Modulation of Cancer Cell Proliferation by Cell Survival Signal Akt and Tumor Suppressive Energy Sensor AMP-activated Protein Kinase in Colon Cancer Cells Treated with Resveratrol

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Abstract It has been well known that resveratrol inhibits the proliferation of various cancer cells. AMP-activated protein kinase (AMPK), a sensor of cellular energy status, has emerged as a potent target for cancer prevention and/or treatment. It has been found that the activation of AMPK by resveratrol was crucial for the inhibition of HT-29 colon cancer cells. Resveratrol strongly inhibited phosphorylation of Akt. The possibility whether AMPK activation was essential to the inhibition of p-Akt was investigated in resveratrol-treated cancer cells. The inhibitory effect of resveratrol on Akt was not observed when AMPK activities were blocked by the treatment with AMPK siRNA at a relatively lower level of resveratrol. However, the higher concentrations of resveratrol inhibited Akt without the activation of AMPK. Therefore, it was concluded that resveratrol could modulate Akt AMPK-dependently or AMPK-independently. The inhibition of Akt along with the activation of AMPK may contribute to the unraveling anti-cancer mechanism of resveratrol.

Keywords: Akt, AMP-activated kinase, LY294002, resveratrol, HT-29 colon cancer cell

Introduction

Cancer is one of the most frequently occurring diseases in the world. Especially, colon cancer patients are increasing steadily in Asian and Western countries. Colon cancer seems alarming because of the relative high death or reoccurrence rates and anti-cancer agent resistancy (1). Many phytochemicals exhibit anti-inflammatory and anti-cancer effects through the regulation of intracellular signaling pathway. Resveratrol, one of the grape polyphenol, is known to exert anti-cancer and anti-inflammatory effects (2). Resveratrol induced inhibition of anti-apoptotic signaling pathway such as phosphatidylinositol-3 kinase (PI3K)/AKT pathway, mitogen-activated protein kinase (MAPK) pathway or by the regulation of nuclear factor-κB (NF-κB), Bcl-Xl, and extracellular-signal-regulated kinase (ERK)1/2 (3-6).

AMP-activated protein kinase (AMPK), a serine/threonine protein kinase, emerges as one of the most significant molecules in intracellular signaling pathways. AMPK regulates cellular energy consumption, glucose homeostasis, and apoptosis (7). Metformin, a well-known anti-diabetes drug, is known to be an activator of AMPK signaling pathway. Metformin inhibits cell proliferation by up-regulating AMPK in several cancers such as breast, colon, and pancreatic cancer cells (8-10). AMPK regulates the activation of tumor suppressor gene, p53 which induces cell cycle arrest (11). Also AMPK inhibits cancer cell survival signaling pathway such as PI3K/Akt signaling pathway (12).

Recently, a report that LKB1/AMPK pathway inhibits Akt activity in metformin-treated breast cancer cells suggests there is the connection between AMPK and Akt regulation (13). Akt, a serine/threonine protein kinase, regulates intracellular signals involved in metabolism, apoptosis, growth, and survival. Akt is consisted of 3 families Akt1, Akt2, and Akt3 (14). Among them, Akt1 is importantly participated in the proliferation of many cancer cells, and it
carries important functions of tumor development and angiogenesis. Activation of Akt enhances NF-κB, BCL-2 family, and murine double minute (MDM2) activities (15).

The reports explaining the individual regulations of AMPKα1 or Akt respectively under the treatment of resveratrol are noticeable in the control of various cancer cells. However, the regulation of both AMPKα1 and Akt, and the connection between AMPKα1 and Akt have not been investigated. In this study, it has been shown that resveratrol induced apoptosis via AMPKα1-Akt pathway in HT-29 colon cancer cells. Additionally, it is suggested that there exist 2 pathways to regulate Akt by resveratrol—namely, AMPKα1-dependent and AMPKα1-independent.

