2′-Benzoyloxyccinnamaldehyde (BCA) is a promising antitumor agent which induces cancer cells apoptosis via reactive oxygen species (ROS) generation. BCA shows more effective antiproliferation in MDA-MB-435 than in MCF-7 breast cancer cells. DJ-1 has been known to protect cells against oxidative stress as an antioxidant because of its cysteine residues sensitive to oxidative stress. In the present study, we evaluated the mechanism of DJ-1 for cell protection from oxidative stress after BCA treatment in MCF-7 cell. BCA upregulates the expression of DJ-1 in MCF-7 cells. However, DJ-1 expression decreased continuously for 24 h after BCA treatment in MDA-MB-435 cells. DJ-1 knockdown sensitized MCF-7 cells to BCA, on the contrary, DJ-1 overexpression induced MDA-MB-435 cells less sensitive to BCA. Confocal microscopic observation showed that only in MCF-7 cells BCA increased the overlapped signal between mitochondria and DJ-1. Mitochondrial membrane potential (MMP) was decreased in MDA-MB-435 cells by BCA, and DJ-1 overexpression inhibited BCA-induced MMP decrease in these cells. On the contrary, DJ-1 knockdown in MCF-7 induced MMP perturbation by BCA. These findings suggest that DJ-1 upregulation protects MCF-7 cells from BCA via inhibiting mitochondrial damage.

Key words 2′-benzoyloxyccinnamaldehyde; breast cancer cell; DJ-1; mitochondrial membrane potential

2′-Benzoyloxyccinnamaldehyde (BCA) was originally synthesized as a derivative of 2′-hydroxyccinnamaldehyde (HCA), a natural compound isolated from the bark of Cinnamomum cassia Blume. These compounds are characterized as antioxidant agents in diverse cancer cell lines inducing apoptosis via reactive oxygen species (ROS) generation and caspase-3 activation. BCA/HCA-induced apoptosis has also been associated with the inhibition of proteasome activity. BCA significantly blocked tumor growth in a nude mouse without body weight loss, making it a good candidate drug for cancer therapy.9

We investigated the effect of BCA on breast cancer cells, and found that BCA inhibits cell proliferation more effectively in MDA-MB-435 than in MCF-7 cells. We therefore tried to clarify the molecular mechanism of BCA-induced cell death underlying the differential chemosensitivity of MCF-7 and MDA-MB-435 cells.

DJ-1, originally identified as a novel oncogene in collaboration with ras, belongs to the peptidase C56 family of proteins. It has been generally known that loss-of-function DJ-1 mutations can cause early-onset Parkinson’s disease. Mitochondrial membrane perturbation, oxidative stress, and proteasome dysfunction are among the several hypotheses suggested to explain the molecular basis of neuronal damage. DJ-1 acts as a redox-sensitive chaperone and as a sensor for oxidative stress; it also protects cells against oxidative stress in neuronal injury. Moreover, DJ-1 protects several kinds of cells such as pancreatic, neuronal, leukemic, and lung carcinoma against oxidative stress-induced apoptosis. Higher DJ-1 expression level is clearly associated with renal carcinoma and the onset of hepatocellular carcinoma. Previous studies related to the cell protective molecular mechanisms of DJ-1 have shown that it induces glutathione (GSH) synthesis and scavenges H₂O₂ by cysteine oxidation during oxidative stress. Furthermore, DJ-1 upregulates p53 tumor suppressor protein via Topors-mediated sumoylation and affects the c-Jun N-terminal kinase (JNK) signaling pathway.

The relevance of DJ-1 subcellular compartmentalization to its cytoprotective activity was evaluated in neuronal cells. DJ-1 is mainly localized to the cytoplasm, but under oxidative stress, translocates to the mitochondria within 3 h. They also confirmed that oxidative stress-induced early translocalization of DJ-1 to mitochondria is important in its protective role. The biochemical function and subcellular localization of DJ-1 protein was also clarified in human neuroblastoma cell. They suggested that DJ-1 might contribute to the proper function of mitochondria in physiological conditions. Consistent with this suggestion is that DJ-1 knockout mice are more sensitive to the toxic effects of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine which impairs mitochondrial respiration.

