Ferulic Acid Protects Human Umbilical Vein Endothelial Cells from Radiation Induced Oxidative Stress by Phosphatidylinositol 3-Kinase and Extracellular Signal-Regulated Kinase Pathways

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Ferulic acid (FA) has been demonstrated to have a remarkable antioxidant activity, the mechanism of FA of protecting human umbilical vein endothelial cells (HUVECs) from radiation induced oxidative stress was investigated in the present study. The oxidative protection of FA was assessed by cellular glutathione (GSH) content, nicotinamide adenine dinucleotide phosphate (NADPH) levels, and reactive oxygen species (ROS) analysis. Nuclear factor erythroid 2-related factor 2 (Nrf2) nuclear translocation was detected using Western blotting. The upstream signaling pathway involved in FA mediated Nrf2 activation was determined by signaling inhibitors. FA significantly increased the transcription of antioxidant related genes such as GCLC (glutamate-cysteine ligase catalytic subunit), GCLM (glutamate-cysteine ligase regulatory subunit), NQO1 (NADPH quinone oxidoreductase-1) and heme oxygenase-1 (HO-1) mRNA in radiated cells, and these changes involved in a significant increase of the intracellular GSH content and the expression of NAPDH. FA evidently promoted Nrf2 translocation into nuclei and increased the intracellular GSH and NAPDH levels in radiated cells. Phosphatidylinositol 3-kinase (PI3K) and extracellular signal regulated kinase (ERK) pathways were associated with FA-induced Nrf2 activation. The results suggested that FA-induced Nrf2 activation play key role in cytoprotective effect of FA against oxidative stress via PI3K and ERK signaling pathways.

Key words ferulic acid; radiation; nuclear factor erythroid 2-related factor 2; oxidation

Chronic oxidative stress plays pivotal role in radiation-induced tissue dysfunction, associated with cell inflammation and vascular injury. It has long been recognized that the damaging effects of ionizing radiation are brought about by both direct and indirect mechanisms. The direct action produces disruption of sensitive molecules in the cells, whereas the indirect effects result from its interaction with water molecules, which results in the production of reactive oxygen species (ROS) and their subsequent action on subcellular structures, and antioxidants have been used in preventing or mitigating the severity of radiation-induced late effects. Protection against ROS-mediated cell injury requires maintenance of endogenous thiol pools, most importantly, by reduced glutathione (GSH) and reduced nicotinamide adenine dinucleotide phosphate (NADPH). GSH provides the reducing equivalents necessary for the conversion of hydrogen peroxide and lipid peroxides to water and lipid alcohols, respectively. GSH also plays an important role in protection against oxidation of protein sulfhydryl groups. The rate-limiting reaction in GSH biosynthesis is catalysed by glutamate-cysteine ligase (GCL), which consists of a catalytic subunit (GCLC) and a regulatory subunit (GCLM). NADPH is of crucial importance for protection tissues against oxidative injuries. NAPDH preserves cellular redox potential by providing reducing equivalents for glutathione reductase and thioredoxin reductase, which maintain glutathione and thioredoxin in the biologically reduced state.

Protection against ROS-mediated cell injury could be achieved through the induction of phase 2 detoxifying enzymes and antioxidant enzymes such as glutathione S-transferase, NADPH quinone oxidoreductase-1 (NQO1), and heme oxygenase-1 (HO-1), a process that is mediated mainly by the antioxidant response element (ARE) within the promoter regions of these genes. Nuclear factor erythroid 2-related factor 2 (Nrf2) plays a key role in ARE-mediated gene expression. Under normal condition, Nrf2 is sequestered in the cytoplasm by an actin-binding protein, Kelch-like ECH associating protein 1 (Keap1), and upon exposure of cells to inducers such as oxidative stress, Nrf2 dissociates from Keap1, translocates to the nucleus, binds to AREs, and transactivates phase 2 detoxifying and antioxidant genes. Nrf2–ARE pathway plays a pivotal role in the cellular defense against the cellular damage caused by ROS.

