Caffeic Acid Protects Hydrogen Peroxide Induced Cell Damage in WI-38 Human Lung Fibroblast Cells

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Cytoprotective effect of caffeic acid (3,4-dihydroxy cinnamic acid) on human lung fibroblast (WI-38) cells against hydrogen peroxide induced damage was investigated. Caffeic acid was found to scavenge intracellular reactive oxygen species, and 1,1-diphenyl-2-picrylhydrazyl radical, and thus prevented lipid peroxidation. The caffeic acid protected cell damage of WI-38 cells exposed to hydrogen peroxide (H₂O₂), via the activation of extracellular signal regulated kinase protein. Caffeic acid increased the activity of catalase and its protein expression. Hence, from the present study, it is suggestive that caffeic acid protects WI-38 cells against H₂O₂ damage by enhancing the cellular antioxidant activity.

Key words caffeic acid; oxidative stress; catalase

Caffeic acid (3,4-dihydroxy cinnamic acid), a natural product containing a catechol group with an α,β -unsaturated carboxylic acid chain (Fig. 1), is a widespread phenolic acid, naturally found in many agricultural products such as fruits, vegetables, wine, olive oil, and coffee. Caffeic acid has been reported to possess a wide spectrum of biological effects such as antioxidant activity,^{1,2)} inhibition of enzyme activities (lipoxygenase, glutathione *S*-transferase, xanthine oxidase),^{3–5)} antitumor activity,^{6,7)} anti-inflammatory effect,^{8,9)} and inhibition of HIV replication.^{10,11)} More recently, caffeic acid has been demonstrated to modulate cellular response on oxidative challenge.^{2,12–14)}

Reactive oxygen species (ROS) are known to cause oxidative modification of DNA, proteins, lipids and small cellular molecules. ROS are associated with tissue damage and are the contributing factors for inflammation, aging, cancer, arteriosclerosis, hypertension and diabetes.^{15–21)} The cells have developed a variety of antioxidant defense mechanisms against ROS. Enzymatic defense mechanisms involve superoxide dismutase, which catalyzes dismutation of superoxide anion to hydrogen peroxide, catalase, which converts hydrogen peroxide into molecular oxygen and water, and glutathione peroxidase, which destroys toxic peroxides.

In the present study, we have investigated the protective effect of caffeic acid on cell damage induced by hydrogen peroxide and the possible mechanism of cytoprotection.

MATERIALS AND METHODS

Reagents Caffeic acid (3,4-dihydroxy cinnamic acid)

Fig. 1. Chemical Structure of Caffeic Acid

was purchased from Wako Pure Chemical Ind., Ltd. (Tokyo, Japan) and freshly dissolved in dimethylsulfoxide and final concentration of dimethylsulfoxide did not exceed 0.02%. 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical, 2',7'-dichlorodihydrofluorescein diacetate (DCF-DA), and Hoechst 33342 were purchased from Sigma Chemical Company (St. Louis, MO, U.S.A.). PeroXOquant™ quantitative peroxide assay kit was purchased from Pierce (Rockford, IL, U.S.A.). Primary rabbit polyclonal anti-ERK 2 (42 kDa ERK) and phospho-ERK1/2 (phosphorylated 44 kDa/42 kDa ERK) (Thr 202/Tyr 204) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). The other chemicals and reagents were of analytical grade.

Cell Culture Lung is one of the organs suffered from high concentration of oxygen and exposed high level of oxidative stress. So lung has good defense systems against oxidative stresses.^{22,23)} To study the effect of caffeic acid on oxidative stress induced by hydrogen peroxide, we used human lung fibroblasts (WI-38 cells). The WI-38 cells from the American Type Culture Collection were maintained at 37 °C in an incubator with a humidified atmosphere of 5% CO₂ and cultured in RPMI containing 1% non-essential amino acid, 10% heat-inactivated fetal calf serum, streptomycin (100 µg/ml) and penicillin (100 units/ml).