Moreover, downregulation of DJ-1 gene sensitizes cells to oxidative stress in HEK293 or neuronal cells. Recently, it was reported that DJ-1 maintains mitochondrial functions under oxidative stress in human dopaminergic cells and loss of DJ-1 leads to mitochondrial phenotypes such as reduction of membrane potential and increases of fragmentation. These data suggest that DJ-1 may have roles in association with mitochondria in response to oxidative stress.

Lines of evidence have suggested that mitochondrial dysfunction plays essential roles in the pathogenesis of Parkinson’s disease. However, there are little evidence for DJ-1 cell protection and subcellular localization in cancer cells. In the present study, therefore, we evaluated the molecular mechanism of DJ-1 for differential cell protection from BCA in MCF-7 breast cancer cells.
MATERIALS AND METHODS

Chemicals and Reagents 3-[4,5-dimethyl-2-thiazolyl]-2,5-diphenyl-2H-tetrazolium bromide (MTT) and propidium iodide were purchased from Sigma (St. Louis, MO, U.S.A.). Dulbecco’s modified Eagle’s medium (DMEM), RPMI, fetal bovine serum (FBS) and penicillin/streptomycin antibiotics were from Gibco (Invitrogen Corporation, CA, U.S.A.). Qiazo was from Qiagen (Valencia, CA, U.S.A.) and 2 X SYBR green polymerase chain reaction (PCR) master mix from Applied Biosystems (Foster, CA, U.S.A.). RNase A was from USB (Cleveland, OH, U.S.A.). Mouse monoclonal anti-DJ-1 and rabbit polyclonal anti SPI antibodies were purchased from Abcam (Cambridge, U.K.). Horse-radish peroxidase (HRP)-conjugated mouse monoclonal anti-β-actin antibody was from Santa Cruz (Santa Cruz, CA, U.S.A.). HRP conjugated goat anti-rabbit and anti-mouse secondary antibodies were from Pierce (Rockford, IL, U.S.A.). Alexa Fluor goat anti-mouse, anti-rabbit immunoglobulin G (IgG) fluorescence secondary antibodies and MitoTracker Green, a green-fluorescent mitochondrial stain were from Molecular Probes (Eugene, Oregon, U.S.A.). ECL-plus Western Blotting Detection Reagent was from Amersham Biosciences (St. Giles, Buckinghamshire, U.K.). All other reagents were obtained from standard commercial sources.

Cell Culture and Cell Viability Analysis Human breast cancer cell lines MCF-7, MDA-MB-231 and MDA-MB-435 were maintained in DMEM and RPMI media, respectively, containing 10% FBS, 100 units/mL penicillin and 1 µg/mL streptomycin. Cells were grown at 37°C in a 5% CO₂ humidified atmosphere.

Cells (5000 cells/well) were seeded into 96-well plates. On the following day, cells were treated with different concentrations of BCA (0–60 µM) in a fresh medium and incubated for another 24 h. Cell viability was then evaluated using MTT assay and the absorbance read at 570 nm using an enzyme-linked immunosorbent assay (ELISA) microplate reader (Molecular Devices, Downington, PA, U.S.A.).

Cell Cycle Analysis To investigate the effect of BCA on cell cycle progression, DNA content was analyzed using flow cytometry analysis. Cells were treated with different concentrations of BCA for 24 h. Washed cells were trypsinized and resuspended in phosphate buffered saline (PBS) (1×10⁶/500 µL), then fixed with ice cold 70% ethanol overnight. Fixed cells were then washed twice with PBS containing 1% fetal bovine serum. Cells were treated with 100 µg/mL RNase A and incubated at 37°C for 30 min. Propidium iodide was then added to a final concentration of 50 µg/mL for DNA staining. Cells were immediately analyzed by flow cytometry using FACSscan (BD Biosciences, San Jose, CA, U.S.A.).

