

## Ferulic acid induces heme oxygenase-1 *via* activation of ERK and Nrf2

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**ABSTRACT:** This study investigated the effect of ferulic acid (FA) on the up-regulation of heme oxygenase-1 (HO-1) in lymphocytes and the molecular mechanisms involved. Lymphocytes were treated with FA (0.001-0.1  $\mu$ M) for certain times. Cell viability, the activity and level of expression of HO-1, and signal pathways were analyzed. FA significantly up-regulated HO-1 expression both at the level of mRNA and protein in lymphocytes. Moreover, FA induced NF-E2-related factor (Nrf2) nuclear translocation and transcriptional activity, which is upstream of FA-induced HO-1 expression. In addition, lymphocytes treated with FA exhibited activation of extracellular regulated kinase (ERK) and treatments with U0126 (an ERK kinase inhibitor) attenuated the FA-induced activation of Nrf2, resulting in a decrease in HO-1 expression. Zinc protoporphyrin (ZnPP, a HO-1 inhibitor) markedly suppressed cytoprotection from radiation-induced cell damage by FA. Results suggested that the ERK signaling pathway controlled the anti-oxidation of FA by regulating the expression of the antioxidant enzyme HO-1.

**Keywords:** Ferulic acid, radiation, oxidation, heme oxygenase-1

### 1. Introduction

Ionizing radiation is known to induce oxidative stress through generation of reactive oxygen species (ROS), resulting in an imbalance of pro-oxidant and anti-oxidant activity and ultimately resulting in cell death (1,2). There has been considerable public and scientific interest in the use of phytochemicals derived from dietary components to combat oxidative stress (3,4). Ferulic acid (FA) is a phytochemical commonly found in many herbs used in

traditional Chinese medicine (5). FA exhibits a wide range of therapeutic effects against various diseases like cancer, diabetes, and cardiovascular and neurodegenerative diseases (6,7). A wide spectrum of benefits to human health has been attributed to this phenolic compound, at least in part, because of its strong antioxidant activity (8). FA effectively scavenges superoxide anion radicals and inhibits lipid peroxidation (9). It possesses antioxidant properties by virtue of the phenolic hydroxyl group in its structure (10). The hydroxy and phenoxy groups of FA donate electrons to quench free radicals (10). The phenolic radical in turn forms a quinone methide intermediate, which is excreted *via* bile (7).

Recent studies have highlighted the important biological effects of heme oxygenase-1 (HO-1) reaction products that have antioxidant functions (11). HO-1 is a crucial factor in the response to oxidative injury, a major result of which is the degradation of heme to biliverdin, iron, and carbon monoxide (12). Upregulation of many phase-2 detoxifying and antioxidant enzymes, including HO-1, is mediated by antioxidant response elements (AREs) (13). NF-E2-related factor (Nrf2) is responsible for the activation of ARE-driven antioxidant gene expression (14). Recent studies have demonstrated that the activation of the mitogen-activated protein kinases (MAPKs) contributes to the induction of HO-1 and the modulation of ARE-driven gene expression *via* Nrf2 activation (15).

A previous report by the current authors showed FA was a nontoxic and effective radioprotectant in animal studies (16). FA has been found to protect lymphocytes from radiation-induced early damage (17), and this effect may be mediated by scavenging ROS (18). However, the detailed mechanism of FA's role in protection from radiation-induced cellular damage needs to be clarified. This study investigated the effect of FA on the up-regulation of HO-1 in lymphocytes and the molecular mechanisms involved.

### 2. Materials and Methods

#### 2.1. Drugs and reagents

FA (purity > 99%) was purchased from the Chinese National Institute for the Control of Pharmaceutical and Biological

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Products (Beijing, China). [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium]bromide (MTT), zinc protoporphyrin (ZnPP), and U0126 were purchased from Sigma-Aldrich (St. Louis, MO, USA). Antibodies against Nrf2, extracellular regulated kinase (ERK), phospho-ERK, and  $\beta$ -actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies against Nrf2 and HO-1 were purchased from BioTek (Winooski, VT, USA). A reverse transcription (RT) system and polymerase chain reaction (PCR) system were purchased from Takara Biotechnology (Dalian) (Dalian, Liaoning, China). Polyvinylidene difluoride membranes, and enhanced chemiluminescence kits were purchased from Amersham (Arlington Heights, IL, USA). RPMI 1640 medium and fetal bovine serum were purchased from Invitrogen (Carlsbad, CA, USA). Trizol was purchased from Invitrogen (Carlsbad, CA, USA). Nuclear and cytoplasmic extraction kits and electrophoretic mobility shift assay (EMSA) kits were from Pierce (Rockford, IL, USA). Other analytically pure reagents were from Promega (Madison, WI, USA).