Ferulic acid (FA), a phenolic phytochemical found in many traditional Chinese medicine, is a strong membrane antioxidant and known to positively affect human health. It effectively scavenges superoxide anion radical and inhibits the lipid peroxidation because of its strong antioxidant activity. FA is also used as a radioprotector during radiotherapy to stimulate hematopoiesis, immunity and DNA repair in irradiated mice. Although radiation protection and ROS protection of FA have received considerable attention, little is known about the mechanism of FA on ROS protection, and a better understanding of this may provide important insights into radiation prevention. We therefore tested the ability of FA to protect oxidation and its mechanism. The present study demonstrated that FA-induced Nrf2 activation is the major regulatory pathway of cytoprotective gene expression against oxidative stress via phosphatidylinositol 3-kinase (PI3K) and extracellular signal regulated kinase (ERK) signaling pathways.
MATERIALS AND METHODS

Reagents and Materials  FA (purity >99%) was purchased from Chinese National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). SP600125 (a c-Jun N-terminal kinase (JNK) inhibitor), PD98059 (an ERK inhibitor), and SB203580 (a p38 inhibitor) were purchased from Calbiochem (La Jolla, CA, U.S.A.). LY294002 (a PI3K inhibitor), Staurosporine (a protein kinase C (PKC) inhibitor), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). Antibody against Nrf2 was from Biotek, antibody against actin, Lamin B was purchased from Santa Cruz Biotechnology, U.S.A. The Reverse Transcription System and the polymerase chain reaction (PCR) system were purchased from Promega (Madison, WI, U.S.A.).

Cell Cultures and Radiation  Human umbilical vein endothelial cells (HUVECs) were obtained from Cascade Biologies (Portland, OR, U.S.A.) as cryopreserved primary cultures, and grown in culture flasks in endothelial cell growth medium M200 (Cascade Biologies) supplemented with 2% LSGS (low serum growth supplement; Cascade Biologies) according to the Cascade Biologies’ recommended protocol. The growth medium was changed every other day until cells reached confluence. Cells of passages 3 and 4 were grown in 10 Gy of 60Co source (dose rate of 1.64 Gy/min), and then uniformly radiated at room temperature with FA in serum free medium for the indicated period. After reaching 80—90% confluence on a 96-well plate, cells were washed once with serum-free medium and then incubated with FA in serum free medium for the indicated periods. During the last 30 min of incubation, 10 mmol/l 2,7'-dichlorofluorescein diacetate (DCFH-DA) (Sigma) was added to the culture plate at an emission wavelength of 535 nm and an excitation wavelength of 490 nm with a multwell microplate reader.

Assessment of Cell Viability  For evaluation of cytotoxicity, cells were seeded at 5000 cells/well into 96-well culture plates and grown for 24 h. The cells were incubated with various concentrations of the agents in serum-free medium for 48 h, and then 20 µl of MTT was added to each well and further incubated for 4 h allow the conversion of MTT into formazan crystals, then, after washing with phosphate-buffered saline (PBS), the cells were lysed with dimethyl sulfoxide (DMSO), and the absorbance at 570 with a VictorI 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, which were considered 100%.

Measurement of Intracellular ROS Levels  Intracellular ROS were assessed with the ROS-sensitive fluorophore 2,7'-dichlorofluorescein diacetate (DCFH-DA) (Sigma). After reaching 80—90% confluence on a 96-well plate, cells were washed once with serum-free medium and then incubated with FA in serum free medium for the indicated periods. During the last 30 min of incubation, 10 mmol/l 2,7'-dichlorofluorescein (DCF) was added to the culture plate at an emission wavelength of 535 nm and an excitation wavelength of 490 nm with a multwell microplate reader.

