nrf2 (4). In several types of human cancers, recent studies have demonstrated that high Nrf2 expression results in enhanced resistance to toxic effects of chemotherapeutic drugs and that suppression of endogenous Nrf2, either by transfecting Nrf2-specific siRNA or overexpressing Keap1, renders these cells more susceptible to therapy (5, 6). A number of Nrf2 downstream target genes, such as HO-1, Prx1, GCL and Trx, may also contribute to the observed Nrf2-dependent chemoresistance and cancer promotion (7). These observations suggest that the Nrf2 signal pathway may function as a cell survival pathway that protects cancer cells against drug-induced cell death. Therefore, if Nrf2 inhibitors as adjuvant to chemotherapeutic drugs can be identified to maximize cancer cells death, this could have significant therapeutic potential. We recently found that human mesothelioma MSTO-211H cells regulate Nrf2 level at multiple

*Corresponding author. Tel: +82-41-570-2442; Fax: +82-41-579-6080; E-mail: m1037624@sch.ac.kr
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steps, including de novo transcription, protein synthesis, and posttranslation (8). Although a higher capacity of MSTO-211H cells in Nrf2 regulation may provide a selective advantage for survival, the possibility that Nrf2 plays a protective role against other chemotherapeutic drugs has not been investigated in mesothelioma cells.

Based on these findings, the present study was designed to investigate the combined effects of chemotherapeutics clofarabine and chemopreventive agent resveratrol on MSTO-211H cells and the role of Nrf2 in protecting cells against injury.

RESULTS

Intracellular Nrf2 levels and cell viability in resveratrol or sulforaphane-treated cells

The sensitivity of MSTO-211H cells to resveratrol and sulforaphane was examined using the XTT assay after exposure to these agents for 48 h. Exposure of cells to two compounds resulted in reduction of cell viability in a time- and dose-dependent manner (Fig. 1A and B). Resveratrol treatment reduced the cell viability more strongly than treatment with sulforaphane. The IC_{20} calculated from values measured directly after 24 h treatment with resveratrol and sulforaphane was 22.5 μM and 67.3 μM, respectively, which decreased after post-treatment for 48 h to 16.1 μM and 26.7 μM, respectively. We used 15 μM as an effective concentration of resveratrol in further studies on Nrf2 induction. The treatment of cells with sulforaphane for 24 h markedly increased the Nrf2 protein level, but resveratrol only minimally increased the Nrf2 protein content when compared with the untreated control (Fig. 1C).

Effects of clofarabine and resveratrol, alone and in combination, on cell viability and Nrf2 induction

To evaluate the efficacy of resveratrol or sulforaphane as a chemo-potentiator in combination with the chemotherapeutic agent clofarabine on the growth of MSTO-211H cells, we treated the cells with two phytochemicals and clofarabine, alone and in combination. After treatment with subtoxic doses of clofarabine (40 nM) and resveratrol (15 μM) for 48 h, cell growth inhibition was enhanced by a combination of the two, resulting in 50.01% inhibition compared with the additive effect of the agents (26.8%). In contrast, combination with clofarabine and sulforaphane was neither synergistic nor additive (Data not shown).

To investigate whether the synergistic inhibition of mesothelioma cell growth by the combination of clofarabine and resveratrol is associated with the Nrf2 activity, we assessed the effect of resveratrol and clofarabine, alone or in combination, on the nuclear accumulation of Nrf2 protein and its expression. The transcriptional activity of Nrf2 was confirmed by measuring the level of HO-1 protein, a target gene product for Nrf2. Fig. 2B clearly demonstrates that nuclear localization of Nrf2 was potently inhibited by their combination, whereas it was increased slightly in cells treated with clofarabine or resveratrol alone. Similar findings were also observed in experiment using whole cell lysates. In time-response experiment, the decrease of Nrf2 protein level was evident at 8 h after the com-

