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Role of NADPH Oxidase-Mediated Generation of Reactive Oxygen Species in the Mechanism of Apoptosis Induced by Phenolic Acids in HepG2 Human Hepatoma Cells

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Although plant-derived phenolic acids have been reported to have anti-cancer activity, the exact mechanism is not completely understood. In this study, we investigated the role for reactive oxygen species (ROS) as a mediator of the apoptosis induced by caffeic acid (CA) and ferulic acid (FA), common phenolic acids in plants, in HepG2 human hepatoma cells. CA and FA reduced cell viability, and induced apoptotic cell death in a dose-dependent manner. In addition, they evoked a dose-related elevation of intracellular ROS. Treatment with various inhibitors of NADPH oxidase (diphenylene iodonium, apocynin, neopterine) significantly blunted both the generation of ROS and the induction of apoptosis induced by CA and FA. These results suggest that ROS generated through activation of NADPH oxidase may play an essential role in the apoptosis induced by CA and FA in HepG2 cells. These results further suggest that CA and FA may be valuable for the therapeutic management of human hepatomas.

Key words: Caffeic acid, Ferulic acid, Apoptosis, HepG2 cell, NADPH oxidase, Reactive oxygen species

INTRODUCTION

Caffeic acid (CA) and ferulic acid (FA) are abundant plant-derived phenolic acids found in fruits, vegetables, wine, olive oil, coffee, etc. (Mathew and Abraham, 2004; Shahidi and Naczk, 1995). These phenolic compounds elicit several interesting biological responses: antioxidant activities (Graf, 1992; Vieira *et al.*, 1998), inhibitory activities of enzymes such as lipoxygenases, cyclooxygenase, glutathione S-transferase and xanthine oxidase (Chan *et al.*, 1995; Koshihara *et al.*, 1984; Michaluart *et al.*, 1999; Mirzoeva *et al.*, 1996; Schefferlie and van Bladeren, 1993), anti-inflammatory properties (Michaluart *et al.*, 1999), anti-HIV replication activities (Fesen *et al.*, 1993), and antitumor activities (Frenkel *et al.*, 1993; Tanaka *et al.*, 1993). Furthermore, CA efficiently inhibited the development of azoxymethane-induced tumors in the colon of rats and phorbol-induced tumors in mouse skin (Frenkel *et al.*, 1993; Rao *et al.*, 1993). Recently, Chung *et al.* (2004) reported that CA inhibited the growth and metastasis of hepatocarcinoma cells through the inhibition of nuclear factor-κB (NF-κB) and matrix metalloproteinase-9 (MMP-9) activities. However, the exact mechanism of their anticancer activity is not completely understood.

Apoptosis is characterized by condensation of nuclear chromatin, loss of plasma membrane phospholipid asymmetry, activation of proteases and endonucleases, enzymatic cleavage of the DNA into oligonucleosomal fragments, and segmentation of the cells into membrane-bound apoptotic bodies (Kidd, 1998). Apoptosis plays a critical role in the maintenance of tissue homeostasis by the selective elimination of excessive cells (Song and Steller, 1999). In particular, genetic mutations culminating in disturbances of apoptosis or derangement of apoptotic signaling pathways are an essential factor of carcinogenesis (Lowe and Lin, 2000; Wang, 1999). On the other hand, the induction of apoptosis in cancer cells is one of the most important methods for cancer treatment (Kornblau, 1998).

Excessively produced reactive oxygen species (ROS) may result in cellular damage through interactions with cellular macromolecules and structures (Yu, 1994). ROS appear to act as important signaling molecules in the process of apoptotic cell death (Bohler *et al.*, 2000) and

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cell differentiation (Suzukawa *et al.*, 2000) *via* activation of proteases and nucleases (Yu, 1994), altered gene expression (Schiaffonati and Tiberio, 1997), and changes in membrane permeability (Yu, 1994).

The main purposes of the present study were to investigate: (i) whether CA and FA induce apoptotic cell death in HepG2 human hepatoma cells, (ii) whether ROS are involved in the mechanism of apoptosis induced by CA and FA, and (iii) whether NADPH oxidase specifically mediates the production of ROS associated with the mechanism of apoptosis induced by CA and FA.

