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Resveratrol Downregulates Acetyl-CoA Carboxylase α and Fatty Acid Synthase by AMPK-mediated Downregulation of mTOR in Breast Cancer Cells

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Abstract Overexpression of HER2 in breast cancer cells is considered to induce the expression of acetyl-CoA carboxylase α (ACACA) and fatty acid synthase (FASN) through activation of mammalian target of rapamycin (mTOR) signaling pathway. Resveratrol, a red wine polyphenol, has been shown to induce apoptosis in several cancers by interfering in several signaling pathways. Present study elucidated the mechanism by which resveratrol downregulates ACACA and FASN in breast cancer cells. Resveratrol activated AMP-activated protein kinase (AMPK) and downregulated mTOR in BT-474 cells. These effects of resveratrol were mimicked by AICAR, an AMPK activator, and exogenously expressed constitutively active AMPK, while they were abolished by a dominant-negative mutant of AMPK. The downregulation of mTOR was not accompanied with changes in Akt, the upstream regulator of mTOR. These findings indicate that the downregulation of ACACA and FASN by resveratrol is mediated by the downregulation of mTOR signaling pathway via activation of AMPK.

Keywords: resveratrol, fatty acid synthase, acetyl-CoA carboxylase, mammalian target of rapamycin, AMP-activated protein kinase, breast cancer

Introduction

One of the characteristic phenotypes of virtually all aggressive cancers is an increased *de novo* fatty acid synthesis (1). A number of studies have demonstrated a close linkage of increased fatty acid synthesis to the abnormally high levels of fatty acid synthase (FASN) in various tumors and their preneoplastic lesions (2). FASN catalyzes the biosynthesis of palmitic acid that is used for the synthesis of triacylglycerol as a storage fuel molecule as well as membrane lipids including phospholipids and sphingolipids (3). The increased proliferation of tumor cells which require exacerbated lipogenesis is reflected by an inevitable increase of FASN expression. Meanwhile, acetyl-CoA carboxylase α (ACACA), the rate-limiting enzyme for *de novo* synthesis of fatty acids, has been reported to be overexpressed in advanced breast cancers and to play an important role in breast carcinomas development (4,5). The expression of these two lipogenic enzymes, ACACA and FASN, is closely related to the aggressiveness of cancers as well as to the development, maintenance, and cell cycle progression of human cancers (5-7). In the previous study, it was reported that the expression of FASN and ACACA is increased by human epidermal growth factor receptor 2 (HER2) in breast cancer cells, and that activation of mammalian target of rapamycin (mTOR) by HER2 plays an important role in their inductions through the selective translational activation of their mRNAs (8).

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Resveratrol attracted our attention due to its wide range of pharmacological properties, including anti-inflammatory, antioxidant, antiplatelet, immunomodulatory, neuroprotective, and cancer-chemopreventive effects (9,10). Resveratrol (3,4',5-trihydoxystilbene) is one of graphe polyphenols (11) and consists of the principal pharmacologically active component of red wine (12) whose intake inversely correlates to the incidence of cardiovascular disorders and cancers (13,14). A particular interest in resveratrol was driven by the study carried out by Provinciali *et al.* (15) who reported that supplementation of resveratrol delayed the development of spontaneous mammary tumors, reduced the mean number and size of mammary tumors, and diminished the number of lung metastases in HER2 transgenic mice. They proposed that the antitumor effect of resveratrol is related to the downregulation of HER2 expression and the induction of apoptosis in breast cancer cells. Recent studies have demonstrated that resveratrol can activate AMP-activated protein kinase (AMPK) *in vitro* (16-18) and *in vivo* (19). AMPK is a heterodimeric protein kinase that acts as an energy sensor to modulate glucose and lipid metabolism (20). Activation of AMPK enhances processes that increase ATP generation and inhibit others that consume ATP (21). One of the downstream target of activated AMPK is reported to be mTOR, a positive effector of cell growth and division (22,23). It has been reported that suppression of protein synthesis and mTOR signaling is mediated by AMPK in muscle or liver (24-26). However, neither of these studies provided any information as to whether resveratrol-mediated AMPK activation involves

modulation of mTOR signaling.

In this study, we showed that resveratrol decreased the expression of ACACA and FASN without significant changes in their mRNA in BT-474 cells which overexpressed HER2. We demonstrated that these changes were mediated by the downregulation of mTOR which resulted from activation of AMPK by resveratrol.