DJ-1 Short Interfering RNA (siRNA) or pcDNA-flag-DJ-1 Transfection Cells were transfected with DJ-1 siRNA (siDJ-1-1 sense: 5'-GGCGUUUGAAACAUUUAAACATT-3', antisense: 5'-UGUUAAUGUUUACAGCTG-3', and siDJ-1-2 sense: 5'-CGACGCAUCUUGAGAAAATT-3', antisense: 5'-UUUUCUAAUGUGUAGCGCA-3') (Qiagen, Valencia, CA, U.S.A.). Briefly, 1×10⁵ cells were seeded in 60-mm plates and the media replaced on the following day with serum-free media just prior to transfection. Cells were then transfected with DJ-1 siRNA at a final concentration of 10 nm using Lipofectamine™ 2000 (L2K, Invitrogen, Carlsbad, CA, U.S.A.) as a transfecting agent in Opti-MEM (Invitrogen). After 12 h, serum-free media containing the transfection mixture were replaced with fresh serum-containing media. For each transfection, a mock no transfection control (L2K only) is included. To investigate the effect of DJ-1 siRNA on cell viability, a proper number of cells were transfected in 96-well plates for three days and MTT assay was performed. For exogenous DJ-1 overexpression, pcDNA-flag-DJ-1 vector (provided by Dr. Yook at Yonsei University, Seoul, Korea) or pcDNA-flag empty vector were transfected under the same conditions.

Quantitative Real Time PCR To investigate the effect of BCA on DJ-1 mRNA expression, real time qRT-PCR analysis was performed. In brief, 1×10⁵ cells were plated in 60-mm dishes and after 24 h, the cells were treated with BCA for 24 h more. Total RNA was extracted with Qiazo (Qiagen, Valencia, CA, U.S.A.) according to the manufacturer’s instructions. Five micrograms of extracted RNA was reverse transcribed into cDNA using a first strand cDNA synthesis kit (Applied Biosystems, Fosters city, CA, U.S.A.), and the resulting cDNA was diluted 10-fold and kept at −20°C until use. The real time RT-PCR primers were designed using Primer Express 1.5 software (Applied Biosystems) as follows: DJ-1 forward, 5’-GTC ATTGTCCTGATGCCACG-3’ and DJ-1 reverse, 5’-TCAAGTAAATCTCTGTGCCC-3’; glyceraldehyde-3-phosphate dehydrogenase (GAPDH) forward, 5’-AGATCATCAGCAATGCTCT-3’ and GAPDH reverse, 5’-ATGGCAGCTGACTGT GTCATG-3’. DJ-1 expression was normalized with GAPDH as a housekeeping gene. Real time PCR was carried out using the ABI Prism 7500 sequence detection system (Applied Biosystems, U.S.A.). Ten microliters of SYBR Green PCR master mix, 4 µL of diluted cDNA, and 200 nM primer set were used for amplification in 20 µL reaction mixture. All samples were amplified in triplicate in a 96-well plate and the cycling conditions being as follows: 2 min at 50°C, 10 min at 95°C, and 40 cycles at 95°C for 15 s followed by 1 min at 60°C. The values of Acy whole threshold (ACt) were calculated by normalizing the average Ct value of each treatment compared to its opposite endogenous control (GAPDH) and then calculating 2−ΔΔCt for each treatment. Statistical analysis of the data was performed as previously described.28) These experiments were repeated three times.

Western Blot Analyses Cells were washed twice with cold PBS, after which 200 µL of PRO-PREP protein extraction solution (Intron, Daejon, South Korea) was added. Cell lysates were centrifuged and protein concentrations were estimated using the Coomassie protein assay reagent (Thermo Scientific, Rockford, IL, U.S.A.). Forty micrograms of protein samples were electrophoresed on 10–15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels. Proteins were transferred to nitrocellulose membranes, which were blocked in 5% skim milk in TBS (25 mM Tris base and 150 mM NaCl) for 2 h at room temperature, and then incubated with primary antibody overnight at 4°C. Horseradish peroxidase-conjugated secondary antibody was used at 1:5000 dilutions for 1 h at room temperature and then washed three times in TBST (TBS and 0.1% Tween 20). The target proteins were detected with ECL detection reagents and the relative intensity of the bands was analyzed using Image-J software.