MATERIALS AND METHODS

Materials

The HepG2 human hepatoblastoma cell line was purchased from American Type Culture Collection (Rockville, MA). Powdered Eagle's minimum essential medium (MEM), trypsin solution, sodium pyruvate, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), propidium iodide (PI), ribonuclease A and all salt powders were obtained from Sigma Chemical CO. (St. Louis, MO). CA and FA were purchased from Aldrich Chemical Co. (Milwaukee, WI, U.S.A.). 2',7'-Dichlorofluorescin diacetate (DCFH-DA) was from Molecular Probes, Inc. (Eugene, OR). Fetal bovine serum (FBS) and antibiotics (penicillin and streptomycin mixture) were purchased from GIBCO (Grand Island, NY). DCFH-DA was prepared as stock solutions in dimethyl sulfoxide (DMSO), then diluted with aqueous medium to the final desired concentrations. The stock solution of drugs was sterilized by filtration through 0.2 μm disc filters (Gelman Sciences: Ann Arbor, MI).

Cell culture

HepG2 cells were grown at 37°C in a humidified incubator under 5% CO_2/95% air in MEM supplemented with 10% FBS, 200,000 IU/L penicillin, 200 mg/L of streptomycin and 1 mM sodium pyruvate. Culture medium was replaced every other day. After attaining confluence the cells were subcultured following trypsinization.

Cell viability assay (MTT staining)

Cell viability was assessed by the MTT staining method (van de Loosdrecht *et al.,* 1991). Cells from 4- to 5-day-old cultures were seeded in 24-well plates at a density of 5×10^4 cells/well. The volume of the medium in the wells was 1 mL. In control experiments, cells were grown in the same media containing drug-free vehicle. After incubation with drug for 48 h, 100 μL of MTT (5 mg MTT/mL in H_2O) were added and cells incubated for a further 4 h. Two hundred microliters of DMSO was added to each culture and mixed by pipetting to dissolve the reduced MTT crystals. Relative cell viability was obtained by scanning with a microplate reader (Molecular Devices, Menlo Park, CA) with a 540 nm filter.

Flow cytometry assays

For flow cytometry analysis, HepG2 cells were collected and washed twice with phosphate buffered saline (PBS), pH 7.4. After fixing in 80% ethanol for 30 min, cells were washed twice and resuspended in PBS buffer (pH 7.4) containing 0.1% Triton X-100, 5 mg/L PI, and 50 mg/L ribonuclease A for DNA staining. Cells were then analyzed by a FACScan (BIO-RAD, Hercules, CA). At least 20,000 events were evaluated. All histograms were analyzed using WinBryte software (BIO-RAD, Hercules, CA) to determine percentage of nuclei with the hypodiploid content indicative of apoptosis (Bombeli *et al.*, 1997).

Measurement of intracellular ROS

Relative changes in intracellular ROS in HepG2 cells were monitored using a fluorescent probe, DCFH-DA (LaBel *et al.*, 1992). DCFH-DA diffuses through the cell membrane readily and is hydrolyzed by intracellular esterases to nonfluorescent 2', 7'-dichlorofluorescin (DCFH), which is then rapidly oxidized to highly fluorescent 2', 7'-dichlorofluorescein (DCF) in the presence of ROS. The DCF fluorescence intensity is proportional to the amount of ROS formed intracellularly (Shen *et al.*, 1996). Cells were washed twice and resuspended at a concentration of 4×10^5 cells/mL in Hank's solution. For loading DCFH-DA into the cells, cells were incubated with the dye for 2 h at a final concentration of 5 μM at 37°C. Fluorescence was monitored at 530 nm with an excitation wavelength of 485 nm in a stirred quartz cuvette.

Data analysis

All experiments were performed four times. Data are expressed as mean ± standard error of the mean (SEM) and were analyzed using one way analysis of variance (ANOVA) and Student-Newman-Keul's test for individual comparisons. P values less than 0.05 are considered statistically significant.

RESULTS

Induction of apoptotic cell death by CA and FA

The effects of CA and FA on HepG2 viability were examined using MTT staining. CA and FA decreased cell viability in a dose-dependent manner (Fig. 1). Significant cytotoxicity was induced by CA and FA from 5 μM and 10 μM, respectively. CA and FA reduced cell viability to 50% at 10 and 28 μM, respectively. A flow cytometry assay was used to determine whether cell death was occurring by apoptosis (Bombeli *et al.*, 1997). CA and FA increased the number of cells with hypodiploid nuclei, indicative of