Materials and Methods

Cell culture and reagents HT-29 cells were obtained from the American Type Culture Collection (USA) and grown in RPMI 1640 medium containing 10% fetal bovine serum, 100 mg/L streptomycin, and 100 U/mL penicillin at 37°C in 5% CO2 atmosphere. Resveratrol was purchased from Sigma-Aldrich (USA). Compound C was obtained from Calbiochem (USA) and LY294002 obtained from Tocris Bioscience (USA). Specific antibodies that recognize the phosphorylated forms of AMPKα1 Thr172, ACC Ser79, and Akt Ser473, and β-actin were obtained from Cell Signaling Technology (USA).

Measurements of cell viability Cells were seeded on 96-well micro plates at 4,000 cells/well and then they were incubated with resveratrol at the dose- and time-dependent treatment. The medium was removed and the cells were then incubated with 100 µL of (4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma-Aldrich) solution [2 mg/mL MTT in phosphate-buffered saline (PBS)] for 4 hr. Optical densities of the solutions were determined by an enzyme-linked immunosorbent assay (ELISA) reader.

Apoptosis detection Apoptosis was measured by FITC-Annexin V apoptosis detection kit (BD PharmingenTM, USA). For AnnexinV/PI staining after treatment with resveratrol or LY294002, cells were harvested by trypsinization, washed with ice-cold PBS, and suspended in a binding buffer at a density of 1×10^6 cells/mL. Cells were stained with Annexin V-FITC and propidium iodide (PI) and analyzed by flow cytometry (Becton-Dickinson Biosciences, USA).

Western blot analysis The cells were washed with PBS, scraped into lysis buffer [50 mM Tris-HCl (pH 7.4), 1% NP-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM ethylenediamine tetraacetic acid (EDTA), 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM sodium orthovanadate, 1 mM NaF, 1 mg/mL aprotinin, 1 mg/mL leupeptin, and 1 mg/mL pepstatin], and subjected to Western blot analysis with specific antibodies.

Transgenic transfection with small interfering RNA (siRNA) Small interfering RNAs (siRNAs) were synthesized by Dharmacon Research (USA). For transient transfection, the cells were seeded on a 6-well plate at the density of 2.5×10^5 cell/mL with antibiotic-free medium. After incubation overnight, the targeting siRNAs were transfected using DharmaFECT4 transfection reagent (Dharmacon, USA) according to the manufacturer’s instructions. After incubation for 72 hr, the cells treated with resveratrol for 6 hr and analyzed by Western blot.

Immunofluorescence staining The cells were seeded on a 12-well plate with cover glasses. After treatment of resveratrol, the cells were fixed in 3.7% formaldehyde for 20 min at room temperature (RT) and permeabilized in 0.2% Triton X-100 for 20 min at RT. Then cells were blocked with 1% bovine serum albumin for 1 hr. Next, the cells were incubated overnight with primary antibody of phospho-AKT. After washing, the cells were incubated with Alexa488-conjugated anti-mouse IgG (Molecular Probes, USA) for 1 hr at RT. Next, cell nuclei were stained with 10 µM of Hoechst33342 for 10 min and then observed by a confocal microscope (Carl Zeiss, USA).

Results and Discussion

Effect of resveratrol on cell proliferation and induction of apoptosis in HT-29 colon cancer cells The effects of resveratrol on the growth of HT-29 cells was examined. Cells were treated with different concentration (50-200 µM) and different time (3-24 hr) of resveratrol and cell viability was measured by MTT assay. As shown in Fig. 1A and 1B, resveratrol decreased cell viability in a dose- and time-dependent manner. Resveratrol and Akt inhibitor LY294002 have known to induce cancer cell apoptosis (16). In order to investigate the effects of resveratrol and LY294002 on induction of apoptosis, cells were treated with 100 µM of resveratrol or 20 µM of LY294002 for 24 hr and apoptosis was examined by Annexin V/PI staining. As shown in Fig. 2, resveratrol induced apoptosis, and under the inhibition of Akt activity by LY294002, increased apoptotic cell death was also observed. AMPK is the most important signaling molecule that regulates intracellular energy homeostasis and possibly apoptosis. AMPK is activated in response to cellular energy consumption, heat shock, oxidative stress, and hypoxia (17,18). Akt is one of...