Immunocytochemical Analysis Cells were cultured in 6-well plates containing cover slides. On the following day, the cells were treated with 30 µM BCA and after 24 h were
Cells were immediately fixed in 4% paraformaldehyde for 1 h and then washed with PBS. Cells were cleared in 0.1% Triton X-100 in 0.1% sodium citrate at pH 6 for 5 min, followed by three washes in PBS. Cells were blocked by adding Tris buffered saline solution (TBS) containing BSA (0.05 M TBS + 3 drops of albumin serum) for 1 h at room temperature. To assess the mitochondrial translocalization of DJ-1 protein after BCA treatment, cells were immunostained with DJ-1 antibody and MitoTracker Green probe, and the fluorescence images were observed under Confocal laser scanning microscope (LSM 510 Pascal, Carl Zeiss, Thornwood, NY, U.S.A.).

Mitochondrial Membrane Potential Analysis
MMP was measured using a lipophilic cationic probe, 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethyl-benzimidazolocarbocyanineiodide (JC-1). After incubating cells (10000/well) in a 96-well plate with 30 µM of HCA for 1 to 12 h, cells were stained with 10 mg/mL JC-1 at 37°C for 10 min. JC-1 detects a decrease in MMP, which fluoresces green. However, at high concentrations, aggregation occurs and both the absorption and emission spectra shifts to a longer wavelength, which fluoresces red. In cells, aggregate formation increases linearly with increasing membrane potential. A 485 nm filter was used in the fluorescence microplate reader for excitation of JC-1. In the first run of each plate, a 590 nm emission filter was used to detect a total orange fluorescence (JC-1 aggregates) of the sample. A second run was performed on each plate with a 525 nm emission filter to detect green fluorescence (JC-1 monomers). The relative ratio of A590 (red) : A525 (green) was calculated as an indicator of MMP. A decrease in MMP led to a decrease in the ratio of red to green fluorescence.

Statistical Analysis
The differences in mean values among groups were evaluated and expressed as the means ± S.D. Averages were drawn and the statistical calculations were carried out by Student t-test using Microsoft Excel-2007 software.
RESULTS

BCA Decreased Cell Viability and Modulates Cell Cycle Progression  The effect of BCA on cell viability was investigated using MTT assay in breast cancer cell lines. Although the origin of MDA-MB-435 cell line has been disputed, it has many properties similar to those of other aggressive breast cancer cell lines.29 Cell viability was inhibited in all three cells after BCA treatment in a dose-dependent manner, though antiproliferation was more effective in MDA-MB-435 (IC50, 24.5 µM) and MDA-MB-231 (IC50, 27.7 µM) when compared to MCF-7 cells (IC50, 57.25 µM) (Fig. 1A). We evaluated the effect of BCA on cell cycle progression in MCF-7 and MDA-MB-435 cells. BCA-induced apoptosis (sub-G1) is stronger in MDA-MB-435 cells (Fig. 1B). The cell population in the sub-G1 phase increased from 3.3% in the control to 35.8% in the BCA-treated MDA-MB-435 cells. Under the same experimental conditions, BCA increased the sub-G1 apoptotic cells from 2.4 to 19.1% in MCF-7 cells.

BCA Induces Differential Expression of DJ-1 in Both Cell Lines  To investigate the effect of BCA on DJ-1 expression, we analyzed early time-dependent expression change of DJ-1 protein under BCA treatment. The basal expression level of DJ-1 was higher in MDA-MB-435 than in MCF-7 (Fig. 2A). In MCF-7 cells, 30 µM BCA increases DJ-1 expression 1.5-fold within 3–6 h (Fig. 2B). However in MDA-MB-435 cells, there was a remarkable decrease of DJ-1 expression after only 3–6 h at the same condition (Fig. 2B). SP1, a transcription factor activating DJ-1 promoter, shows similar patterns of expression found in DJ-1.

