FEBS Letters 584 (2010) 3185–3192

journal homepage: www.FEBSLetters.org

The role of enoyl-CoA hydratase short chain 1 and peroxiredoxin 3 in PP2-induced apoptosis in human breast cancer MCF-7 cells

Xiang Liu, Renqing Feng *, Liying Du

College of Life Sciences, Peking University, Beijing 100871, PR China

ARTICLE INFO

Article history:
Received 12 April 2010
Revised 25 May 2010
Accepted 2 June 2010
Available online 10 June 2010

Edited by Quan Chen

Keywords:
Breast cancer
Apoptosis
4-Amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine
Enoyl-CoA hydratase short chain 1
Peroxiredoxin 3

ABSTRACT

We show that 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine (PP2) induces apoptosis and down-regulates the expression of enoyl-CoA hydratase short chain 1 (ECHS1) and peroxiredoxin 3 (PRDX3) in human breast cancer MCF-7 cells. The decrease of ECHS1 and PRDX3 was validated by Western blot and quantitative real-time reverse transcription-PCR in MCF-7 and other carcinoma cells. Knockdown and over-expression of ECHS1 and/or PRDX3 further supported the key role of ECHS1 and PRDX3 in regulation of PP2-induced apoptosis. These results suggest a possible apoptotic pathway whereby down-regulation of ECHS1 and PRDX3 potentiates PP2-induced apoptosis in MCF-7 cells.

1. Introduction

Breast cancer is now the most prevalent cancer in women worldwide [1]. Although endocrine therapy brings improvement to breast cancer, it inevitably results in recurrence with uncontrolled growth and metastasis [2,3]. c-Src, a non-receptor tyrosine kinase, plays key roles in mediating many functions during the progression of cancer. c-Src itself is weakly oncogenic [4], but phosphorylation of c-Src activates downstream targets, and therefore it can regulate many cellular processes. Because of its pivotal roles in many signal pathways that regulate cell growth, adhesion, migration and survival, c-Src may be characterized as a therapeutic target for cancers [5–7].

Recent interest in c-Src as a therapeutic target for cancer has led to the development of small molecule inhibitors [8,9]. Among these inhibitors, PP2 (4-amino-5-(4-chlorophenyl)-7-(*t*-butyl) pyrazolo[3,4-*d*]pyrimidine) was first identified as a pyrazolopyrimidine compound that fits into the ATP binding site of c-Src and therefore is a potent and selective inhibitor of the c-Src family kinases [10]. PP2 has been reported to induce apoptosis in murine B cell leukemia [11]. PP2 has also been reported to potentiate the apoptosis in human neuroblastoma cells [12]. Although many characters of PP2-induced apoptosis have been described, the molecular mechanism remains unknown. In addition, PP2 has not been studied for its effect on mitochondrial protein expression in breast cancer cells.

In the present study, we observed that PP2 induced apoptosis in MCF-7 cells with a concomitant decrease of mitochondrial membrane potential (MMP) and an alteration of Bcl-2 family proteins, suggesting the possible mitochondria-mediated apoptotic pathway. Using two-dimensional electrophoresis and mass spectrometry, we identified two significantly down-regulated mitochondrial proteins, enoyl-CoA hydratase short chain 1 (ECHS1) and peroxiredoxin 3 (PRDX3) after c-Src suppression by PP2. RT-PCR and Western blot analysis demonstrated the down-regulation of ECHS1 and PRDX3 at the transcription and protein levels in MCF-7 and other carcinoma cells. In addition, we provide evidence that ECHS1 and PRDX3 may play a key role in PP2-induced apoptosis by using knockdown and over-expression strategy. These findings provide important insights into c-Src suppression by PP2. Thus, for the first time ECHS1 and PRDX3 are shown to be down-regulated after c-Src suppression.

Abbreviations: PP2, 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine; ECHS1, enoyl-CoA hydratase short chain 1; PRDX3, peroxiredoxin 3; MMP, mitochondrial membrane potential

* Corresponding author. Address: Department of Biochemistry and Molecular Biology, College of Life Sciences, Peking University, Beijing 100871, PR China. Fax: +86 10 62751526.
E-mail address: rqfeng@pku.edu.cn (R. Feng).

0014-5793/$36.00
doi:10.1016/j.febslet.2010.06.002

2. Materials and methods

2.1. Cell culture

All cell lines used in this work were obtained from American Type Culture Collection (Manassas, VA). Cells were seeded at an initial concentration of 10^5 cells/ml in DMEM (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS, Hyclone, Pittsburgh, PA), 3.75 g/L sodium bicarbonate, 100 U/mL penicillin and 100 μg/mL streptomycin. Cells were cultured at 37 °C in a humidified incubator containing 5% CO_2.

2.2. Cell growth assay

MCF-7 cells were seeded in 96-well plates (Costar, Corning, NY) with complete culture medium (DMEM containing 10% FBS) and grown for 24 h. Cells were then cultured with different concentrations of PP2 (Merck, Whitehouse Station, NJ) or without PP2 for 48 h. At the end of the treatment period, 20 μL 3-(4,5)-dimethylthiahiazo (−z−y1)-3,5-di-phenytetrazoliumromide (MTT, Amresco, Solon, OH) was added to each well and cells were incubated at 37 °C for 3 h. The reacting product formazan crystals were dissolved in 200 μL DMSO (Sigma, St. Louis, MO) after removing the supernatant. After shaking plates for 15 min, the optical density of the wells was measured at the wavelength of 570 nm with Microplate Reader (Bio-Rad, Hercules, CA). Each concentration of PP2 was performed in three wells and cell growth assay was repeated for three times.