Assay for Cellular Glutathione Content  Intracellular GSH levels were determined by a glutathione reductase-coupled 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) assay. Briefly, each cell pellet was sonicated in 150 ml PBS. Lysates were mixed with 2.5% metaphosphoric acid and centrifuged at 14000 g for 5 min. The supernatant fraction was diluted 20-fold with assay buffer. In each well, diluted samples were mixed with DTNB and glutathione reductase. After 5 min incubation, NADPH were added to each well and the initial reaction rates were measured at 405 nm by the microtiter plate reader. Several concentrations of pure GSH were also assayed in the same plate to establish a calibration curve for calculation of GSH content in the samples. The concentration of GSH was expressed as nanomoles per milligram protein.

Measurement of NADPH Levels  NADPH was measured by a modification of the technique described previously. In brief, cell extracts are separated into three aliquots (A1, A2, A3). A1 is untreated, A2 is treated with glucose-6-phosphate dehydrogenase (G6PDH) that converts all of the NADP+ to NADPH, A3 is treated with glutathione reductase that converts all of the NADPH to NADP+, and then the absorbance at 340 nm is measured. A1—A3 is the NADPH content of the extract.

RNA Isolation and Reverse Transcription-Polymerase Chain Reaction (RT-PCR)  At the indicated times, the cells were collected and total RNA was extracted using Trizol (Invitrogen, Carlsbad, U.S.A.) according to the manufacturer’s instructions. For RT-PCR analysis, 1 µg total RNA was reverse-transcribed using RT-PCR kits (Promega, Madison, WI, U.S.A.) and then amplified with specific primers. The following primers with the predicted size were used for amplification: Nrf2: (F) 5'-GGC AGG AGA AGA GTA TGA TGT-3', (R) 5'-GTG GGC AGA TCC ACT GGT TT-3'; NQO1: (F) 5'-GGG TGG TTT GAG AGA GTG-3', (R) 5'-GTC GCC TGG AAT AGA TGT TTT-3'; HO-1: (F) 5'-CAG CAC TAC TGA AAG CTT CCT CTC C-3', (R) 5'-AGT GCT GAT CTG GGA TTT TCC T-3'; GCLM: (F) 5'-ACC TGG CCT CCT GCT GTG TG-3', (R) 5'-CTG CGG TGA GCT GTG GGT GT-3'; GCLC: (F) 5'-ACC TGC AGA CCG GGA ACC TG-3', (R) 5'-CGC AGT AGC CAC AGA GGC ACC-3'; glyoxaldehyde-3-phosphate dehydrogenase (GAPDH): (F) 5'-CCA TCA TTC ACA ACA CGA GTG TTA-3', (R) 5'-CAA AGT TGT CAT GGA TGA CC-3'. The cycle number was determined from a linear amplification curve as being within the linear amplification range. The PCR conditions were as follows: 28 cycles of 94 °C for 45 s, 55 °C for 45 s and 72 °C for 45 s. Amplification products were visualized on 1.5% agarose gel and visualized by ethidium bromide staining and quantified densitometrically (GAPDH as a loading control) using Quantity One software (Bio-Rad, Hercules, CA, U.S.A.).

Preparation of Nuclear Extract and Nrf2 Analysis  Nuclear and cytosolic extracts from HUVECs were prepared using a nuclear and cytoplasmic extraction kit (Pierce, Rockford, U.S.A.), the cells were harvested at the indicated times, and were then lysed with cytoplasmic lysis buffer, after 5 min of centrifugation at 16000 g, the supernatant was harvested as cytosolic protein extracts, and the pellets were re-suspended in nuclear extraction buffer and incubated on ice for 50 min, and centrifuged at 16000 g for 10 min, and the supernatant was then harvested as nuclear protein extracts. Nrf2 levels in nuclear and cytosolic were determined by Western
Western Immunoblotting Western blot analysis was performed by a modification of the technique described previously. The cells were harvested at the indicated times and suspended in a lysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 0.1% sodium dodecyl sulfate (SDS), 0.5% sodium deoxycholate, 100 μg/ml phenylsulfonyl fluoride, 2 μg/ml apro- tinin, 1 μg/ml pepstatin, and 10 μg/ml leupeptin), and placed on ice for 30 min. The supernatant was collected after centrifugation at 15000 g for 20 min at 4 °C. The protein concentration was determined using the Bio-Rad protein assay (Bio-Rad Lab, Hercules, CA, U.S.A.) with bovine serum albumin (BSA) as the standard. The whole lysates (50 μg) were resolved on a 10% SDS-polyacrylamide gel. The fractionated proteins were electrophoretically transferred to an immobilon polyvinylidene difuride membrane (Amersham, Arlington Heights) and probed with the appropriate antibodies. The blots were developed using an enhanced chemoluminescence (ECL) kit (Amersham).

