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The contribution of activating transcription factor 3 to apoptosis of human colorectal cancer cells by Protocatechualdehyde, a naturally occurring phenolic compound

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ABSTRACT

Protocatechualdehyde (PCA) is one of the important compounds found in barley, green cavendish bananas and grapevine leaves. PCA shows anti-cancer activities in breast, leukemia and colorectal cancer cells. Previous study reported that PCA exerts anti-cancer activity through down-regulating cyclin D1 and HDAC2 in human colorectal cancer cells. However, the underlying mechanisms for the expression of activating transcription factor 3 (ATF3) by PCA has not been studied. Thus, we performed *in vitro* study to investigate if treatment of PCA affects ATF3 expression and ATF3-mediated apoptosis in human colorectal cancer cells. PCA decreased cell viability in a dose-dependent manner in HCT116 and SW480 cells. In addition, PCA reduced cell viability in MCF-7, MDA-MB-231 and HepG-2 cells. Exposure of PCA activated the levels of ATF3 protein and mRNA in HCT116 and SW480 cells. Inhibition of ERK1/2/ by PD98059 and p38 by SB203580 inhibited PCA-induced ATF3 expression and transcriptional activation. ATF3-knockdown inhibited PCA-induced apoptosis and cell viability. In addition, ATF3 overexpression enhanced PCA-mediated cleavage of PARP. These findings suggest that inhibition of cell viability and apoptosis by PCA may be result of ATF3 expression through ERK1/2 and p38-mediated transcriptional activation.

Introduction

Cancer has been regarded as a major public health problem in many parts of the world including the United States [1]. Among cancers, colorectal cancer is the third leading cause of cancer-related death in both males and females in the United States [1]. Although surgery is regarded as the most effective treatment for colorectal cancer, cancer chemoprevention using dietary factors has received attention as an effective approach to reduce colorectal cancer-related mortality. For last two decades, many researchers have tested and reported anti-cancer activities of natural products in dietary factors such as fruits, vegetables and teas [2].

Protocatechualdehyde (3,4-dihydroxybenzaldehyde, PCA) is a naturally occurring polyphenol found in barley [3], green cavendish bananas [4] and grapevine leaves [5]. PCA has been reported to have anti-atherosclerosis [6], anti-oxidant and anti-inflammatory effect [7]. In addition, PCA has anti-cancer activities in human breast cancer cells [8] and leukemia cells through inhibition of casein kinase II activity [9] and colorectal cancer cells through downregulating cyclin D1 and HDAC2 [10]. However, the other potential mechanisms of PCA for an anti-cancer activity have not been studied so far.

Activating transcription factor 3 (ATF3)[2] is a member of the ATF/CREB subfamily of the basic-region leucine zipper (bZIP) family and exhibits dual functions (tumor suppressor or tumor promoter), depending on cancer cell types. In human colorectal cancer cells, ATF3 is repressed [11] and its overexpression cancer induces apoptosis [12], which means that ATF3 may function as a tumor

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[2] *Abbreviations used:* ATF3, activating transcription factor 3; DMEM, Dulbecco's modified Eagle medium; PCA, protocatechualdehyde; FBS, fetal bovine serum; PBS, phosphate-buffered saline; BCA, bicinchoninic acid; HRP, horse radish peroxidase; IgG, immunoglobulin G; HUVEC, human umbilical vein endothelial cells.

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suppressor in human colorectal cancer. On the other hand, ATF3 shows to function as tumor promoter in hepatocytes through inducing DNA synthesis and cyclin D1 expression [13], and in breast cancer through enhancing cancer cell-initiating features [14].

In this study, we aimed to elucidate the role of ATF3 in PCA-induced apoptosis and for the first time, we demonstrated that PCA activates ATF3 overexpression through p38 and ERK1/2 activation and ATF3 expression may be required for PCA-induced apoptosis.

Materials and methods

Chemicals

Cell culture media, Dulbecco's Modified Eagle medium (DMEM)/F-12 1:1 Modified medium (DMEM/F-12) was purchased from Lonza (Walkersville, MD, USA). Protocatechualdehyde (PCA) and 3-(4,5-dimethylthizaol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were purchased from Sigma Aldrich (St. Louis, MO, USA). SB203580, PD98059 were purchased from Calbiochem (San Diego, CA, USA). ATF3 antibody and ATF3 siRNA were purchased from Santa Cruz Biotechnology, Inc (Santa Cruz, CA, USA). Antibodies against β-actin and poly (ADP-ribose) polymerase (PARP), and control siRNA were purchased from Cell Signaling (Bervely, MA, USA). ATF3 promoter constructs (−1420/+34, −718/+34, −514/+34, −318/+34, −147/+34 and −84/+34, pATF3-514 del Ftz and pATF3-514 del CRE) were kindly provided by Dr. S-H Lee (University of Maryland College Park, Maryland, USA). All chemicals were purchased from Fisher Scientific, unless otherwise specified.