Materials and Methods

Reagent Resveratrol and 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR), were purchased from Sigma-Aldrich (St. Louis, MO, USA). MTS assay kit was purchased from Promega (Madison, WI, USA). LY294002 was purchased from Alexis (San Diego, CA, USA). Rapamycin was purchased from Cell Signaling Technology (Danvers, MA, USA). Polyclonal antibodies against phosphorylated ACACA (Ser^{79}), Akt, phosphorylated Akt (Ser^{473}), AMPK, and phosphorylated AMPKα (Thr^{172}) were purchased from Cell Signaling Technology. The other antibodies used in the current study were as described previously (8). Adenovirus expressing a constitutively active (T172D) or a dominant-negative mutant of AMPK (D157A) were kindly provided by Dr. Joohun Ha (Dept. Biochemistry and Molecular Biology, Kyung Hee University School of Medicine, Seoul, Korea).

Cell culture Human breast cancer cell lines, BT-474, MCF-7, MDA-MB-231, and SK-BR-3, were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were maintained in the respective media (Invitrogen, Carlsbad, CA, USA) as recommended by the ATCC at 37°C in a humidified atmosphere of 5% CO_2 in air. For the treatment of cells with resveratrol or AICAR, BT-474 cells were set up at 5×10^5 cells/well in a 6-well plate in Dulbecco's modified Eagle's medium (DMEM) containing 100 units/mL penicillin and 100 μg/mL streptomycin sulfate supplemented (medium A) with 10% fetal bovine serum (FBS) on day 0. On day 1, the cells were washed twice with phosphate-buffered saline (PBS), and changed to the fresh medium supplemented with resveratrol or AICAR at the indicated concentration. On day 3, cells were harvested and processed for the immunoblot analyses as described below. For adenoviral infection, BT-474 cells were incubated with recombinant adenovirus at a multiplicity of infection of 10 in serum-free medium at 37°C. Culture medium was replaced with fresh medium A supplemented with 10% FBS 2 hr after transduction. After overnight incubation, the cells were treated with or without 50 μM resveratrol for 24 hr and processed for the immunoblot analyses as described below.

Cell viability assay Cells (4×10^3 cell/well) were set up on a 96-well culture plate on day 0. On day 1, the medium was replaced with fresh medium supplemented with 10% FBS and resveratrol at the indicated concentration. On day 4, cell viability was determined using the CellTiter 96 Aqueous Non-radioactive Cell Proliferation Assay kit (Promega) according to the manufacturer's protocol.

Immunoblot analyses Immunoblot analyses were performed as described previously (8). Briefly, the cells treated as indicated in the figure legends were harvested and lysed in 2× sodium dodecyl sulfate (SDS) loading buffer, then briefly sonicated. Lysates were cleared by centrifugation at 10,000×g for 10 min at 4°C. Supernatants were collected and protein concentrations were determined by Bradford assay (Bio-Rad, Hercules, CA, USA). The proteins were subjected to electrophoresis on SDS-polyacrylamide gels and were transferred to Protran nitrocellulose membranes (Schleicher & Schuell, Keene, NH, USA). The membranes were blocked in PBS containing 0.05%(v/v) Tween-20 (PBST) and 5%(w/v) non-fat dried milk, and probed with primary antibodies as indicated in the figures. Immunoreactive bands were visualized by horseradish perxodiase-conjugated goat anti-rabbit antibody (Pierce, Rockford, IL, USA) using the SuperSignal West Pico Chemiluminescent Substrate System (Pierce). The cell cultures were repeated more than 3 times in order to evaluate the resveratrol effects. The analysis of each cell lysate by Western blot was repeated more than twice to rule out the experimental artifacts.