## 2.2. Cell culture and treatment

AHH-1 cells, kindly donated by Dr. Ping-Kun Zhou (Beijing Institute of Radiation Medicine, Beijing, China), were cultured routinely in RPMI 1640 medium, supplemented with 10% heat inactivated fetal bovine serum, in a humidified atmosphere of 5% CO<sub>2</sub> at 37°C. The cells were then uniformly irradiated at room temperature with a <sup>60</sup>Co  $\gamma$  source (Beijing Institute of Radiation Medicine, Beijing, China) at a dose rate of 2.51 Gy/min.

## 2.3. Cell viability analysis

An MTT assay was used to determine the effect of FA on the viability of AHH-1 cells. Cells were seeded at a density of  $1 \times 10^5$  cells/well in 96-well plates, with the indicated concentrations of FA, and then subjected to  $\gamma$ -irradiation at 3 Gy 12 h later. Cells were then cultured for 48 h, 20  $\mu$ L of MTT was added to each well, and cells were further incubated for 4 h allowing the conversion of MTT into formazan crystals. After centrifugation, the supernatant was aspirated, 200  $\mu$ L dimethyl sulfoxide was added to each well to dissolve the formazan crystals, and the absorbance was read at 540 nm with a Victor 1420 Multilabel Counter (Wallac, Turku, Finland). The reduction in optical density (OD) caused by radiation and drug treatment was used as a measurement of cell viability, normalized to cells incubated in control medium, that was considered to be 100%.

## 2.4. HO-1 assay

An HO-1 assay was performed with an ELISA Kit by adding 0.1 mL volume of culture medium to a 96-well micro-titration plate coated with HO-1 antibody and incubating those plates for 90 min at 37°C. The liquid

in the wells was discarded and plates were treated with 0.1 mL of biotinylated antibody for HO-1 for 60 min at 37°C. Plates were washed with PBS three times, 0.1 mL of streptavidin-peroxidase solution was added, and plates were incubated for 30 min at 37°C. After plates were washed with PBS five times, 0.09 mL tetramethylbenzidine was added, and plates were incubated for 18 min at 37°C. The reaction was then terminated with stop buffer. The absorbance was measured at 450 nm with a microplate reader after incubation for 5 min at room temperature, and wells without cells served as blanks. The amount of HO-1 secreted from cells was expressed as the mean concentration quantified by the binding activity of antibodies.

## 2.5. Semiquantitative RT-PCR analysis

RT-PCR analysis was performed by modifying a previously described technique (19). At certain timepoints, cells were collected and total RNA was extracted according to the manufacturer's instructions. For RT-PCR analysis, 1  $\mu$ g total RNA was reverse-transcribed using RT-PCR kits. PCR was used to amplify target cDNA with the following conditions: 32 cycles of 94°C for 45 sec, 55°C for 45 sec, and 72°C for 45 sec. The following primers and predicted sizes of PCR products were used: 5-GCAGAGGGTGATAGAAGAGG-3(sense) and 5-GTAAGGACCCATCGGAGAA-3(antisense) for HO-1; 5-TCATTGACCTCAACTACATG-3(sense) and 5-CAAAGTTGTCATGGATGACC-3(antisense) for GAPDH.

## 2.6. Western blotting assay

Western blotting analysis was performed by modifying a previously described technique (20). After treatment, the cells were washed twice in PBS and suspended in a lysis buffer and then placed on ice for 30 min. The supernatant was collected after centrifugation at  $15,000 \times g$  for 20 min at 4°C. Whole lysates (50  $\mu$ g) were resolved on a 10% sodium dodecyl sulphate-polyacrylamide gel. The fractionated proteins were electrophoretically transferred to an immobilon polyvinylidene difluoride membrane and probed with the appropriate antibodies. The blots were developed using an enhanced chemiluminescence kit. In all immunoblotting experiments, the blots were reprobbed with anti-actin antibody, which served as a control for protein loading.