Cell culture and treatment

Human cells lines such as colorectal cancer cells (HCT116 and SW480), breast cancer cells (MCF-7 and MDA-MB-231) and hepatocellular carcinoma cells (HepG-2) and colon normal cells (CCD-18co) were purchased Korean Cell Line Bank (Seoul, Korea). Cells were grown in DMEM/F-12 supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin. The cells were maintained at 37 °C under a humidified atmosphere of 5% CO_2. PCA was dissolved in dimethyl sulfoxide (DMSO) and treated to cells. DMSO was used as a vehicle and the final DMSO concentration was not exceeded 0.1% (v/v).

MTT assay

Cell viability was measured using MTT assay system. Briefly, cells were plated onto 96-well plated and grown overnight. The cells were treated with 0, 50, 100 and 200 μM of PCA for 24 and 48 h. Then, the cells were incubated with 50 μl of MTT solution (1 mg/ml) for the additional 2 h. The resulting crystals were dissolved in DMSO. The formation of formazan was measured by reading absorbance at a wavelength of 570 nm.

SDS–PAGE and Western blot

After PCA treatment, cells were washed with 1× phosphate-buffered saline (PBS), and lysed in radioimmunoprecipitation assay (RIPA) buffer (Boston Bio Products, Ashland, MA, USA) supplemented with protease inhibitor cocktail (Sigma Aldrich) and phosphatase inhibitor cocktail (Sigma Aldrich), and centrifuged at 15,000×*g* for 10 min at 4 °C. Protein concentration was determined by the bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL, USA). The proteins were separated on SDS–PAGE and transferred to PVDF membrane (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The membranes were blocked for non-specific binding with 5% nonfat dry milk in Tris-buffered saline containing 0.05% Tween 20 (TBS-T) for 1 h at room temperature and then incubated with specific primary antibodies in 5% nonfat dry milk at 4 °C overnight. After three washes with TBS-T, the blots were incubated with horse radish peroxidase (HRP)-conjugated immunoglobulin G (IgG) for 1 h at room temperature and chemiluminescence was detected with ECL Western blotting substrate (Amersham Biosciences) and visualized in Polaroid film.

Reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was prepared using a RNeasy Mini Kit (Qiagen, Valencia, CA, USA) and total RNA (1 μg) was reverse-transcribed using a Verso cDNA Kit (Thermo Scientific, Pittsburgh, PA, USA) according to the manufacturer's protocol for cDNA synthesis. PCR was carried out using PCR Master Mix Kit (Promega, Madison, WI, USA) with primers for human ATF3 and human GAPDH as follows: human ATF3: 5′-gtttgaggattttgctaacctgac-3′, and reverse 5′-agctgcaatcttatttctttctcgt-3′; human GAPDH: forward 5′-acccag aagactgtggatgg-3′ and reverse 5′-ttctagacggcaggtcaggt-3′.

Transient transfections

Transient transfections were performed using the PolyJet DNA transfection reagent (SignaGen Laboratories, Ijamsville, MD, USA) according to the manufacturers' instruction. HCT116 and SW480 cells were plated in 12-well plates at a concentration of 2×10^5 cells/well. After growth overnight, plasmid mixtures containing 0.5 μg of ATF3 promoter linked to luciferase and 0.05 μg of *pRL-null* vector were transfected for 24 h. The transfected cells were cultured in the absence or presence of PCA for the indicated times. The cells were then harvested in 1× luciferase lysis buffer, and luciferase activity was normalized to the *pRL-null* luciferase activity using a dual-luciferase assay kit (Promega).

Transfection of small interference RNA (siRNA)

The cells were plated in six-well plates and incubated overnight. HCT116 cells were transfected with control siRNA and ATF3 siRNA for 48 h at a concentration of 100 nM using TransIT-TKO transfection reagent (Mirus, Madison, WI, USA) according to the manufacturer's instruction. Then the cells were treated with 100 μM of PCA for 48 h.

Cell death assay

Cell death was performed using Cell Death Detection ELISA^{PLUS} Kit (Roche Diagnostics, Indianapolis, IN, USA) according to the manufacturer's instruction. Briefly, ATF3 siRNA-transfected HCT116 cells were treated with 0 and 100 μM of PCA for 48 h. After the PCA treatment, the cytosol was prepared using Nuclear Extract Kit (Active Motif). Equal amounts of cytosolic extracts, immunoreagent containing anti-histone-biotin, and anti-DNA-POD were added to microplate well and incubated for 2 h under shaking. After washing, the ABTS solution was added to each well for 20 min and then the ABTS stop solution was added. The absorbance was recorded at 405 nm and 490 nm in an enzyme-linked immunosorbent assay plate reader (Bio-Tek Instruments Inc.).

Statistical analysis

Statistical analysis was performed with the Student's unpaired *t*-test, with statistical significance set at $^*P < 0.05$.