1,1-Diphenyl-2-picrylhydrazyl (DPPH) Radical Scavenging Activity Various concentrations of caffeic acid were added to 1 × 10^{−4} M solution of DPPH in methanol, and the reaction mixture was shaken vigorously. After 1 h, the amount of residual DPPH was determined spectrophotometrically at 520 nm.²⁴⁾

Intracellular Reactive Oxygen Species (ROS) Measurement and Image Analysis The DCF-DA method was used to detect the intracellular ROS level.²⁵⁾ DCF-DA diffuses into cells, where it is hydrolyzed by intracellular esterase to polar 2',7'-dichlorodihydrofluorescein. This non-fluorescent fluorescein analog gets trapped inside the cells and is oxidized by intracellular oxidants to a highly fluorescent, 2',7'-dichloro-

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fluorescein. The WI-38 cells were seeded in a 96 well plate at 1×10^4 cells/well. Sixteen hours after plating, the cells were treated with various concentrations of caffeic acid and 30 min later, 1 mM H_2O_2 was added to the plate. The cells were incubated for an additional 30 min at 37 °C. After addition of 25 μ M of DCF-DA solution for 10 min, the fluorescence of 2',7'-dichlorofluorescein was detected at 485 nm excitation and at 535 nm emission using a PerkinElmer LS-5B spectrofluorometer. For image analysis for production of intracellular ROS, the WI-38 cells were seeded on coverslip loaded 6 well plate at 2×10^5 cells/well. Sixteen hours after plating, the cells were treated with caffeic acid and 30 min later, 1 mM H_2O_2 was added to the plate. After changing media, 100 μ M of DCF-DA was added in the well and was incubated for an additional 30 min at 37 °C. After washing with PBS, stained cells were mounted onto microscope slide in the mounting medium (DAKO, Carpinteria, CA, U.S.A.). Images were collected using the LSM 510 program on a Zeiss confocal microscope.

Measurement of Hydrogen Peroxide Level of H_2O_2 was determined by PeroXOquant™ quantitative peroxide assay kits, which detect H_2O_2 based on oxidation of ferrous to ferric ion in the presence of xylenol orange.²⁶⁾

Lipid Peroxidation Inhibitory Activity Lipid peroxidation was assayed by thiobarbituric acid reaction.²⁷⁾ The WI-38 cells were seeded in a culture dish at 1×10^6 cells/dish. Sixteen hours after plating, the cells were treated with various concentrations of caffeic acid. One hour later, 1 mM H_2O_2 was added to the plate, and was incubated for further 1 h. The cells were then washed with cold PBS, scraped and homogenized in ice-cold 1.15% KCl. One hundred microliters of the cell lysate was mixed with 0.2 ml of 8.1% SDS, 1.5 ml of 20% acetic acid (adjusted to pH 3.5) and 1.5 ml of 0.8% thiobarbituric acid. The mixture was made up to a final volume of 4 ml with distilled water and heated to 95 °C for 2 h. After cooling to room temperature, 5 ml of *n*-butanol and pyridine mixture (15 : 1, v/v) was added to each sample, and the mixture was shaken well. After centrifugation at $1000 \times g$ for 10 min, the supernatant fraction was isolated, and the absorbance was measured spectrophotometrically at 532 nm.

Cell Viability The effect of caffeic acid on the viability of the WI-38 cells was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, which is based on the reduction of a tetrazolium salt by mitochondrial dehydrogenase of the viable cells.²⁸⁾ The WI-38 cells were seeded in a 96 well plate at 1×10^4 cells/well. Sixteen hours after plating, the cells were treated with various concentrations of caffeic acid. One hour later, 1 mM H_2O_2 was added to the plate and incubated at 37 °C for an additional 24 h. Fifty microliters of the MTT stock solution (2 mg/ml) was then added to each well to attain a total reaction volume of 200 μ l. After incubating for 4 h, the plate was centrifuged at $800 \times g$ for 5 min and the supernatants were aspirated. The formazan crystals in each well were dissolved in 150 μ l of dimethylsulfoxide and A_{540} was read on a scanning multi-well spectrophotometer.

Nuclear Staining with Hoechst 33342 The WI-38 cells were placed in a 24 well plate at 2×10^5 cells/well. Sixteen hours after plating, the cells were treated with 50 μ M of caffeic acid and after further incubation for 1 h, 1 mM H_2O_2 was added to the culture. After 24 h, 1.5 μ l of Hoechst 33342

(stock 10 mg/ml), a DNA specific fluorescent dye, was added to each well (1.5 ml) and incubated for 10 min at 37 °C. The stained cells were then observed under a fluorescent microscope, which was equipped with a CoolSNAP-Pro color digital camera to examine the degree of nuclear condensation.