## 2.7. EMSA assay

Nuclear extracts were prepared using a nuclear and cytoplasmic extraction kit, and EMSAs were carried out using a lightshift chemiluminescent gel shift kit in accordance with the manufacturer's instructions. Briefly, oligonucleotide 5'-TTTTCTGCTGACTCAAGGTCCG-3' containing the ARE binding site was biotin-

labeled using a 3'-end labeling kit and the DNA probe was incubated with 2  $\mu$ g of the nuclear extract at room temperature for 20 min. The protein-DNA complexes were then separated on a 6% polyacrylamide gel and electrically transferred to a nylon membrane for chemiluminescence band detection. The specificity of the binding was examined using competition experiments, in which a 200-fold excess of the unlabeled oligonucleotide with the same sequence was added to the reaction mixture prior to the addition of the biotin-labeled oligonucleotide.

### 2.8. Plasmid construction

A plasmid to harbor the ARE-promoter was constructed by subcloning the sequence "CCG CTC GAG GTG ACA AAG CAC CCG TGA CAA AGC ACC CGT GAC AAA GCA CCC GTG ACA AAG CAC CCG AAG CTT GGG" to PGL4 vector *via* restriction enzyme sites of *Xho* I and *Hind* III. The orientation of all constructs was verified using sequencing and restriction analysis.

### 2.9. Transient transfection of ARE promoter and luciferase assay

Cells were sub-cultured at a density of  $1 \times 10^5$  cells in 96-well plates to maintain approximately 60-80% confluence. The cells were transiently transfected with the plasmid harboring the ARE-promoter using the transfection reagent in accordance with the manufacturer's instructions. After transfection for 6 h at 37°C, cells were supplied with new culture media with FA (0.001-0.1  $\mu$ M) and then incubated overnight. Cells were washed three times with PBS and lysed with reporter lysis buffer. Twenty  $\mu$ L of cell extract was mixed with 100  $\mu$ L of the luciferase assay reagent at room temperature, and the mixture was placed in a luminometer to measure the light produced.

### 2.10. Statistical analysis

All experiments were performed at least three times. The data were presented as means  $\pm$  S.E.M. (standard error of the mean). Statistical comparison of groups was done with one-way variance analysis and a least significant difference *t* test using SPSS 16.0. The significance level was defined as  $p < 0.05$ .

## 3. Results

### 3.1. Effect of FA on HO-1 expression and activity

HO-1 plays an important role in the course of the cellular defense against oxidative stress, so this study investigated whether the antioxidant activity of FA is related to HO-1 expression. AHH-1 cells exposed to FA for 24 h showed a concentration-dependent increase in

HO-1 protein expression (Figure 1A). The enhanced HO-1 activity correlated with increased HO-1 expression (Figure 1E). Treatment of cells with 0.1  $\mu$ M FA resulted in a time-dependent enhancement of HO-1 mRNA and protein expression (Figures 1B and 1D) with a similar increase in HO-1 activity (Figure 1F).