Statistical Analysis The data are presented as mean±S.E.M., and statistical comparisons between groups were performed using 1-way ANOVA followed by the Student t-test.

RESULTS

Effects of FA on ROS Production Induced by Radiation
To examine whether FA could affect ROS production induced by radiation, we measured intracellular ROS levels using DCFH-DA. As shown in Fig. 1, radiation significantly enhanced ROS production, while FA significantly attenuated radiation induced ROS production in a concentration-dependent manner. FA alone treated cells exposed to sham radiation did not show significantly effect on ROS production. As shown in Fig. 2, the time of incubation and concentration of FA used in these experiments had no effect on the viability as determined by MTT assay.

Effects of FA on Radiation-Induced Redox Status in Radiated HUVECs
To clarify whether the effect of FA was related to the alteration of intracellular redox status, we examined intracellular GSH and NADPH levels in HUVECs. As shown in Fig. 3, intracellular GSH and NADPH levels was significantly down-regulated by radiation, pretreatment with FA for 18 h provided significant protection against down-regulation of intracellular GSH and NADPH levels caused by radiation in a concentration-dependent manner. While FA alone treated cells exposed to sham radiation did not show significantly effect on GSH and NADPH levels.

Effects of FA on Nrf2 Translocation and Nrf2-Mediated Genes in Radiated HUVECs
To determine the molecular mechanism of FA on intracellular GSH and NADPH, the effect of FA on Nrf2 translocation was examined. Confluent HUVECs were pre-incubated for 18 h with the indicated concentrations of FA, the cells were exposed to radiation and cultured for 1 h, and the levels of Nrf2 protein in the cytosolic and the nuclei were detected by Western blotting. As shown in Fig. 4, Nrf2 protein level in cytosolic decreased by FA in a concentration-dependent manner, while Nrf2 protein level in nuclei increased by FA in a concentration-dependent manner. This result demonstrated FA evidently promoted

Fig. 1. Effects of FA on Gamma-Radiated Induced Intracellular ROS Generation in HUVECs
Confluent HUVECs were pre-incubated for 18 h with the indicated concentrations of FA. The cells were then exposed to gamma-radiated and cultured for 1 h. ROS were quantitatively analyzed as described in Materials and Methods. The ratio of ROS generation is indicated on the ordinate and related to the value for the control with no additives.

* p<0.05 significantly different from control cells not treated with radiation or FA, ## p<0.05 significantly different from gamma-irradiated cells not treated with FA.

Con=control; Rad=radiation.

Fig. 2. Effects of FA on Cell Viability as Determined by MTT Assay
Confluent HUVECs were pre-incubated for 18 h with the indicated concentrations of FA. The cells were then exposed to gamma radiation and cultured for 30 h. Cell viability was determined by MTT assay.

Fig. 3. The Effects of FA on Intracellular GSH and NADPH Levels
Confluent HUVECs were pre-incubated for 18 h with the indicated concentrations of FA. The cells were then exposed to gamma radiation and cultured for 6 h. (A) Assays of intracellular GSH levels were performed as described in Materials and Methods. (B) Assays of intracellular NADPH levels were performed as described in Materials and Methods. Results were given as mean±S.E.M. of six independent experiments.

* p<0.05 significantly different from control cells not treated with radiation and FA, ## p<0.05 significantly different from control cells not treated with radiation or FA, # p<0.05 significantly different from gamma-irradiated cells not treated with FA.