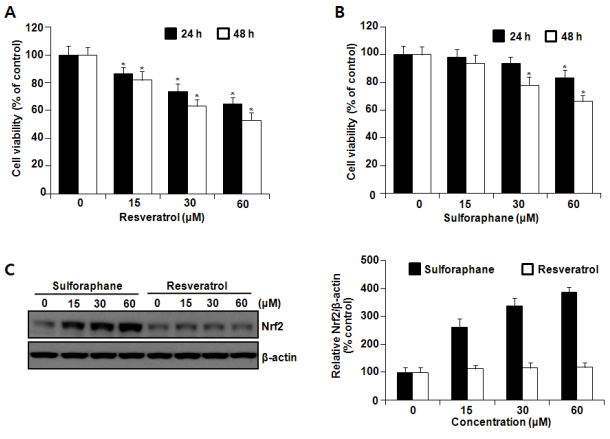

Fig. 1. Effects of resveratrol and sulforaphane on cell viability and Nrf2 levels. MSTO-211H cells were treated with various concentrations (0-60 μM) of resveratrol and sulforaphane for 48 h. (A and B) The percentage viability was then determined by using the XTT assay. (C) Cell lysates were analyzed by immunoblotting with anti-Nrf2 antibody. The normalized intensity of Nrf2 versus β-actin is presented. Error bars represent the mean ± SEM for three independent experiments. *P < 0.05 compared with respective controls.

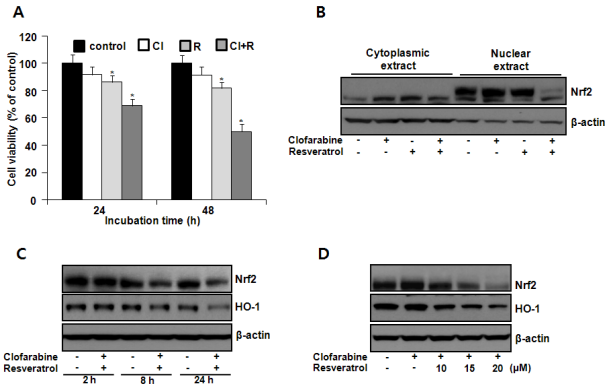

Fig. 2. Effects of clofarabine and resveratrol on cell viability and Nrf2 levels. MSTO-211H cells were treated with clofarabine (40 nM) and resveratrol (15 μM), alone or in combination, for the indicated times. (A) The percentage viability was then determined by using the XTT assay. Cl, clofarabine; R, resveratrol. (B) Nuclear and cytoplasmic extracts and (C) Whole cell lysates after 24 h of treatment were then analyzed by immunoblotting. (D) Cells were treated with clofarabine (40 nM) and resveratrol (10, 15, and 20 μM) for 24 h, after which whole lysates were analyzed by immunoblotting. Error bars represent the mean ± SEM for three independent experiments. *P < 0.05 compared with respective controls.

bined treatment and augmentation lasted for at least 24 h. Such effect was accompanied by a decrease in HO-1 level (Fig. 2C). In combination with clofarabine, the ability of resveratrol to reduce the amount of Nrf2 and HO-1 proteins was also evident in the dose-dependent experiment (Fig. 2D).

Effect of clofarabine and resveratrol, alone or in combination, on cell viability in Nrf2 knockdown or overexpressed cells

To investigate the biologic relevance between Nrf2 and drug resistance, we examined whether Nrf2 overexpression or knockdown affected sensitivity to combined treatment. When flag-Nrf2-transfected cells were treated with clofarabine and resveratrol in combination, the cell viability was significantly increased compared with that of cells transfected with the empty vector (Fig. 3A). Transfection of flag-Nrf2 markedly increased flag-Nrf2 level, which was accompanied by up-regulation of HO-1 protein expression (Fig. 3B). These results clearly showed that ectopically expressed flag-Nrf2 has significant biological activity as a transcriptional factor and increases the resistance of MSTO-211H cells to the combination treatment. Next, to knockdown the endogenous Nrf2 level, cells were transiently transfected with control siRNA and an Nrf2-specific siRNA. As shown in Fig. 3C, when cells transfected with Nrf2-specific siRNA were treated with clofarabine and resveratrol in combination, cell viability was significantly decreased to approximately 65% compared with that of control siRNA (78%), and expression of endogenous Nrf2 and HO-1 proteins was also effectively suppressed (Fig. 3D).

Involvement of the PI3K/Akt signaling in cytotoxicity induced by the combination of clofarabine and resveratrol

To further clarify the possible upstream antioxidative signaling pathway involved in the down-regulation of the Nrf2 and HO-1 proteins, we examined whether the combination of clofarabine and resveratrol could modulate the activation of Akt and Erk. In the time-response experiment, Akt phosphorylation was found to be inhibited during the first 30 min after treatment, and then remained decreased below control levels throughout 24 h (Fig. 4A). Pretreatment with Ly294002, a PI3K inhibitor, augmented the decrease in HO-1 level by their combination, whereas no obvious changes were observed in Nrf2 levels. However, PD98059, a MEK inhibitor, slightly increased the Nrf2 level (Fig. 4B). Next, we investigated whether the supplementation of antioxidant NAC protected cells against synergistic cytotoxic effect of clofarabine and resveratrol. As shown in Fig. 4C, pretreating the cells with NAC 1 h before the combination treatment enhanced cell viability from approximately 54.1% of that of control cells at 72 h treatment to 67.7%.