### 3.2. FA increased the transcriptional activity of Nrf2, nuclear translocation, and ARE-binding

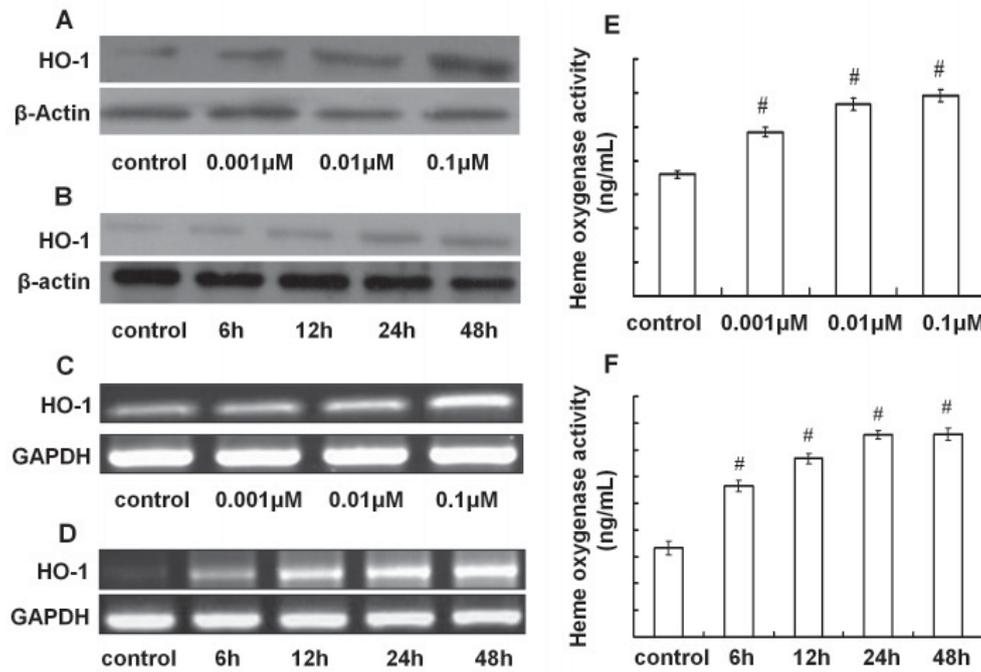
Several investigators have defined Nrf2 as a major transcription factor regulating ARE-driven phase 2 gene expression. Therefore, this study attempted to determine whether FA activated Nrf2 in association with its HO-1 up-regulation. Activation of Nrf2 was determined by Western analysis of nuclear extracts from AHH-1 cells treated with different doses of FA. As shown in Figure 2A, FA treatment caused increased Nrf2 accumulation in the nuclear fraction. To elucidate the role of Nrf2 in transcriptional activation of ARE, EMSA was performed using the oligonucleotide harboring the HO-1 specific ARE sequence. Incubation of the nuclear extract from AHH-1 cells after FA treatment (0.1  $\mu$ M) with biotin-labeled ARE oligonucleotide resulted in the enhanced ARE-binding activity of Nrf2 (Figure 2B). To verify the functional relevance of Nrf2 binding to the ARE sequence of HO-1, an ARE-promoter construct containing the Nrf2 binding DNA consensus site linked to a luciferase reporter gene was used. As illustrated in Figure 2C, FA increased the transcriptional activity of Nrf2. These results further suggested that Nrf2 mediates the FA-induced activation of the HO-1 promoter.

### 3.3. FA activates Nrf2 via phosphorylation of ERK

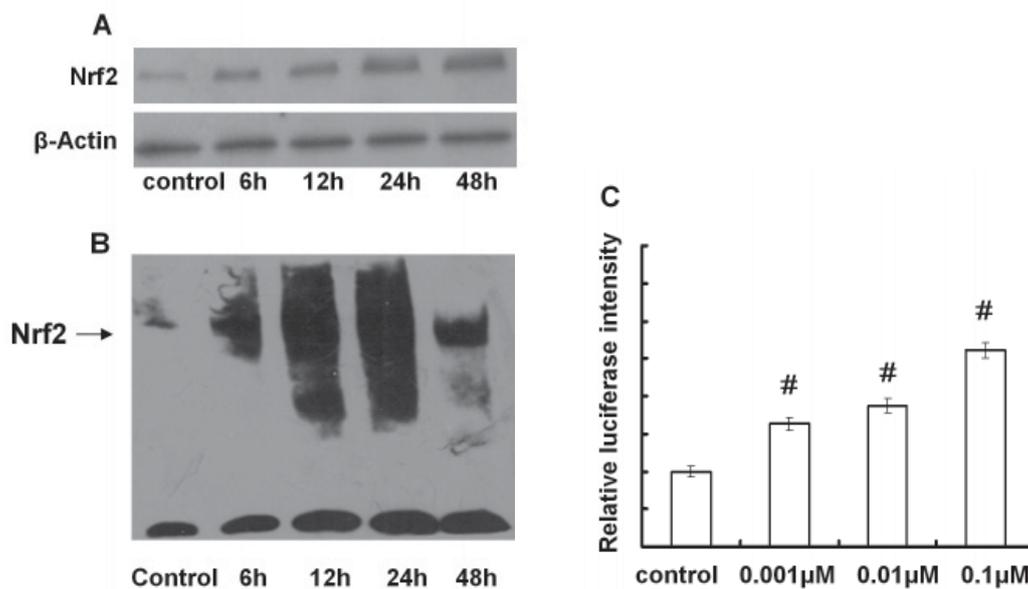
To further elucidate the upstream signaling pathway involved in FA mediated Nrf2 activation and induction of HO-1, this study examined the activation of ERK, a major signal molecule involved in cell survival despite oxidative stress. Results showed that FA enhanced ERK phosphorylation, and U0126, a specific ERK kinase inhibitor, inhibited this activation (Figure 3A). U0126 treatment resulted in marked suppression of FA, causing HO-1 induction (Figure 3B) and the nuclear accumulation of Nrf2 (Figure 3C). Similarly, U0126 inhibited the transcriptional activity of Nrf2 (Figure 3D).