Reverse transcription (RT)-polymerase chain reaction (PCR) Total RNAs were isolated from cultured cells using TRIzol reagent (Invitrogen) according to the manufacturer's protocol. cDNAs were synthesized from 5 μg of total RNA using oligo(dT) and Superscript reverse transcriptase II according to the manufacturer's protocol. The resulting cDNA were used as a template for PCR amplification of the target genes. The sequences of the primers used are as follows: *FASN*, 5' primer, 5'-GTCCTGGGAGGAGTGTAA ACAG-3', and 3' primer, 5'-GTCCCTGTGATCCTTCTTC ATC-3'; *ACACA*, 5' primer, 5'-TGGTCTCTTTCCGGACC TTTGAAG-3', and 3' primer, 5'-TCCTCCTCAAACT TATCCCTTGCTCGGA-3'; glyceraldehydes-3-phosphate dehydrogenase (*GAPDH*), 5' primer, 5'-CCCCTTCATTG ACCTCAACTAC-3', and 3' primer, 5'-GAGTCCTTCCA CGATACCAAAG-3'. The amplified products were analyzed on 1.5% agarose gels containing ethidium bromide and visualized by ultraviolet (UV) light. The cell cultures were repeated more than 3 times in order to evaluate the resveratrol effects.

Results and Discussion

Resveratrol decreases viability of breast cancer cells overexpressing HER2 In the first sets of experiments, we determined the effects of resveratrol on the viability of 4 different breast cancer cell lines, BT-474, MCF-7, MDA-MB-231, and SK-BR-3, in which the relative amounts of HER2 differ form each other (8). As shown in Fig. 1, resveratrol significantly decreased viability of all cell lines examined at 100 μM based on the MTS cell viability assay after treatment for 72 hr. At concentrations up to 50 μM, resveratrol increased the viability of MCF-7 cells which express the estrogen receptor (27). This augmentation of the viability is supposed to be due to the structural similarity of resveratrol to estrogen (9). HER2 in breast cancer cells is reported to increase the expression of 2 lipogenic enzymes, ACACA and FASN, and this increase in lipogenic enzymes plays an important role on cell survival (8,28). Interestingly, viabilities of BT-474 and SK-BR-3 which overexpress HER2 were decreased more efficiently by resveratrol than

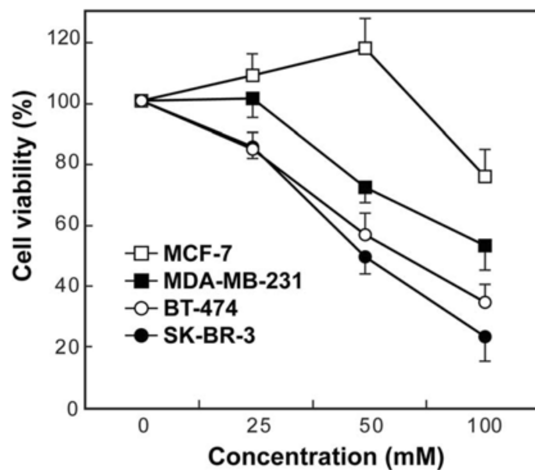

Fig. 1. The effect of resveratrol on cell viability in breast cancer cells. The cell viability was measured by the MTS assay 72 hr after treatment, and represented as % of viability of the cells compared to that of vehicle-treated cells (0 μM). Each value represents the mean±SD of triplicate incubations. Similar results were obtained in 2 independent experiments.