2.3. Protein extraction for two-dimensional gel electrophoresis (2-DE)

MCF-7 cells were cultured for 30 h in DMEM containing 0.2% FBS for the synchronization of cell cycle, which was also evaluated by FACS analysis. Cells were collected before PP2 treatment or after 10 μM PP2 treatment for 12 h, 24 h and 30 h. Cells were washed three times with washing buffer (0.25 M sorbitol, 0.01 M Tris, pH 7.5) and centrifuged at 500×*g* for 10 min at 4 °C. The cell pellet was resuspended in lysis buffer (8 M urea, 2 M thiourea, 4% CHAPS, 40 mM DTT, containing protease inhibitor cocktail, Roche, Mannheim, Germany). Total cell lysates were centrifuged at 16 000×*g* for 1 h at 4 °C. The supernatant was collected and purified with 2-D Clean-Up Kit (Amersham Pharmacia Biotech, Piscataway, NJ). Protein concentration was determined by 2-D Quant Kit (Amersham Pharmacia Biotech).

2.4. 2-DE and mass spectrometry analysis

2-DE was carried out according to the protocol described before [13,14]. Briefly, protein samples (70 μg for analytical gel, 1 mg for preparative gel) were loaded on 24 cm Immobiline 4–7 linear DryStrips (Amersham Pharmacia Biotech) and IEF was run at 30 V for 8 h, 50 V for 4 h, 100 V for 1 h, 300 V for 1 h, 500 V for 1 h, 1000 V for 1 h, and 8000 V for 12 h. Then second dimensional SDS–PAGE was carried out in the Ettan Dalt Six Elect Unit 230 (Amersham Pharmacia Biotech) at 2 W/gel for 45 min and then 15 W/gel until the dye front reached the bottom of the gel. The analytical gels for quantification were silver stained and the preparative gels were stained with PhastGel Blue R (CBB R350, Amersham Pharmacia Biotech). Spot detection, quantification and analysis were performed using Imagemaster 2D Software (version 4.01, Amersham Pharmacia Biotech). The "Total Spot Volume Normalization" method was used in order to correct the differences in sample loading or stain intensity among gels. Spots of interest were excised from 2-D gels that were stained with CBB R350 and followed by in gel digestion. All mass spectra from MALDI-TOF MS were obtained on an Ultraflex TOF/TOF (Bruker Daltonics, Bremen, Germany) in positive ion mode at an accelerating voltage of 20 kV using CHCA as the matrix. The spectra were internally calibrated using trypsin autolysis products. All PMFs obtained were used to search the NCBI database using the MASCOT search engine (http://www.matrixscience.com/) with a tolerance of ±100 ppm and one missed cleavage site.

2.5. Western blot analysis

Twenty micrograms of cell lysates of each sample was separated by 12.5% SDS–PAGE. Proteins were transferred onto PVDF membrane (Millipore) and incubated with primary antibodies at optimal dilution at 4 °C overnight, followed by incubating with secondary antibody (HRP-conjugated goat anti-mouse IgG was used, Jackson ImmunoResearch, West Grove, PA) at 37 °C for 1 h. The immunoblot was visualized with Enhanced Western Luminescent Detection Kit (Vigorous, Beijing, China). All Western blots were performed at least three times for each experiment. The relative amount of immunoblot band was measured by densitometry using Imagemaster 2D Software.

Monoclonal antibodies to peroxiredoxin 3, Bcl-2 and Bax were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal antibody to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was obtained from Proteintech Group (Chicago, IL). Monoclonal antibody to enoyl-CoA hydratase short chain 1 was a generous gift from Dr. Jian'en Gao (Beijing Proteome Research Center, China).

2.6. Quantitative real-time reverse transcription-PCR (qRT-PCR) analysis

Total RNA was extracted by TRIzol reagent (Invitrogen) according to the suggested protocol. RNA (5 μg) was used to synthesize the first-strand cDNA with the Superscript first-strand synthesis system (Invitrogen) for RT-PCR according to the manufacturer's recommendation. Gene-specific primers for RT- and qRT-PCR analyses were synthesized commercially at AuGCT Biotechnology (Beijing, China) as follows, *ECHS1*: 5′-CGCTGCTGTCAATGGCTATG-3′ (forward) and 5′-CTTGGCGTCCTGGGCTGAGA-3′ (reverse); *PRDX3*: 5′-GCCGTTGTCAATGGAGAGTTC-3′ (forward) and 5′- GCAAGATGGCTAAAGTGGGAA-3′ (reverse). Human *GAPDH* gene was used as internal control (forward primer, 5′-GGGAGCCAAAAGGGTCATCATC-3′; reverse primer, 5′-CCATGCCAGTGAGCTTCCCGTTC-3′). The qRT-PCR was performed using the SYBR Green qPCR Kits (NEB, Ipswich, MA) and a DNA Engine Opticon continuous fluorescence detection system (Bio-Rad) according to the manufacturer's instruction. All results were obtained from at least three independent experiments using different RNA samples.

2.7. Analysis of apoptosis by flow cytometry

After different treatment, apoptotic cells were detected by staining with FITC-conjugated Annexin V and propidium iodide (PI) using a commercially available apoptosis detection kit (Biosea, Beijing, China). Briefly, 2×10^5 cells were collected, washed three times with PBS and resuspended in binding buffer. After incubation for 15 min with FITC-conjugated Annexin V and PI according to the manufacturer's instructions, apoptotic cells were analyzed by FACS Calibur flow cytometer. The Summit software (version 4.0, DakoCytomation, CO) was used to determine the number of apoptotic cells.

2.8. Measurement of mitochondrial membrane potential (MMP)

Mitochondrial membrane potential was measured using fluorescent probe tetramethylrhodamine, methyl ester, perchlorate (TMRM, Invitrogen). After different treatment, 20 nM TMRM was