Catalase Activity The WI-38 cells were seeded at 1×10^6 cells/dish, and at 16 h after plating, the cells were treated with 50 μ M of caffeic acid for 1 h. The harvested cells were suspended in 10 mM phosphate buffer (pH 7.5) and then lysed on ice by sonicating twice for 15 s. Triton X-100 (1%) was then added to the lysates and was incubated for 10 min on ice. The lysates were centrifuged at $5000 \times g$ for 30 min at 4 °C to remove the cellular debris. The protein content of the supernatant was determined by Bradford method,²⁹⁾ with bovine serum albumin as the standard. Fifty micrograms of protein was added to 50 mM phosphate buffer (pH 7) containing 100 mM (v/v) H_2O_2 . The reaction mixture was incubated for 2 min at 37 °C and the absorbance was monitored at 240 nm for 5 min. The change in absorbance with time was proportional to the breakdown of H_2O_2 .³⁰⁾ The catalase activity was expressed as units/mg protein and one unit of enzyme activity was defined as the amount of enzyme required to breakdown 1 μ mol H_2O_2 /min.

Western Blot The WI-38 cells were placed in a plate at 1×10^6 cells/dish. Sixteen hours after plating, the cells were treated with 50 μ M of caffeic acid. The cells were harvested at the indicated times, and washed twice with PBS. The harvested cells were then lysed on ice for 30 min in 100 μ l of lysis buffer [120 mM NaCl, 40 mM Tris (pH 8), 0.1% NP 40] and centrifuged at $13000 \times g$ for 15 min. Supernatants were collected from the lysates and protein concentrations were determined. Aliquots of the lysates (40 μ g of protein) were boiled for 5 min and electrophoresed in 10% SDS-polyacrylamide gel. Blots in the gels were transferred onto nitrocellulose membranes (Bio-Rad, Hercules, CA, U.S.A.), which were then incubated with primary rabbit monoclonal anti ERK2, anti phospho ERK1/2, and primary sheep monoclonal anti catalase antibodies. The membranes were further incubated with goat anti rabbit or anti sheep immunoglobulin G-horseradish peroxidase conjugates (Pierce, Rockland, IL, U.S.A.). Protein bands were detected using an enhanced chemiluminescence Western blotting detection kit (GE Healthcare, Little Chalfont, Buckinghamshire, U.K.), and then exposed to X-ray film.

Statistical Analysis All the measurements were made in triplicate. The results were subjected to an analysis of the variance (ANOVA) using the Tukey test to analyze the difference. $p < 0.05$ were considered significantly.

RESULTS

Radical Scavenging Activity of Caffeic Acid The radical scavenging effect of caffeic acid on the intracellular ROS and DPPH free radical scavenging activities was measured. The intracellular ROS scavenging activity of caffeic acid was 37, 54 and 61% at concentrations of 0.5, 5 and 50 μ M, respectively (Fig. 2A). In the presence of 2 mM of *N*-acetylcysteine (positive control), there was 87% of ROS inhibition (data not shown). As shown in Fig. 2B, the fluorescent intensity of DCF-DA staining was enhanced in H_2O_2 treated WI-38 cells. However, caffeic acid reduced the red fluorescence

Fig. 2. Effect of Caffeic Acid on Scavenging Intracellular ROS and DPPH Radicals

The intracellular ROS generated was detected by DCF-DA method (A) and by confocal microscopy (B). Representative confocal images illustrate the increase in red fluorescence intensity of DCF produced by ROS in H₂O₂ treated WI-38 cells as compared to control and the lowered fluorescence intensity in H₂O₂ treated WI-38 cells in the presence of caffeic acid (original magnification×400). (C) The amount of DPPH radicals was determined spectrophotometrically at 520 nm. * Significantly different from control ($p < 0.05$).

intensity by H₂O₂ treatment, reflecting a reduction of ROS generation. The DPPH radical scavenging activity of caffeic acid as shown in Fig. 2C was 2, 21, and 57% at concentrations of 0.5, 5 and 50 μM, respectively when compared to 91% of scavenging activity in the presence of 2 mM *N*-acetyl-cysteine (data not shown).

