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Celastrol suppresses breast cancer MCF-7 cell viability via the AMP-activated protein kinase (AMPK)-induced p53–polo like kinase 2 (PLK-2) pathway

Ji Hae Kim, Jung Ok Lee, Soo Kyung Lee, Nami Kim, Ga Young You, Ji Wook Moon, Jie Sha, Su Jin Kim, Sun Hwa Park, Hyeon Soo Kim *

Department of Anatomy, Korea University College of Medicine, Seoul, Republic of Korea

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ABSTRACT

Celastrol, an anti-oxidant flavonoid that is widely distributed in the plant kingdom, has been suggested to have chemopreventive effects on cancer cells: however, the mechanism of this process is not completely understood. In this study, we found that celastrol suppressed the viability of breast cancer MCF-7 cells in an AMP-activated protein kinase (AMPK)-dependent fashion. Celastrol also induced an increase in reactive oxygen species (ROS) levels, leading to AMPK phosphorylation. Protein kinase C (PKC) zeta was also shown to play a role in celastrol-induced ROS generation. In addition, celastrol increased phosphorylation of the pro-apoptotic effector, p53. Inhibition of AMPK blocked celastrol-mediated p53 phosphorylation. Moreover, celastrol increased the expression of tumor suppressor polo like kinase-2 (PLK-2) in a p53-dependent manner. Neither celastrol-induced PLK-2 induction nor celastrol-mediated apoptosis inducing factor poly(ADP-ribose) polymerase-2 (PARP-2) induction was observed in p53 knock-out cells. Furthermore, add-back of PLK-2 resulted in an increase in both celastrol-mediated PARP-2 induction and celastrol-induced apoptotic index sub G1 population. Together, these results suggest that celastrol may have anti-tumor effects on MCF-7 cells via AMPK-induced p53 and PLK-2 pathways.

1. Introduction

Celastrol is a pentacydic triterpenoid. Several studies have demonstrated that celastrol has therapeutic potential in many diseases, including autoimmune diseases, asthma, chronic inflammation and neurodegenerative disease [1–4]. Celastrol can also inhibit the growth of tumor cells, e.g. prostate cancer [5], leukemia [6], and melanoma [7]. Studies that have examined its therapeutic effects have shown that celastrol affects NF-κB signaling [8], angiogenesis [9], heat-shock protein (HSP) 90 [10], and extracellular signal-regulated kinase (ERK) [11]. These findings led us to determine whether celastrol can modulate cancer apoptosis, and, if so, through which mechanism.

AMP-activated protein kinase (AMPK) belongs to a family of serine/threonine kinases. AMPK is a cellular fuel sensor that monitors the AMP/ATP ratio during maintenance of cellular homeostasis [12]. Several metabolic stressors, including hypoxia, exercise, and starvation, have been shown to lead to activation of AMPK [13,14]. AMPK also induces apoptosis in several cell types [15–18]. These results suggest that AMPK signaling could be a potential therapeutic target for cancer.

Due to its pivotal role in tumorigenesis, cell death and survival, p53 is one of the most widely studied proteins. It is a transcription factor that mediates cell response to many types of harmful stress [19]. Activation of the expression of genes that induce cell cycle regulation, DNA repair, senescence, and cell death by p53 in cells exposed to a variety of insults, including DNA damage, hypoxia, and oxidative, is also well established [20]. Metabolic alterations is a determinate of the response of cancer cells to variable nutrient conditions, as well as their regulation of cell proliferation [21], indicating that p53 plays an important role in metabolic shifting in cancer cells. One study reported that AMPK resides within the complex signaling process that connects cell metabolism with cell proliferation [22]. Nevertheless, the metabolic pathways that delineate cell proliferation remain an exciting area of investigation in cancer research.

In light of earlier reports suggesting that AMPK plays a role in cellular apoptosis, a significant amount of attention has been focused on inhibition of tumor cell growth. In the current study, we investigated the effects of celastrol on the activity of AMPK, in an effort to explore new therapeutic possibilities for the use of AMPK as an anti-tumor agent.