### 3.4. Effect of FA on cell damage induced by oxidative stress

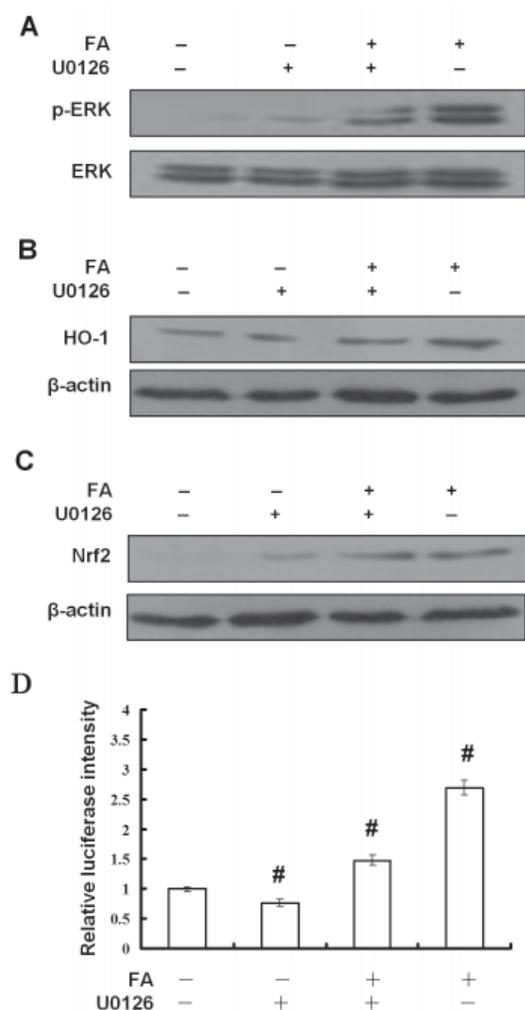
The protective effect of FA from oxidative stress was examined in AHH-1 cells irradiated with  $\gamma$ -rays. Cells were pretreated with FA for 24 h before being irradiated with 3 Gy of radiation. Cell viability was determined with an MTT assay 48 h later. FA increased cell survival in a dose-dependent manner (Figure 4A). In order to ascertain whether increased HO-1 activity due to FA is



**Figure 1.** Effect of FA on HO-1 mRNA, protein expression, and activity in AHH-1 cells. **(A)** Effect of FA on HO-1 protein expression in a concentration-dependent manner. AHH-1 cells were treated for 24 h with the indicated concentrations of FA and the expression of HO-1 was detected with specific antibody. **(B)** Effect of FA on HO-1 protein expression at the times indicated. AHH-1 cells were treated with FA at a concentration of 0.1  $\mu$ M and the expression of HO-1 protein was detected with specific antibody at the times indicated. **(C)** Effect of FA on mRNA of HO-1 in a concentration-dependent manner. AHH-1 cells were treated for 24 h with the indicated concentrations of FA and the mRNA of HO-1 was detected by RT-PCR. **(D)** Effect of FA on mRNA of HO-1 at the times indicated. AHH-1 cells were treated with FA at a concentration of 0.1  $\mu$ M and the mRNA of HO-1 was detected at the times indicated. **(E)** Effect of FA on HO-1 activity in a concentration-dependent manner. HO-1 activity was measured at 24 h after exposure to various concentrations of FA. Each bar represents the mean  $\pm$  S.E.M. in three experiments. # Significantly different from control ( $p < 0.05$ ). **(F)** Effect of FA on HO-1 activity at the times indicated. Cells were treated with 0.1  $\mu$ M FA and HO-1 activity was measured at the times indicated. Data are expressed as the means  $\pm$  S.E.M. in triplicate experiments. # Significantly different from control ( $p < 0.05$ ).