DISCUSSION

The chemopreventive properties of resveratrol are mediated by modulating numerous cell signaling pathways, which regulate cell survival or apoptosis (9). Among its wide range of bio-

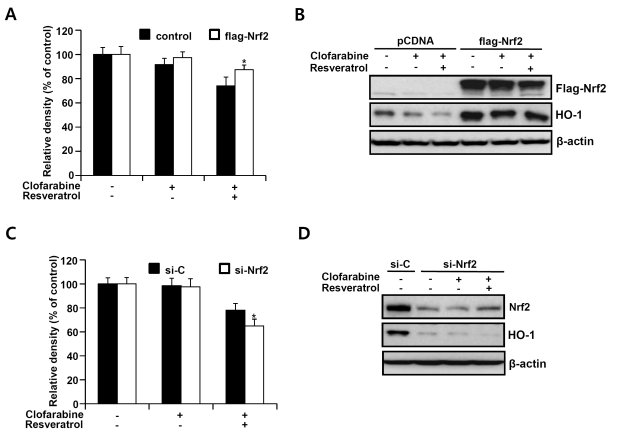

Fig. 3. Effects of Nrf2 overexpression or knockdown on cell viability and Nrf2 level. MSTO-211H cells were transfected with expression vectors for flag-Nrf2 or pCDNA3 as a control for 24 h prior to incubation with clofarabine (40 nM) and resveratrol (15 μM), alone or in combination, for an additional 24 h, after which they were processed for XTT assay (A) or immunoblotting (B). Cells were transfected with 10 nM Nrf2-targeting siRNA (si-Nrf2) or Stealth RNAi control (si-C) for 24 h prior to incubation with clofarabine (40 nM) and resveratrol (15 μM), alone or in combination, for an additional 24 h, after which they were processed for XTT assay (C) or immunoblotting (D). *P < 0.05 compared with respective controls.

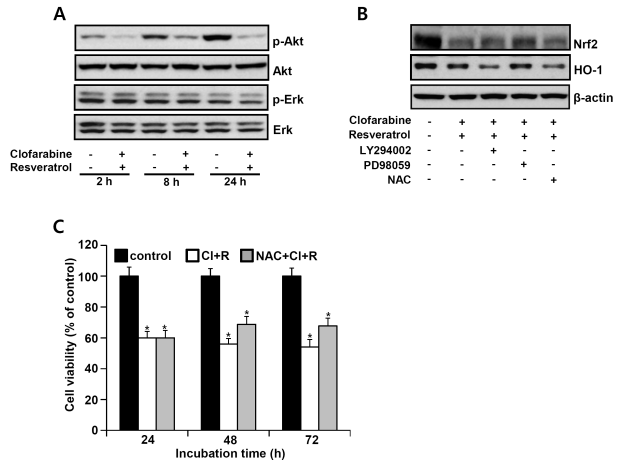

Fig. 4. Effects of clofarabine and resveratrol on Akt and Erk1/2 phosphorylation. (A) MSTO-211H cells were treated with clofarabine (40 nM) and resveratrol (15 μM) for the indicated times. (B) Cells were pretreated with or without Ly294002 (20 μM), PD98059 (50 nM), and NAC (5 mM) for 1 h prior to combined treatment for 24 h. Cell lysates were analyzed by immunoblotting using anti-p-Akt, anti-p-Erk, anti-Nrf2, and anti-HO-1 antibodies. (C) Cells were treated with or without NAC (5 mM) for 1 h prior to the combination treatment for 24 h. The percentage of viable cells was then determined by the trypan blue exclusion assay. *P < 0.05 compared with respective controls.

logical activities, the antioxidative role of resveratrol has been well characterized in many studies and is most likely associated with its ability to induce antioxidant molecules. However, enhancement of antioxidative capacity by resveratrol may also produce untoward effects that favor the survival of cancer cells and the development of drug resistance since it may undermine the free radical mechanisms of chemotherapy. Therefore, identification of specific compounds that target the Nrf2-dependent protective response can be one of the ways to overcome chemoresistance and to enhance the efficacy of therapy.