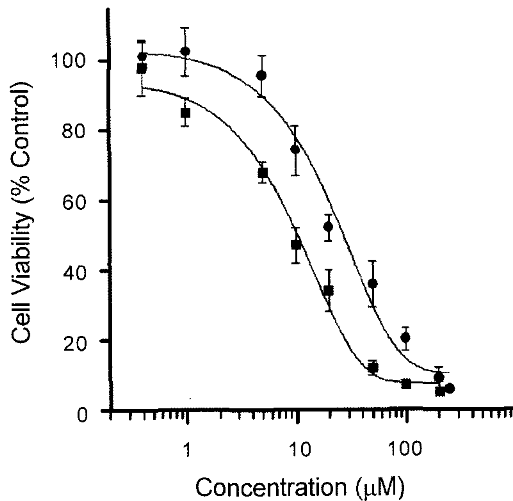

Fig. 1. Effects of CA (closed rectangle) and FA (closed circle) on cell viability in HepG2 human hepatoma cells. Cells were incubated with CA and FA for 48 h. Cell viability was measured by MTT staining. Results are expressed as the percent change of control conditions (media without drug). Data points represent the mean values of four replicates, with bars indicating SEM.

apoptosis, in a dose-dependent manner (Fig. 2). The concentrations of CA and FA that gave a 50% of maximum response (EC50) were 5 and 16 μM, respectively. These results clearly showed that CA and FA induced apoptotic cell death in HepG2 cells.

Role of NADPH oxidase in the generation of ROS and apoptosis induced by CA and FA

To explore the role of ROS in the mechanism of CA- and FA-induced apoptosis, we examined of their effects on intracellular ROS production using DCF fluorescence. CA and FA enhanced the generation of ROS in a concentration-dependent fashion (Fig. 3). We then focused on whether this ROS production required NADPH oxidase, which generates ROS following treatment with

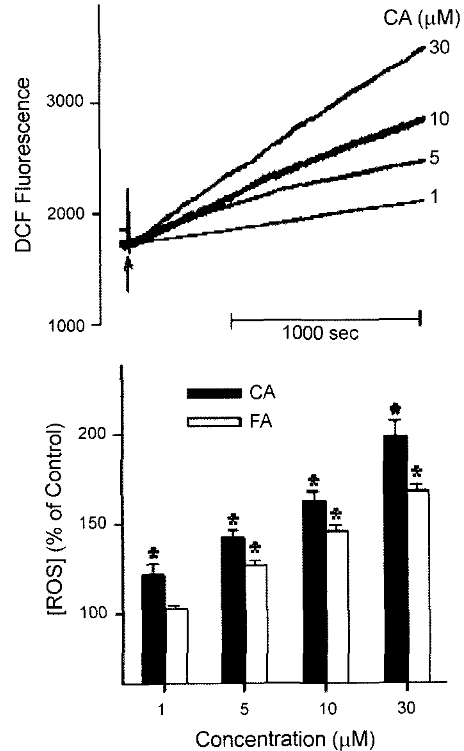

Fig. 3. CA and FA enhance ROS production in a dose-dependent manner in HepG2 human hepatoma cells. Time-dependent changes in ROS levels measured by DCF fluorescence (above). The arrow shows the time point for addition of CA. Results are expressed as percent increase compared to the initial DCF fluorescence intensity (below). The data represent the mean values of four replicates, with bars indicating SEM. *P<0.05 compared to control (drug-free medium).

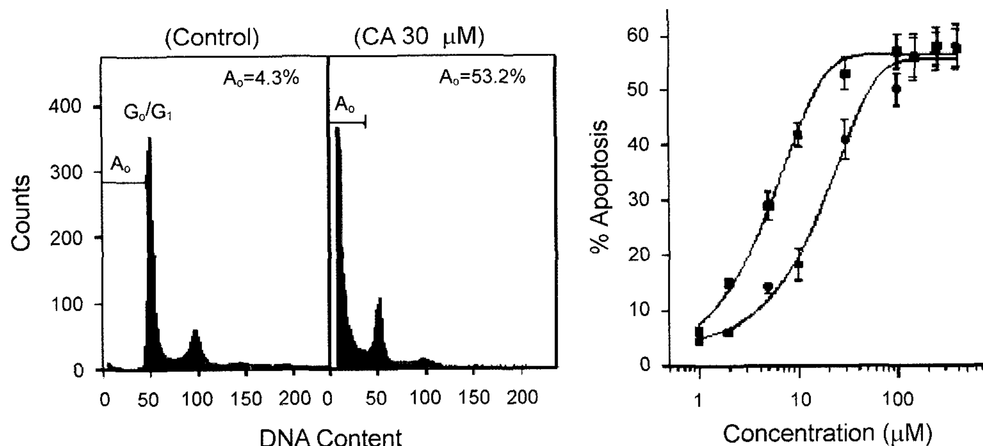

Fig. 2. CA and FA induce apoptotic cell death of HepG2 human hepatoma cells. (Left panel) Cells were treated with CA (30 μM) for 48 h, stained with PI and analyzed by flow cytometry as described in *Materials and Methods*. The region to the left of the Go/G1 peak, designated Ao, was defined as cells undergoing apoptosis-associated DNA degradation. (Right panel) Cells were incubated with CA (closed rectangle) or FA (closed circle) for 48 h. The data represent the mean values of four replicates, with bars indicating SEM.