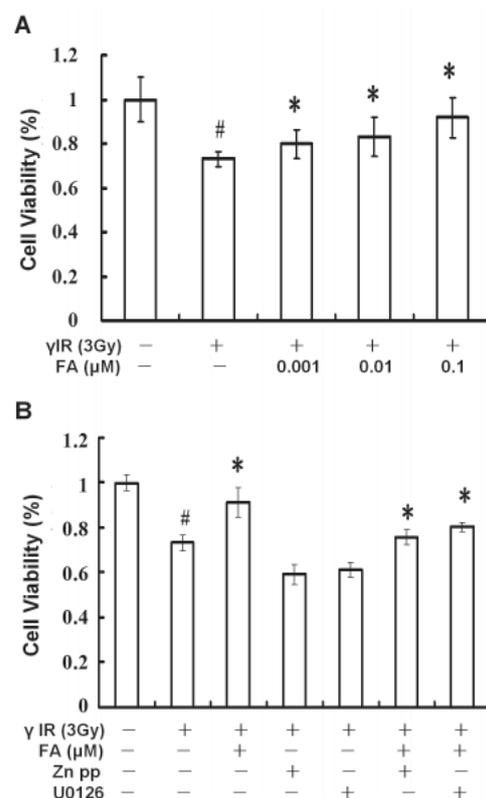


**Figure 2.** Effect of FA on Nrf2 expression, its translocation into the nucleus, and its transcriptional activity in AHH-1 cells. **(A)** Effect of FA on the protein levels of nuclear Nrf2. Nuclear extract was prepared from AHH-1 cells treated with 0.1  $\mu$ M FA for the times indicated. **(B)** Effect of FA on the ARE-binding activity of Nrf2 in AHH-1 cells. Nuclear extract was prepared from AHH-1 cells treatment with 0.1  $\mu$ M FA for the times indicated. **(C)** Effect of FA on the transcriptional activity of ARE in AHH-1 cells. AHH-1 cells were transfected with an ARE-luciferase construct or control vector. After standing overnight, cells were treated with FA, cell lysates were mixed with a luciferase substrate, and luciferase activity was measured with a luminometer. The control group transfected with control vector and treated without FA. Data are expressed as the means  $\pm$  S.E.M. in triplicate experiments. # Significantly different from control ( $p < 0.05$ ).



**Figure 3. Induction of HO-1 and activation of Nrf2 by FA via phosphorylation of ERK.** (A) Effect of U0126 on FA-induced phospho-ERK expression. After treatment with U0126, cell lysates were electrophoresed, and ERK and phospho-ERK were detected with specific antibodies. (B) Effect of U0126 on FA-induced HO-1 expression. Cells were incubated with 0.1  $\mu$ M FA in the absence or presence of U0126 for 4 h. Cell lysates were electrophoresed and HO-1 was detected with specific antibody. (C) Effect of U0126 on FA-induced Nrf2 expression. Nuclear extract was prepared from AHH-1 cells treated with 0.1  $\mu$ M of FA in the absence or presence of U0126 for 4 h and subjected to Western blotting to measure Nrf2 protein expression. (D) Effect of U0126 on FA-induced transcriptional activity of ARE in AHH-1 cells. AHH-1 cells were transfected with the ARE-luciferase construct. After standing overnight, cells were treated with FA in the absence or presence of U0126, cell lysates were mixed with a luciferase substrate, and luciferase activity was measured with a luminometer. Data are expressed as the means  $\pm$  S.E.M for three experiments. # Significantly different from untreated cells ( $p < 0.05$ ).

related to cytoprotection from oxidative stress, AHH-1 cells were pretreated with ZnPP, the inhibitor of HO-1. The protective effect of FA was attenuated (Figure 4B), illustrating that HO-1 induction may be partly determined by the protective effect of FA. Furthermore, cytoprotection from radiation-induced cytotoxicity by FA was reduced by U0126 (Figure 4B), suggesting the ERK signaling plays a role in FA-mediated HO-1 gene induction and cytoprotection.