Previously, we reported that MSTO-211H cells have a higher capacity to increase the amount and activity of Nrf2 through a ROS-mediated pathway in response to changes in the cellular redox environment (8). In the present study, we found that resveratrol was even more effective in inducing cytotoxicity than sulforaphane in MSTO-211H cells, whereas it was less effective in up-regulating the Nrf2 protein level. Based on this finding, we hypothesized that the difference in cell viability after treatment with resveratrol and sulforaphane might arise from the difference in the abilities to induce Nrf2 expression between two compounds. Accumulating data in cancer studies indicate that Nrf2 is directly involved in the resistance against various drugs, such as cisplatin in lung cancer cells (10), cisplatin and paclitaxel in endometrial cancer (5), and 5-fluorouracil (5-FU) in esophageal squamous cancer (11). Interestingly, many recent reports have shown that in addition to its chemopreventive properties, resveratrol can sensitize cancer cells to a number of chemotherapeutic agents. Combined treatment of 5-FU and resveratrol resulted in a significant decrease in long-term cell survival by inducing a further increase in oxidative stress and inhibiting Akt and STAT3 proteins (12). In another study, resveratrol exhibited a synergistic effect when given with chemotherapeutic agents and reversed multidrug resistance in human oral epidermoid carcinoma cells (13). Thus, it is possible that resveratrol may be one of the agents used in combination with chemotherapy to enhance their cytotoxicity to cancer cells. In accordance with these studies, our data showed that resveratrol, in combination with clofarabine, synergistically increased the cytotoxicity of MSTO-211H cells. This may partly be due to the down-regulation of Nrf2 and the chemoresistant factors modulated by Nrf2. Many Nrf2 downstream genes have been shown to contribute to the observed Nrf2-dependent chemoresistance (7, 14). For instance, treatment of the pancreatic cancer cells with gemcitabine or radiation strongly induced HO-1 expression and enhanced expression was found to be closely associated with the development of cellular resistance to therapy (14).

In this study, we observed that the combined treatment of clofarabine and resveratrol inhibited the Nrf2 signaling pathway by reducing the nuclear localization of Nrf2 and decreasing the Nrf2 and HO-1 protein levels. It is possible that suppression of Nrf2 activation by the combination treatment is mediated by a mechanism that inhibits its stabilization as well as the regulation via its synthesis, thus decreasing the intracellular level of Nrf2 and subsequent transactivation of its downstream target genes. Transient overexpression of Nrf2 protected cells against the cytotoxicity caused by the combination of two agents and its knockdown led to the opposite effects. This is in agreement with an earlier finding that the activation of Nrf2 promoted the survival of cancer cells under a deleterious environment, and that the suppression of Nrf2 activity inhibited cellular proliferation and reduced resistance to the anticancer drugs (10). We also found that Nrf2 overexpression or knockdown potently elicited up-regulation or down-regulation of HO-1 expression, respectively. These findings imply that HO-1 induction in MSTO-211H cells is dependent on Nrf2 signaling, supporting the notion that the HO-1 gene is primarily regulated at the transcriptional level, and its inducibility is linked to the transcription factor Nrf2 (15). Although it is unclear to what extent Nrf2-driven HO-1 expression accounts for the chemoresistance phenomenon, these findings suggest that suppression of Nrf2-dependent protection is one of the mechanism by which resveratrol produced a synergistic inhibitory effect on cell proliferation when used in combination with clofarabine.

Here, we noted the PI3K/Akt signal pathway as a key pathway necessary for cell survival. It has been reported that Akt is constitutively activated in mesothelioma cells (16). Other studies have shown that Akt activity is required for Nrf2-driven HO-1 expression (8) and its inhibition sensitizes chemoresistant mesothelioma cells to various drugs (17), implying a functional link between Akt-mediated chemoresistance and Nrf2. Indeed, Nrf2 is known to be activated by phosphorylation, thereby facilitating the translocation of Nrf2 to the nucleus for its transcriptional activity (18). This prompted us to study the effect of the combined treatment of clofarabine and resveratrol on Akt expression and activity. Expression of the Akt protein was not affected but its phosphorylated form decreased with the combined treatment. Chemical blockage of PI3K/Akt signaling with Ly294002 augmented a decrease in HO-1 level caused by their combination, whereas no obvious changes were observed in Nrf2 levels. This finding suggests the role of the Nrf2-independent pathway in the regulation of HO-1 expression. Similarly, there was evidence for up-regulation of HO-1 expression independent of Nrf2 in response to oleanolic acid (19). Thus, the combined treatment of clofarabine and resveratrol seems to induce downregulation of HO-1 protein via Nrf2-dependent and -independent mechanisms.