Effect of Caffeic Acid on Lipid Peroxidation The ability of caffeic acid to inhibit lipid peroxidation in H₂O₂ treated WI-38 cells was also investigated. The generation of thiobarbituric acid reactive substance (TBARS) was inhibited in the presence of caffeic acid. The inhibitory effect of caffeic acid was 16, 21 and 27% at concentration of 0.5, 5, and 50 μM, respectively (Fig. 3).

Effect of Caffeic Acid on Cell Damage Induced by H₂O₂ The protective effect of caffeic acid on cell survival in H₂O₂ treated WI-38 cells was also measured. Cells were treated with caffeic acid at various concentrations for 1 h, prior to the addition of H₂O₂. The cell viability was determined 24 h later by MTT assay. As shown in Fig. 4A, treat-

Fig. 3. Effect of Caffeic Acid on Inhibition of Lipid Peroxidation

Lipid peroxidation was assayed by measuring the amount of thiobarbituric acid reactive substance (TBARS). * Significantly different from control ($p < 0.05$).

Fig. 4. Protective Effect of Caffeic Acid on H₂O₂ Induced Oxidative Damage of WI-38 Cells

(A) The viability of WI-38 cells was determined by MTT assay. (B) Apoptotic body formation was observed under a fluorescent microscope after Hoechst 33342 staining and apoptotic bodies are indicated by arrows.

ment with caffeic acid induced a dose dependent increase in the cell survival rate; 52% at 0.5 μM, 55% at 5 μM, and 62% at 50 μM as compared to 43% of H₂O₂ treatment. In order to study the cytoprotective effect of caffeic acid on apoptosis induced by H₂O₂, nuclei of WI-38 cells were stained with Hoechst 33342 for microscopy. The microscopic pictures in Fig. 4B showed that the control cells had intact nuclei, and the H₂O₂ treated cells showed significant nuclear fragmentation, characteristic of apoptosis. However, when the cells were treated with caffeic acid for 1 h prior to H₂O₂ treatment, a dramatic decrease in nuclear fragmentation was observed.

Effect of Caffeic Acid on ERK Activation The activation of extracellular signal regulated kinase (ERK) is known to induce cell proliferation.³¹⁾ To better understand the protective mechanism of caffeic acid on WI-38 cells, we examined the activation of the ERK protein by western blot analy-

Fig. 5. Effect of Caffeic Acid on ERK Activation

(A) Cell lysates were electrophoresed and proteins of ERK 2 and phospho-ERK1/2 were detected by their respective antibodies. (B) After treatment with U0126, caffeic acid or/and H₂O₂, the viability of WI-38 cells was determined by MTT assay. * Significantly different from H₂O₂ treated cells ($p < 0.05$).

sis with the phospho-ERK specific antibody. As shown in Fig. 5A, caffeic acid activated phosphorylated ERK at 6 h dramatically. However, there was no change in the total ERK protein level. To determine the effect of ERK inhibitor on protection of caffeic acid from H₂O₂ induced damage, WI-38 cells were pre-treated for 30 min with U0126 (10 nM), a specific inhibitor of ERK kinase, followed by incubation with caffeic acid for 30 min and were then exposed to 1 mM H₂O₂ for 24 h. As shown in Fig. 5B, U0126 treatment abolished the protective activity of caffeic acid in H₂O₂ damaged cells.