Con=control; Rad=radiation.
Fig. 4. Effects of FA on Nrf2 Nuclear Translocation in HUVECs

Confluent HUVECs were pre-incubated for 18 h with the indicated concentrations of FA. The cells were then exposed to gamma-radiation and cultured for 6 h. Total RNA was extracted and RT-PCR was performed and products were resolved by gel electrophoresis. RT-PCR products were normalized to GAPDH products. The intensity of PCR product bands were quantitated by scanning densitometry. The results shown were representative of three independent experiments with similar results. Con=control; Rad=radiation.

Fig. 5. Effects of FA on Nrf2-Mediated Gene Expression in HUVECs

Confluent HUVECs were pre-incubated for 18 h with the indicated concentrations of FA. The cells were then exposed to gamma-radiation and cultured for 6 h. Total RNA was extracted and RT-PCR was performed and products were resolved by gel electrophoresis. RT-PCR products were normalized to GAPDH products. The intensity of PCR product bands were quantitated by scanning densitometry. The results shown were representative of three independent experiments with similar results. Con=control; Rad=radiation.

Nrf2 translocation into nuclei in radiated cells. To further investigate the anti-oxidative effect of FA, we examined the transcriptional levels of Nrf2 target genes by semi-quantitative RT-PCR. As shown in Fig. 5, treatment of the cells with FA indeed enhanced the GCLC, GCLM, NQO1 and HO-1 mRNA expressions in radiated cells, all of which were reported to be regulated by the Nrf2-dependent pathway. While FA alone treated cells exposed to sham radiation did not show significantly effect on Nrf2 translocation and Nrf2-mediated genes.

Effects of Mitogen-Activated Protein Kinases (MAPKs), PI3K, PKC Signaling Pathways on FA-Induced Nrf2 Activation and Redox Status in HUVECs

PI3K, PKC and three major MAPKs: ERK1/2, p38, and JNK play important roles in mediating cellular effects in HUVECs. To further elucidate the upstream signaling pathway involved in FA mediated Nrf2 activation, we determined whether PI3K, PKC and MAPKs signaling pathways were involved in FA-induced Nrf2 activation, cells were pre-treated with Staurosporine, a PKC inhibitor; SB 203580, a p38 inhibitor; SP600125, a JNK inhibitor; PD98059, an ERK inhibitor; LY294002, a PI3K inhibitor. PD98059 and LY294002 inhibited nuclear accumulation of Nrf2 protein induced by FA combination with radiation (Fig. 6A). PD98059 and LY294002 also inhibited increased intracellular GSH (Fig. 6B), NADPH (Fig. 6C) levels induced by FA combination with radiation, while other inhibitors had little effect on Nrf2, GSH, and NADPH in radiated cells.

Effects of PI3K, ERK Signaling Pathways on Induction of Nrf2-Mediated Gene Expression

To confirm the involvement of PI3K and ERK signaling in FA-induced Nrf2 activation, the changes of Nrf2-dependent gene expression were assessed by RT-PCR. As shown in Fig. 7, treatment of the cells with FA enhanced the GCLC, GCLM, NQO1 and HO-1 mRNA expressions in radiated cells, while PD98059 and LY294002 inhibited these changes.