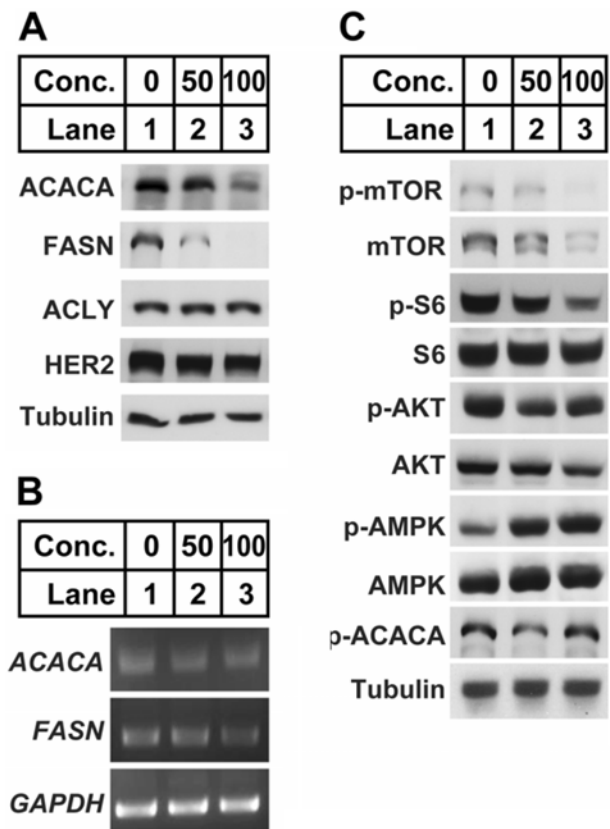

Fig. 2. Changes of lipogenic enzymes, AMPK, and mTOR signaling molecules by resveratrol in BT-474 cells. On day 0, BT-474 cells were set up at a density of 5×10^5 cells/well on 6-well plate, and treated with 50 or 100 μM (Conc., 50 and 100) of resveratrol or vehicle (Conc., 0) on day 1. The cell lysates were prepared and immunoblot analyses were performed 48 hr after treatment (A and C). Three independent experiments were performed and produced similar results. (B) Total RNAs were isolated from the cells treated same as in (A and C) and subjected to RT-PCR with gene specific primers for *ACACA* and *FASN*. After amplification aliquots of PCR were electrophoresed on 1.5% agarose gel and visualized by ethidium bromide staining. GAPDH was used as an invariant control. Similar results were obtained in 3 independent experiments.

that of MCF-7 and MDA-MB-231 in which HER2 expression is low.

Several studies have reported that increased lipogenesis is important in cell viability and that resveratrol has beneficial effects on breast cancer cells, however, the regulation of lipogenic enzymes by resveratrol was barely studied. Most of the studies regarding antitumor effects of resveratrol have been carried out in MCF-7 or MDA-MB-231 cells in which the expression of ACACA and FASN is low accordingly with the low expression of HER2. Our findings suggest that the antitumor effects of resveratrol might be dependent on HER2 expression in breast cancer cells, which led us to determine the effects of resveratrol on the regulation of lipogenic enzymes driven by HER2. Based on previous report that FASN and ACACA were specifically upregulated by endogenous high level of HER2 through mTOR signaling pathway in BT-474 (8), the mechanisms about the downregulations of FASN and ACACA by resveratrol were characterized in BT-474 cells.

Effects of resveratrol on the expression of lipogenic enzymes and on activation of signaling molecules To determine whether resveratrol regulates the expression of lipogenic enzymes in the breast cancer cells, we treated BT-474 cells with resveratrol for 48 hr and the changes of ACACA and FASN were analyzed by immunoblot analysis and RT-PCR. As shown in Fig. 2A, resveratrol markedly decreased the protein levels of ACACA and FASN, while that of ACLY and HER2 remained unchanged. The amounts of mRNAs for ACACA, and FASN were not significantly changed by resveratrol (Fig. 2B). These data suggest that the effect of resveratrol to decrease the viability of BT-474 cells is achieved by the suppression of ACACA and FASN expression in BT-474 cells, which is regulated at a post-transcriptional level. Because the induction of ACACA and FASN in BT-474 cells was reported to be mediated by activation of mTOR (8), we determined whether the downregulation of ACACA and FASN by resveratrol accompanies the changes of phosphoinositide-3-kinase (PI3K)/Akt/mTOR signaling pathway in BT-474 cells. As shown in Fig. 2C, resveratrol significantly downregulated the phosphorylated mTOR (p-mTOR) as well as total amount of mTOR protein. The ribosomal S6 protein which is a direct target of activated mTOR showed decrease in phosphorylation (p-S6) in accordance with decrease in p-mTOR, while the total amount of S6 protein remained unchanged. However, the changes of both the phosphorylated (p-Akt) and the total amount of Akt, which is an upstream activator of mTOR, were hardly observed by treatment of resveratrol. These results suggest that resveratrol downregulates mTOR signaling pathway which is mediated by the mechanism that does not require the Akt and/or upstream PI3K signaling pathway. In contrast, resveratrol increased the phosphorylated AMPK (p-AMPK) while the total amount of AMPK remained unchanged. The phosphorylated