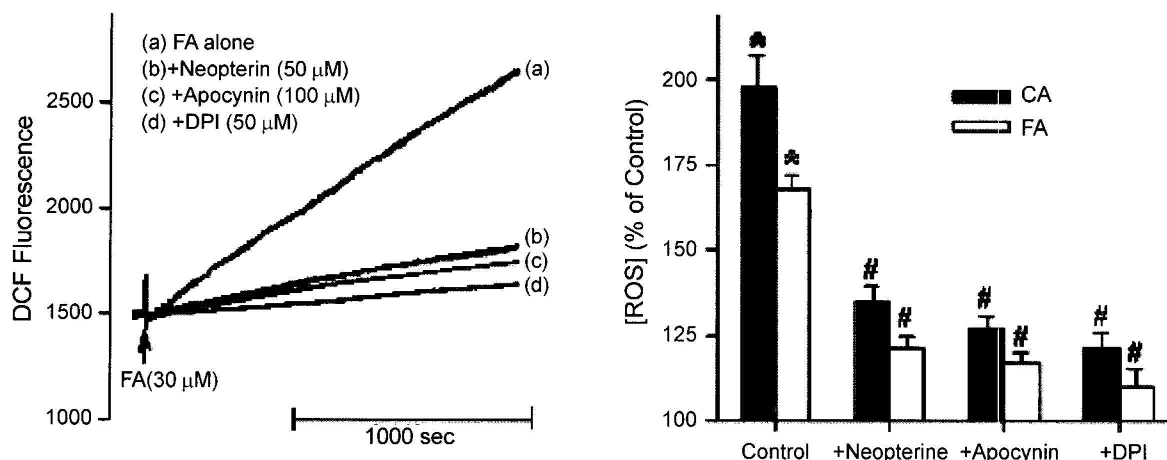

Fig. 4. NADPH oxidase mediates the ROS production induced by CA and FA in HepG2 human hepatoma cells. Time-dependent changes in ROS levels measured by DCF fluorescence (left). The arrow shows the time point for addition of FA (30 μM). DPI, apocynin and neopterine were used as inhibitors of NADPH oxidase. These drugs were given 10 min before FA (30 μM) or CA (30 μM) application. Results are expressed as percent increase compared to the initial DCF fluorescence intensity (right). The data represent the mean values of four replicates, with bars indicating SEM. *P<0.05 compared to control (drug-free medium). #P<0.05 compared to FA or CA alone.

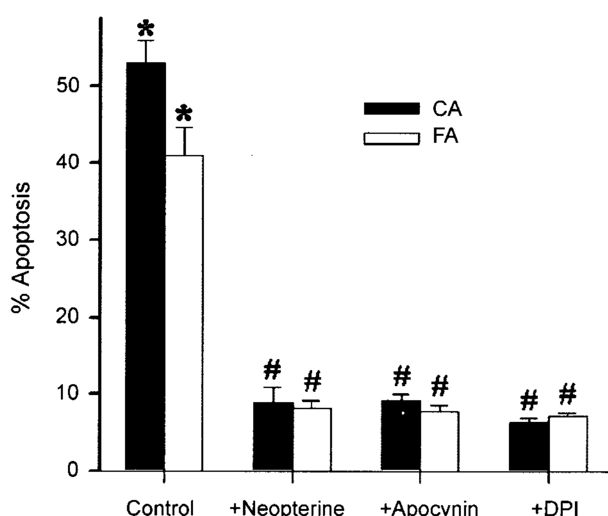

Fig. 5. NADPH oxidase mediates the apoptosis induced by CA and FA in HepG2 human hepatoma cells. Cells were incubated with CA (30 μM) or FA (30 μM) for 48 h, stained with PI, and analyzed by flow cytometry. The number of apoptotic cells was measured by flow cytometry as described in the text. DPI (50 μM), apocynin (100 μM), and neopterine (50 μM) were used as inhibitors of NADPH oxidase. These drugs were given 30 min before CA or FA application. The data represent the mean values of four replicates, with bars indicating SEM. *P<0.05 compared to control (drug-free medium). #P<0.05 compared to FA or CA alone.

an anticancer agent in HepG2 cells (Lee *et al.*, 2000). Treatment with various inhibitors of NADPH oxidase, DPI (O'Donnell *et al.*, 1993), apocynin (Stolk *et al.*, 1994), and neopterine (Kojima *et al.*, 1993), completely suppressed the generation of ROS induced by CA and FA (Fig. 4). These inhibitors also significantly inhibited CA- and FA-induced apoptosis (Fig. 5). Thus, CA and FA may generate ROS through the activation of NADPH oxidase, which induces apoptotic cell death in HepG2 cells.