DISCUSSION

Increased production of ROS induced by radiation, which involves superoxide and hydrogen peroxide, can lead to lipid peroxidation and cell injury.24 To prevent oxidation, antioxidants are used to induce a number of genes encoding antioxidant proteins.25,26 ARE is present in the promoter region of genes encoding antioxidant enzymes such as HO-1, glu-
tathione-S-transferase and NQO1, other antioxidant enzymes such as superoxide dismutase and catalase and non-enzymatic scavengers such as glutathione are also involved in scavenging ROS.\(^2\) Cellular redox potential is largely determined by GSH that is one of the most intensively studied intracellular compounds due to its critical role in cell biochemistry and physiology.\(^3\) This tripeptide is present in a reduced form (GSH) and in two oxidized species: GSSG and GSH mixed disulfide with protein thiols (GSGR). Maintaining an optimal GSH:GSSG ratio in the cell is critical to cell survival, hence, tight regulation of the system is imperative.\(^2\) GSH is required for the maintenance of the thiol redox status of the cell, protection against oxidative damage, detoxification of endogenous and exogenous reactive metals and electrophiles.\(^2\) NADPH is a cofactor in the glutathione reductase catalyzed reaction to regenerate antioxidant GSH and it is also required in maintaining the antioxidant enzyme catalase in its active form.\(^3\) HO-1 converts heme to biliverdin, which in turn is converted to bilirubin that acts as an antioxidant.\(^3\) Induction of such enzymes via activation of the ARE results in enhanced antioxidant capacities. In the present study, FA significantly increased the transcriptional activation of antioxidant related genes, such as GCLC, GCLM, NQO1 and HO-1 mRNA expressions in radiated cells, which involved in a significant increase in the intracellular GSH content and the expression of NAPDH.

Nrf2 is ubiquitously expressed in a wide range of tissues and cell types, and plays an important role in ARE-mediated antioxidant gene expression.\(^4\) In unstressed states, Nrf2 is present in the cytoplasm in association with Keap1, Keap1 normally sequesters Nrf2 in the cytoplasm in association with the actin cytoskeleton, but upon oxidation of cysteine residues, Nrf2 dissociates from Keap1, translocates to the nucleus and binds to ARE sequences leading to transcriptional activation of antioxidant genes.\(^3\) Expression of the Nrf2-dependent proteins is critical to maintain cellular redox homeostasis. Nrf2 translocated into the nuclei from cytosolic induced by FA in radiated cells, which involved in activation of antioxidant genes.

Previous studies suggested that Nrf2 may be regulated at multiple levels by a coordinated process. Cytoplasm-nuclear translocation, upregulated transcription, decreased degradation have been proposed as mechanisms for Nrf2 activation.\(^5,6\) In addition, extensive studies have demonstrated that protein phosphorylation is a potential mechanism for the activation of Nrf2–ARE-mediated pathways.\(^7\) Several cytosolic kinases, including PKC, PI3K, MAPK, and endoplasmic reticulum (ER) localized pancreatic endoplasmic reticulum kinase (PERK).\(^8\) have been shown to modify Nrf2 and to be potentially involved in Nrf2-mediated signal transduction at AREs. Phosphorylation of Nrf2 at Ser-40 through a PKC-based mechanism has been reported to play a critical role in the dissociation of Nrf2 from Keap1, a negative regulator of Nrf2.\(^9\) The PI3K pathway was reported to regulate the rearrangement of actin microfilaments and de-polymerization of actin in the course of nuclear translocation of Nrf2.\(^10\) Furthermore, MAPK pathway activated by ERK was reported to be signaling pathways for Nrf2 activation.\(^11\) Quite recently, it was shown that the accumulation of unfolded proteins in ER activates Nrf2 via the direct phosphorylation of Nrf2 by PERK.\(^12\) In the present study, FA has been shown to induce Nrf2 translocation into nuclei through PI3K and ERK signaling pathways, pretreatment with PI3K or ERK inhibitor resulted in attenuation of Nrf2-mediated gene expression, levels of GSH and NADPH were attenuated by PI3K or ERK inhibitor, showing PI3K and ERK signaling pathways involved in Nrf2–ARE mediated antioxidant action of FA. In agreement with our findings, brazzilin is found to increase heme oxygenase-1 via the PI3K/Akt and ERK pathways,\(^13\) tanshinone IIA is found to induce Nrf2 activation via ERK and protein kinase B (PKB) signaling pathways.\(^14\)

In summary, the present results suggest that FA protects HUVECs against radiation induced oxidative stress through upregulation of GSH and GAPDH. HUVECs treated with FA combination with radiation exhibited inovvement with PI3K and ERK pathway, which lead to nuclear translocation of Nrf2 and its subsequent binding to ARE, and resulted in up-regulating the levels of GSH and GAPDH.

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