2. Materials and methods

2.1. Materials

Antibodies against AMPK, phospho-AMPK (Thr^{172}), phospho-ACC (Ser^{79}), phospho-p53 (Ser^{20}), and ACC were purchased from Cell

Abbreviations: ACC, acetyl-CoA carboxylase; AICAR, 5-aminoimidazole-4-carboxyamide-1-D-ribofuranoside; AMPK, AMP-activated protein kinase; PKC zeta, protein kinase C zeta; PLK-2, polo like kinase-2.

* Corresponding author at: 126-1, Anam-dong 5-ga, Seongbuk-gu, Seoul 136-705, Republic of Korea. Tel.: +82 2 920 6151; fax: +82 2 920 5696.
E-mail address: anatomykim@korea.ac.kr (H.S. Kim).

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Signaling Technology (New England Biolabs, Beverly, MA, USA) and protein kinase C zeta, poly (ADP-ribose) polymerase-2 (PARP-2), and GAPDH were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Polo like kinase 2 (PLK-2) antibody was purchased from GeneTex (Irvine, MA, USA). Phospho-PKC zeta (Thr^{560}) antibody was purchased from Epitomics Inc. (Burlingame, California). P53 antibody was purchased from BD Biosciences (Franklin Lakes, NJ, USA). Horseradish peroxidase (HRP)-conjugated secondary antibodies were obtained from the Kirkegaard and Perry Lab (Gaithersburg, MD, USA). Celastrol, *N*-acetyl-cysteine (NAC) and Hydrogen peroxide (H_2O_2) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Compound C and 5-aminoimidazole-4-carboxy-amide-1-D-ribofuranoside (AICAR) were obtained from Calbiochem (San Diego, CA, USA). Flag-PLK-2 plasmid was provided by Dr. Ingrid Hoffmann (German Cancer Research center, Heidelberg).

2.2. Cell culture

MCF-7, HCT116-p53 wild type and HCT116-p53 knock-out cells were grown in RPMI-1640 (GIBCO™, Auckland, NZ, USA) containing 0.584 g/l of L-glutamate and 4.5 g/l of glucose, 100 g/ml of gentamicin, 2.5 g/l of sodium carbonate and 10% heat-inactivated fetal bovine serum (FBS).

2.3. Immunoblot analysis

Cells were grown in 6-well plates. After treatment with the indicated agents, the medium was aspirated and the cells were washed twice in ice-cold phosphate-buffered saline (PBS), then lysed in 100 μl of lysis buffer (50 mM Tris–Cl [pH 7.4], 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate [SDS], 1 mM ethylenediaminetetraacetic acid [EDTA], and 150 mM NaCl). Lysed samples were briefly sonicated, heated for 5 min at 95 °C, and centrifuged for 5 min. Supernatants were subjected to 8% sodium dodecyl sulfate polyacrylamide electrophoresis (SDS-PAGE) and the separated proteins were transferred to polyvinylidene difluoride membranes. Blots were incubated overnight at 4 °C with primary antibody, washed 6 times in Tris-buffered saline/0.1% Tween 20, and probed for 1 h with the HRP-conjugated secondary antibody at room temperature. An enhanced chemiluminescence (ECL) (Amersham Biosciences, Buckinghamshire, UK) was used for visualization of blots.

2.4. Silencing of AMPKα

MCF-7 cells were seeded in 6-well plates and allowed to grow to a confluency of 70% over 24 h. Transient transfections were performed with transfection reagent (Lipofectamine 2000, Invitrogen, Carlsbad, CA, USA), according to the manufacturer's protocol. Briefly, AMPKα siRNA (NM_001013367; Dharmacon, Lafayette, CO, USA) was bought. One μl of siRNA and 5 μl of transfection reagent (Lipofectamine 2000) were diluted with 95 μl of serum medium (Opti-MEM; Invitrogen) and then mixed. The mixture was incubated for 10 min at room temperature and added drop-wise to each culture well, which contained 800 μl of Opti-MEM (final siRNA concentration, 40 nM). Four hours after transfection, the medium was changed with fresh complete medium. Cells were cultivated for 48 h, lysed, and expression of AMPKα protein was assessed by Western blotting.