DJ-1 Silencing Sensitizes MCF-7 Cells to BCA More Effectively  We investigated the role of DJ-1 in MCF-7 cell protection. We silenced DJ-1 in MCF-7 cells by transfection of two DJ-1 siRNA sets (siDJ-1-1 and siDJ-1-2) for three days. The transfection efficiency was investigated using real time qPCR and western blot analyses (Fig. 3A). The modulatory effect of DJ-1 silencing on cell viability after BCA treatment was investigated using MTT assay. DJ-1 silencing significantly increased chemosensitivity to BCA in MCF-7 cells (Fig. 3B). Further, we found no differential antiproliferation with BCA treatment in siDJ-1-transfected MCF-7 cells and MDA-MB-435 control cells (Fig. 3C).

DJ-1 Overexpression Desensitizes MDA-MB-435 Cells to BCA  We overexpressed DJ-1 in MDA-MB-435 cells by transfecting pcDNA3-flag-DJ-1 vector. The Overexpression efficiency was investigated using western blot analysis (Fig. 4A). DJ-1 overexpression decreased significantly chemosensitivity to BCA in MDA-MB-435 cells (Fig. 4B). Furthermore, there was no significant difference in cell death by BCA treatment between MCF-7 control and DJ-1-overexpressed MDA-MB-435 cells (Fig. 4C).

BCA Induced Early Translocalization of DJ-1 to Mitochondria in MCF-7 Cells  DJ-1 is mainly localize to the cytoplasm, but under oxidative stress, translocates to the mitochondria within 3 h and functions as an antioxidant in neural cell system.21 Therefore, we used confocal microscopy to evaluate the distribution of DJ-1 and mitochondria in both cell lines using MitoTracker dye. BCA treatment for three hours caused an increase in the overlapped signal between mitochondrial staining and DJ-1 in the majority of MCF-7 cells. MDA-MB-435 cells, however, demonstrated a remarkably reduced signal level of overlap when compared to MCF-7 cells (Fig. 5).

DJ-1 Knockdown in MCF-7 or Overexpression in MDA-MB-435 Cells Alleviated the Effect of BCA on MMP  We tried to confirm whether DJ-1 expression by BCA induces MMP perturbation in MCF-7 and MDA-MB-435 cells. BCA induced significant MMP decrease in MDA-MB-435 cells after 3–6 h; on the contrary, there was not significant MMP change in MCF-7 cells under the same experimental condition (Fig. 6A). To evaluate whether these MMP change patterns are derived from differential DJ-1 expression, siDJ-1 or DJ-1-Flag
are transfected in MCF-7 or MDA-MB-435 cells, respectively. In DJ-1-downregulated MCF-7 cells, BCA treatment results in significant MMP decrease as compared with control cells (Fig. 6B). Furthermore, DJ-1-Flag transfection in MDA-MB-435 cells recovered MMP decrease by BCA treatment (Fig. 6C).

DISCUSSION

A previous study has shown that H-ras-transformed cells are more sensitive to treatment with BCA/HCA in NIH/3T3 cells.30) Furthermore, BCA shows a differential antiproliferative effect in K-ras-transformed cells via down-regulation of thiol antioxidants.31) These results suggest that BCA has greater selective antiproliferative effects in oncogene-transformed cancer cells than in normal cells. Though the antiproliferative mechanisms of cinnamaldehyde and its derivatives have been researched extensively, their function in breast cancer remains to be elucidated.

We investigated the effect of BCA on breast cancer cell viability and its possible mechanism of action. As shown in Fig. 1, BCA shows a differential antiproliferation effect on MDA-MB-435 as compared with MCF-7 cells. It has been shown that under oxidative stress, DJ-1 has a role in antioxidant stress to prevent cell death24) and furthermore DJ-1 mediates cisplatin resistance in NSCLC.32) We thus evaluated the expression level of DJ-1 in both cell lines. SP1 protein expression was also evaluated at the same condition because SP1 transcription factor has been known to activate DJ-1 promoter.33) BCA increased the expression of DJ-1 and SP1 proteins...
in MCF-7 cells after 3–6 h. In MDA-MB-435 cells, however, BCA treatment decreased the expression of these two proteins continuously for 24 h (Fig. 2B).