**Figure 4. Protective effect of FA against radiation-induced cell damage.** (A) Effect of FA on cell damage induced by  $\gamma$ -ray irradiation. AHH-1 cells were treated with 0.001, 0.01, and 0.1  $\mu$ M FA, followed 24 h later by  $\gamma$ -irradiation at 3 Gy. After 48 h, cell viability was assessed by the MTT assay. #  $p < 0.05$  compared to the value with the control, \*  $p < 0.05$  compared to the value with 3 Gy radiation alone. (B) AHH-1 cells were pre-incubated with 0.1  $\mu$ M FA for 24 h and HO-1 ZnPP or U0126 for 4 h, followed by exposure to 3 Gy irradiation. Cell viability was assessed by the MTT assay. #  $p < 0.05$  compared to the value with the control, \*  $p < 0.05$  compared to the value with 3 Gy radiation alone.

#### 4. Discussion

Heme oxygenase is a rate-limiting catalyst in the degradation of heme to biliverdin, which is further converted to the antioxidant bilirubin by biliverdin reductase, free iron, and carbon monoxide (12). Three HO isozymes have been identified with distinct genes. Of these, HO-1, a stress-response protein, can be induced by various oxidative-inducing agents. Numerous *in vitro* and *in vivo* studies have shown that the induction of HO-1 is an important mechanism of cellular protection from oxidative injury (21). HO-1-mediated cytoprotection has been shown to be critical for tissues that were vulnerable to oxidative stress (12). Therefore, HO-1 is considered an important target of a number of chemopreventive and cytoprotective agents (11). Growing evidence suggests that HO-1 provides cytoprotection, so modulation of HO-1 expression by a pharmacological agent may represent a novel method of therapeutic intervention. In particular, the identification of a non-cytotoxic inducer of HO-1 may maximize the intrinsic antioxidant potential of cells. The current study demonstrated that

FA significantly up-regulated HO-1 expression both at the level of mRNA and protein in lymphocytes and that this increased expression was accompanied by a gradual increase in HO-1 activity.

Nrf2 is an important transcription factor that regulates ARE-driven gene expression. Nrf2 plays a key role in the transcriptional regulation of *HO-1* gene expression through interaction with ARE (22). Increase nuclear accumulation of Nrf2 plays a key role in the transcriptional regulation of the *HO-1* gene expression through interaction with ARE (23,24). Under normal physiologic conditions, Nrf2 is sequestered in the cytoplasm as an inactive complex with its repressor Keap 1 (25). Upon stimulation by inducers, however, Nrf2 dissociates from Keap 1 and translocates into the nucleus, where it dimerizes with cofactors like small Maf protein and binds to ARE. This leads to mass activation of highly specialized proteins, including HO-1 and other antioxidant enzymes (13). The current study found that FA induced nuclear translocation of Nrf2 and its ARE binding and transcriptional activity in AHH-1 cells. This study is the first to demonstrate that FA induced the expression of HO-1 in AHH-1 cells, at least in part, through activation of Nrf2 signaling.

Numerous studies have demonstrated that protein phosphorylation is a potential mechanism for the activation of Nrf2-ARE-mediated pathways. Several cytosolic kinases, including PKC, PI3K, and MAPK, have shown to modify Nrf2 and to be potentially involved in Nrf2-mediated signal transduction at AREs (25). Furthermore, the MAPK pathway activated by ERK is reported to be a signaling pathway for Nrf2 activation. ERK is reported to phosphorylate Nrf2, which may facilitate the release of Nrf2 from the Keap1-Nrf2 complex and trigger nuclear translocation of Nrf2. There, it forms a heterodimer with small Maf protein (25). Treatment with U0126 prior to exposure to FA reduced Nrf2 nuclear translocation and transcriptional activity of Nrf2, suggesting that Nrf2 might be a direct downstream target of ERK. The present results indicate that the ERK pathway may be a critical component for the activation of Nrf2 in the cellular signaling network, which mediates the transcriptional regulation of *HO-1* gene expression in AHH-1 cells.

In summary, the present results suggest that FA up-regulates HO-1 expression and activity. In addition, evidence indicates that ERK kinases act to mediate the up-regulation of HO-1 by FA. These results offer new insights into the antioxidative mechanisms of FA. Further studies are needed to clarify the molecular mechanisms underlying FA-induced activation of Nrf2 and subsequent induction of HO-1.

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