Effect of Caffeic Acid on Catalase In order to investigate whether the radical scavenging activity of caffeic acid was mediated by the activity of antioxidant enzyme, the catalase activity in caffeic acid treated WI-38 cells were measured. Caffeic acid was found to increase the activity of catalase (Fig. 6A); it was 31 U/mg protein at concentration of 50 μ M as compared to 11 U/mg protein of the control. To confirm the activation of catalase by caffeic acid in terms of protein, the western blot analysis was performed. As shown in Fig. 6B, the protein expression of catalase by caffeic acid was increased. The 3-amino-1,2,4 triazol (ATZ) is known as a specific inhibitor of catalase.³²⁾ To determine the effect of catalase inhibitor on protection of caffeic acid from H₂O₂ induced damage, WI-38 cells were pre-treated with 20 mM of ATZ for 1 h, followed by incubation with caffeic acid for 30 min and were then exposed to 1 mM H₂O₂ for 24 h. As shown in Fig. 6C, ATZ treatment abolished the protective activity of caffeic acid in H₂O₂ damaged cells. It is reported that most of the phenolic compounds interact with commonly used cell culture media to generate H₂O₂.³³⁾ This generates low level of H₂O₂, which can trigger increased levels of antioxidant enzymes. To determine that caffeic acid generates H₂O₂ in culture medium, it was added to cell culture media at a final concentration of 50 μ M and the amount of H₂O₂ was

Fig. 6. Effect of Caffeic Acid on Catalase

(A) The data represent three experiments and are expressed as average enzyme unit per mg protein $\pm$ S.E. * Significantly different from control ($p < 0.05$). (B) Cell lysates were electrophoresed and protein of catalase was detected by its antibody. (C) After treatment with ATZ, caffeic acid or/and H₂O₂, the viability of WI-38 cells was determined by MTT assay. * Significantly different from H₂O₂ treated cells ($p < 0.05$).

Table 1. Generation of H₂O₂ in Cell-Culture Media

[H ₂ O ₂ μ M] present in RPMI media	
Media	0.2 $\pm$ 0.01
Dimethylsulfoxide	0.1 $\pm$ 0.05
Caffeic acid	1.0 $\pm$ 0.1

Caffeic acid at a final concentration of 50 μ M was added to RPMI culture media and incubated at room temperature for 1 h. Generation of H₂O₂ was then measured by the ferrous iron oxidation-xylenol orange assay. Data are means $\pm$ S.E. Dimethylsulfoxide at the same concentration used to dissolve caffeic acid.

measured 1 h later by the ferrous iron oxidation-xylenol orange assay. As shown in Table 1, there was only lowered amount of H₂O₂ in caffeic acid treated media (1 μ M H₂O₂), suggesting that the antioxidant activities in caffeic acid treated cells were not increased by H₂O₂ generated in caffeic acid treated media.

DISCUSSION

The aim of this study was to determine the cytoprotective effect of caffeic acid on oxidative stress induced cell damage. It was observed that upon exposure to H₂O₂, caffeic acid decreased intracellular ROS generation and DPPH radical level. The radical scavenging activity of caffeic acid protected cell damage of WI-38 cells exposed to hydrogen peroxide, *via* the

activation of extracellular signal regulated kinase (ERK) protein. In many cell types, ERK pathway is induced by a variety of extracellular stimuli.³⁴⁾ The phosphorylation of ERK can phosphorylate cytoplasmic and nuclear targets and participates in a wide range of cellular programs including proliferation, differentiation, and movement.^{31,35,36)} The level of phosphorylated ERK in caffeic acid treated WI-38 cells was induced, and treatment of U0126, a specific inhibitor of ERK kinase, suppressed the protective activity of caffeic acid in H₂O₂ damaged cells, suggesting that the protective effect of caffeic acid on cells may also be involved in activating ERK pathway. Catalase plays significant role in effective augmentation of antioxidant defense mechanisms in cells. Superoxide dismutase converts superoxide radicals to hydrogen peroxide and subsequently to water by the activity of catalase. Caffeic acid was found to increase catalase activity, suggesting that scavenging of ROS may be related to the increased antioxidant activity. Recently, it is reported that caffeic acid inhibits lipid peroxidation and exhibits DNA protective effect in normal human peripheral blood mononuclear cells exposed to H₂O₂-induced oxidative stress and this anti-apoptotic activity of caffeic acid is mediated through Bcl-2 independent mechanism.³⁷⁾ The effects of caffeic acid on cell viability might involve dual actions: direct action on oxygen radical scavenging, as shown by DPPH radical scavenging and intracellular ROS scavenging, and indirect action through induction of anti-oxidative enzymes.

In conclusion, caffeic acid exerted intracellular ROS and DPPH radical scavenging activity, promoted cell viability via activation of ERK protein, and enhanced the effects of catalase.

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