To confirm whether differential DJ-1 expression might be a key regulatory factor on chemosensitivity against BCA, we investigated the effect of DJ-1 silencing or overexpression on cell viability after BCA treatment. Differential antiproliferation by BCA treatment disappeared after DJ-1 siRNA transfection in MCF-7 cells (Fig. 3C), suggesting that DJ-1 knockdown enhanced chemosensitivity to BCA in MCF-7 cells. Consistent with our results, it was reported that DJ-1 siRNA sensitizes cell death caused by oxidative and ER stresses as well as proteasome inhibitors. On the contrary, exogenous DJ-1 overexpression inhibited chemosensitivity against BCA in MDA-MB-435 cells, showing similar cell viability with that of MCF-7 cells (Fig. 4C).

To investigate the molecular mechanism of cell protective effect of DJ-1 in MCF-7 cells, we analyzed DJ-1 translocalization to mitochondria after BCA treatment. Previous study in neuroblastoma showed that DJ-1 translocates to mitochondria within 3 h upon oxidant stress, and mitochondrial targeting of DJ-1 provides a significantly stronger cytoprotection. In MCF-7, BCA induced an increase in the overlapped signal between mitochondrial staining and DJ-1 after 3 h (Fig. 5).

Previous study to determine the role of DJ-1 in Parkinson’s disease showed that DJ-1 eliminates hydrogen peroxide by oxidizing itself and that this was a prerequisite for the protection of cells against hydrogen-peroxide-induced cell death. Mitochondrial electron transport chain is one of the major sites for ROS generation, and therefore earlier DJ-1 translocalization after oxidative stress by BCA treatment would be an important mechanism to protect MCF-7 cells. Our data suggest that upregulated DJ-1 translocates to mitochondria within 3 h and might act as an antioxidant to reduce the oxidative stress induced by BCA in MCF-7 cells.

Previous study showed that the mitochondrial outer membrane permeabilization is considered to be the ‘point of no return’ as this event is responsible for executing the apoptotic cascade in numerous cell death pathways. A decrease in MMP has been reported in response to many apoptotic stimuli, and it was shown that cytochrome release from mitochondrial cristae is a consequence of the decline in MMP. Furthermore, MMP generation induces mitochondrial maintenance of its configuration in which most cytochrome c is sequestered in the cristae and is resistant to release by agents that disrupt the mitochondrial outer membrane. Our previous study showed that HCA treatment decreased MMP and at the same time, induced cytochrome c and Bax translocation between cytosol and mitochondrial membrane in SW620 colon cancer cells. Therefore, we evaluated MMP changes after BCA treatment in both cell lines.

BCA induced significant MMP decrease in MDA-MB-435 cells after 3–6 h; on the contrary, there was no significant MMP change in MCF-7 cells under the same experimental condition (Fig. 6A). To confirm whether the stable MMP in MCF-7 cells after BCA treatment is due to DJ-1 overexpression, we transfected DJ-1 siRNA in this cell line. After DJ-1 knockdown in MCF-7 cells, BCA treatment induced significant MMP decrease as compared with that of control cells. This is matching well with the previous data showing that loss of DJ-1 decreased MMP significantly in neuroblastoma cells. In MDA-MB-435 cells, DJ-1-Flag transfection recovered MMP decrease by BCA treatment. These data suggest that DJ-1 overexpression and translocalization to mitochondria in MCF-7 cells cause more resistant for mitochondrial membrane perturbation and finally hinder cell death by BCA treatment.

These data indicate that the remarkable early increase of DJ-1 protein expression in MCF-7 cells may explain why MCF-7 cells are more resistant to BCA compared to MDA-MB435 cells. Furthermore, DJ-1 protein is suggested to be participate in cancer cell protection from oxidative stress via translocalizing into mitochondria and stabilizing mitochondrial permeability. Our data suggest that DJ-1 seems to be a promising target for cancer therapy and its downregulation could serve as a potent measure of the efficiency of
chemotherapeutic and chemosensitizing agents.

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