DISCUSSION

Recently, a great deal of attention has focused on the roles and mechanisms of action of polyphenolic compounds as antioxidants, including CA and FA (Bhimani *et al.*, 1993; van Acker *et al.*, 1996; Vieira *et al.*, 1998). These compounds can inhibit the formation of intracellular H_2O_2 and DNA oxidation by 12-*O*-tetradecanoylphorbol-13-acetate (TPA) treatment of human cells (Huang *et al.*, 1996). Furthermore, CA efficiently inhibits NF-κB activity (Nardini *et al.*, 2001), which is well-correlated with elevated oxidative stress (Wang *et al.*, 2002). However, the results of the present study showed that these phenolic acids, CA and FA, increased the production of ROS, leading to the induction of apoptosis. Although we do not know the exact reason for this discrepancy, many previous reports support our findings: these phenolic compounds elevate intracellular ROS and act as a pro-oxidants (Galati *et al.*, 2002; Sergediene *et al.*, 1999; Stagos *et al.*, 2005; Yamanaka *et al.*, 1997). These compounds have also been shown to exhibit cellular toxicity against tumor cells, which was largely dependent upon the formation of ROS and significantly reduced by treatment with antioxidants (Chiao *et al.*, 1995; Sergediene *et al.*, 1999).

Oxidative stress is proposed as a common mechanism of apoptosis (Simon *et al.*, 2000). ROS play an essential role in apoptosis induced by the anticancer agent tamoxifen in HepG2 cells (Lee *et al.*, 2000). The results of the present study further support the notion that ROS act as a common mediator of apoptosis. The major biological

process leading to oxygen-derived generation of ROS is electron transport associated with mitochondrial membranes (Halliwell, 1989). The membrane-bound NADPH oxidase also appears to play a role in the production of ROS in conjunction with apoptosis induced by an anticancer agent (Lee *et al.*, 2004; Lee *et al.*, 2000). The results of the present study clearly demonstrated, for the first time, that NADPH oxidase mediates the generation of ROS and induction of apoptosis induced by the plant-derived phenolic acids, CA and FA, in HepG2 cells. These results further suggest that NADPH oxidase is important in the production of ROS associated with apoptosis in tumor cells. NADPH oxidase produces ROS during the respiratory burst in neutrophils (Babior, 1995), and is active in non-phagocytic cells, including endothelial cells (Jones *et al.*, 1996), vascular smooth muscle cells (Marshall *et al.*, 1996), neuroepithelial bodies of the lung (Youngson *et al.*, 1997), and type I cells of the carotid body (Kummer and Acker, 1995). Activation of this enzyme proceeds through a multistep assembly at the plasma membrane of several components, including the two subunits of cytochrome b_{558} ($p22^{phox}$ and $gp91^{phox}$), the small GTP-binding proteins (Rac and Rap1A), and the cytosolic factors ($p40^{phox}$, $p47^{phox}$, and $p67^{phox}$) (Babior, 1999). These components are active in HepG2 cells (Cool *et al.*, 1998; Ehleben *et al.*, 1997).

The mechanism by which these phenolic acids activate NADPH oxidase remains unclear, but may involved Ca^{2+}/calmodulin-dependent protein kinase-II. Ca^{2+}/calmodulin-dependent protein kinase-II activates Tiam1 (Fleming *et al.*, 1999), a Rac1-specific exchange factor, leading to Rac1 activation, which is necessary for NADPH oxidase-mediated generation of ROS in HepG2 cells (Cool *et al.*, 1998). Indeed, our preliminary data showed that CA and FA did increase intracellular Ca^{2+} levels (data not shown), which is required for the activation of Ca^{2+}/calmodulin-dependent protein kinase-II (Fujisawa, 2001). In addition, numerous reports have suggested that arachidonic acid is involved in the activation of NADPH oxidase in phagocytic cells (Daniels *et al.*, 1998; Shiose and Sumimoto, 2000). Thus, increased release of arachidonic acid may be a possible mechanism by which CA and FA activate NADPH oxidase. This possibility needs to be tested in a future study.

In conclusion, the plant-derived phenolic acid derivatives, CA and FA, maybe induce HepG2 apoptosis by generation of ROS through activation of NADPH oxidase. These results further suggest that these phenolic acids may be valuable for therapeutic intervention in human hepatomas.

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