In this study, pretreatment with NAC significantly prevented cytotoxicity induced by the combination of clofarabine and resveratrol. This result suggests that impairment of cellular redox capacity to counteract oxidative injury may contribute to cytotoxicity caused by the combined treatment. Ramanathan et al. mentioned that the upregulation of the antioxidant capacity in cancer cells is associated with increased resistance to chemotherapeutic agents (20). Supporting this idea, meso-

thelioma cells containing the highest MnSOD activity has been known to exhibit high resistance to oxidants such as menadione and H2O2, and to cytotoxic drugs, such as epirubicin (21). These findings can partially explain why synergistic inhibition of MSTO-211H cell growth by clofarabine and resveratrol involved suppression of Nrf2 activity and antioxidant NAC supplementation protected cells against cytotoxicity.

In summary, clofarabine and resveratrol cooperatively exerted cytotoxic effects in MSTO-211H cells. The observed synergism was mediated, at least in part, through suppression of the Nrf2 and PI3K/Akt signalings. Although additional studies are necessary to elucidate the mechanism of their synergism, our data suggest the potential importance of Nrf2 targeting on enhancing chemotherapeutic potentials in mesothelioma. A more thorough understanding of the molecular mechanisms underlying chemoresistance in MPM may ultimately improve treatment outcomes for this deadly disease.

MATERIALS AND METHODS

Cell culture and treatment

The human mesothelioma cell line, MSTO-211H, was obtained from the American Type Culture Collection and maintained in RPMI-1640 medium supplemented with 10% FBS, 1 mM glutamine, 100 units of penicillin/ml and 100 μg of streptomycin/ml. Cells were grown to 70% confluence in a monolayer culture in this medium for 24 h before treatment. Except for NAC, resveratrol, sulforaphane, and other inhibitor compounds were dissolved in DMSO. The final concentration of DMSO in culture medium was 0.1% (v/v).

Cell viability assay

Cell viability was measured using the Cell Proliferation kit II (XTT) according to the manufacturer's instruction (Roche Diagnostics). Briefly, cells were seeded in 96-well microtiter plates and then treated with resveratrol and/or clofarabine (Sigma-Aldrich Co) at various concentrations. After incubation, 50 μl of the XTT labeling mixture was added to each well and incubated for an additional 4 h. The formazan dye that formed was measured spectrophotometrically at 450 nM using a Glomax multi detection system (Promega). Statistical comparisons were performed using one-way analysis of variance (ANOVA) followed by a Tukey's post hoc correction for multiple comparisons using SPSS version 17.0 (SPSS Inc.). Data were expressed as the mean ± SEM. Significant differences were considered with values of $P < 0.05$.

Preparation of nuclear and cytoplasmic extracts

Nuclear extracts were prepared according to the instructions provided in the NE-PER® nuclear and cytoplasmic extraction kit (Pierce). Briefly, cells were resuspended in 10 vol of CER I solution, after which they were incubated in a CER II solution on ice for 1 min and homogenized. Nuclei were recovered by centrifugation at 14,000 rpm for 5 min, and the supernatant was kept as the cytoplasmic extract. The nuclear fraction was extracted for 40 min on ice in NER solution. After centrifugation, the supernatant was used as the nuclear extract.

Transient transfection with Nrf2 plasmid and siRNA

RNA interference of Nrf2 was performed using an Nrf2-specific siRNA duplex from Invitrogen (Cat# 12990). Briefly, cells were seeded in 6-well and 96-well plates and transfected at 50% confluency with siRNA duplex using lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's recommendations. Cells transfected with the Stealth RNAi negative control duplex (Invitrogen) were used as controls. For transient expression experiments, cells were transfected with expression vectors for flag-Nrf2 and pCDNA3 (Invitrogen) as a control. After 24 h of transfection, cells were treated with DMSO or with various concentrations of resveratrol and clofarabine.

Western blot analysis

Cell lysates were prepared using RIPA buffer. Proteins (40 μg per lane) were separated on NuPAGE 4-12% bis-tris polyacrylamide gels (Invitrogen) and then electrophoretically transferred to Immuno-Blot PVDF membranes. The membranes were incubated for 2 h at room temperature with a 1:500 dilution of anti-Nrf2 (Santa Cruz Biotechnology), anti-HO-1 (Stressgen Biotechnologies), anti-p-Akt, and anti-p-Erk antibodies (Cell Signaling Technologies). Next, HRP-conjugated secondary antibody was applied at a dilution of 1 : 5,000 and the signal was visualized using an ECL detection kit (Santa Cruz Biotechnology). The blots were then stripped and re-probed with anti-Akt, anti-Erk, or anti-β-actin antibodies as loading controls.

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