2.5. 3-(4, 5-dimethythiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide (MTT) assay

The MTT assay was used as a crude measure of cell viability. MTT is converted by metabolically active cells into a colored water-insoluble formazan salt. MCF-7 cells were seeded at a density of 10×10^4/ml in 96-well plates and grown for 24 h. The growth medium was replaced with serum-free medium 24 h prior to treatment. Subsequently, MTT reagents (10 μl/well [7.5 mg/ml in PBS]) were added and the culture was incubated for 30 min at 37 °C. The reaction was stopped by the addition of acidified triton buffer (0.1 M HCl and 10% [v/v] Triton X-100 [50 μl/well]), and tetrazolium crystals were dissolved by mixing on a plate shaker at room temperature for 20 min. Samples were then analyzed using a plate reader (Bio-Rad 450, Richmond, CA, USA) at a 595 nm test wavelength and a 650 nm reference wavelength. Results are from experiments repeated at least in triplicate.

2.6. Assay of intracellular Reactive Oxygen Species (ROS) by confocal microscope

H_2DCFH-DA (Molecular Probe/Invitrogen, Carlsbad, CA, USA) is a ROS-sensitive probe that can be used for the detection of ROS production in living cells. It diffuses passively into cells, where its acetate groups are cleaved by intracellular esterases, releasing the corresponding dichlorodihydrofluorescein derivatives, H_2DCF. H_2DCF oxidation yields a fluorescent adduction, dichlorofluorescein (DCF), which is trapped inside the cell. Cells were treated with celastrol for 24 h. Cells were incubated at 37 °C with 20 μM H_2DCFH-DA for 30 min and then the fluorescence was measured using confocal microscope (Zeiss LSM 510, Carl Zeiss). Using the argon laser, DCF fluorescence was excited at 488 nm. Emission was detected using a 530 nm long pass filter.

2.7. Detection of intracellular ROS by FACS

Cells were treated with celastrol for 24 h. Cells were washed twice in PBS and incubated with 20 μM H_2DCFH-DA at 37 °C according to the manufacturer's instruction. H_2DCFH-DA was deacetylated intracellularly by nonspecific esterase, followed by further oxidation by ROS to the fluorescence compound 2,7-dichlorofluorescein (DCF). DCF fluorescence was detected using a FACS caliber flow cytometer (Becton-Dickinson) at an excitation wavelength of 488 nm and an emission wavelength of 525 nm. The fluorescence of 10,000 cells was measured by gating the PMN population and analyzed using the Cell Quest program to determine the mean fluorescence.

2.8. Semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted using the Trizol reagents (Life Technologies, MD), according to the manufacturer's instructions. First strand cDNA synthesis was performed using 1 μg of total RNA, which was isolated from frozen tissues at 55 °C for 20 min using a Thermoscript II one-step RT-PCR kit (Invitrogen, Paisly, UK). Amplification of cDNA was performed in the same tube using the Gene Amp System 9700 thermocycler (Applied Biosystems, Warrington, UK). Heating at 94 °C for 5 min was used to inactivate the reverse transcriptase. PCR primers were designed using the following sequence of the PLK-2 primer: forward 5′-TCAGCAACCCAGCAAACACAGG-3′, reverse 5′-TTTCCAGACATCCCCGAAGAACC-3′, beta-actin primer: forward 5′-ACTACCTCATGAAGATCCTCA-3′, reverse 5′-CAGGAGGAGCAATGATCTTGA-3′. The following PCR conditions were used: 35 cycles for 30 s at 94 °C, 30 s at 60 °C, and 30 s at 72 °C, followed by 10 min at 72 °C. The number of PCR cycles was optimized to ensure amplification in the exponential phase. Ten μl samples from each RT-PCR product were removed and analyzed by agarose gel electrophoresis. Bands were stained with ethidium bromide and visualized under ultraviolet light.

2.9. Cell cycle analysis

Cells treated with PI can be utilized to assess not only the stages of the cell cycle (G_0/G_1, S, G_2/M), but also for identification of apoptotic cells (hypodiploid, sub G_0 peak). Half a million cells were pelleted at