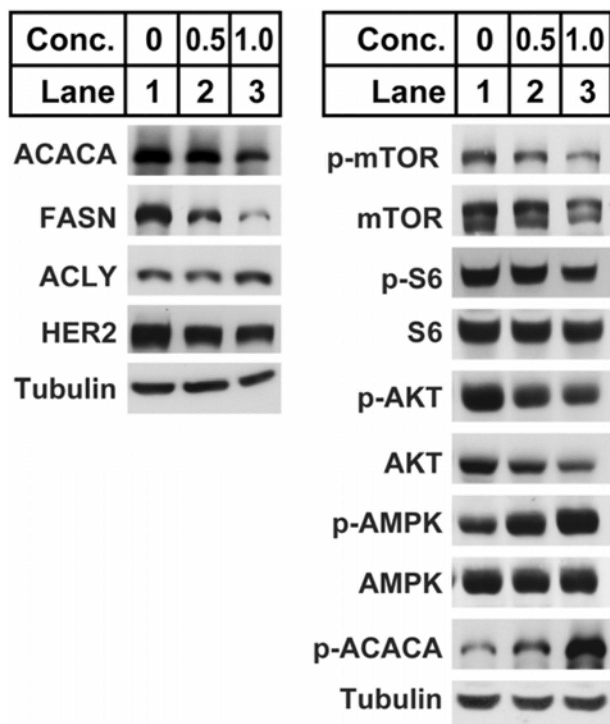

Fig. 3. The effects of AICAR on the on lipogenic enzymes, AMPK, and mTOR signaling molecules in BT-474 cells. BT-474 cells were set up as described in Fig. 1, and treated with 0.5 or 1.0 mM (Conc., 0.5 and 1.0) of AICAR or vehicle (Conc., 0). The cell lysates were prepared and immunoblot analyses were performed 48 hr after treatment with the antibodies as described in Fig. 1. Similar results were obtained in 3 independent experiments.

ACACA (p-ACACA) at Ser[79], which is the target of phosphorylation by AMPK (20) remained constant by resveratrol. However, the relative portion of the amounts of phosphorylated form compared to the total amounts of ACACA was considerably increased by resveratrol. These data agree with the recent reports that resveratrol activated AMPK in myotubus and neuronal cells (16,18). Taken together, these findings suggest that the downregulation of ACACA and FASN by resveratrol is mediated by activation of AMPK and the downregulation of mTOR which is regulated by the mechanism that does not require Akt.

Resveratrol-mediated activation of AMPK downregulates mTOR signaling pathway We next determined whether activation of AMPK by resveratrol downregulates mTOR resulting in the downregulation of ACACA and FASN in BT-474 cells in 2 supportive ways. First, we treated the cells with AICAR, a cell permeable AMPK activator. As shown in Fig. 3, AICAR caused the similar changes of proteins which mimic those observed in BT-474 cells treated with resveratrol. AICAR increased p-AMPK with subsequent increase in p-ACACA, decreased ACACA and FASN without changes in ACLY, decreased p-mTOR and total mTOR with subsequent decrease in p-S6. However, no significant change in Akt was observed by AICAR. These findings support that activation of AMPK resulted in suppression of mTOR, and downregulated expression of ACACA and FASN in BT-474 cells.

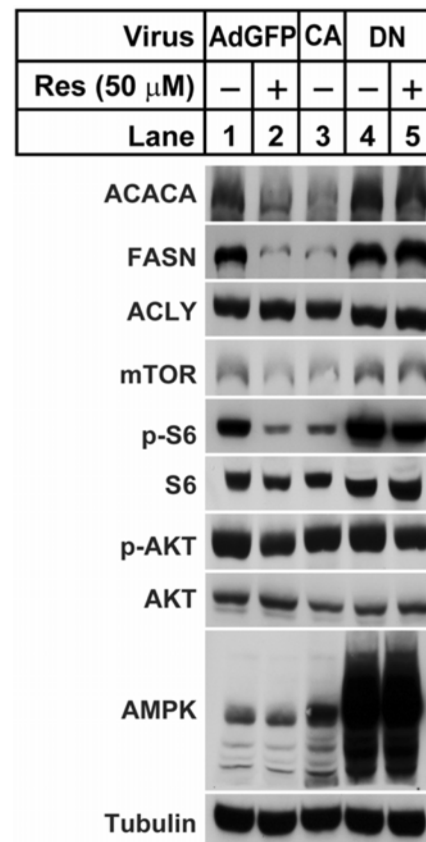

Fig. 4. The effects of adenovirus-mediated expression of AMPK on the cellular events caused by resveratrol in BT-474 cells. BT-474 cells were set up and infected with adenovirus expressing a green fluorescence protein (AdGFP), a constitutively active AMPK (CA), or a dominant-negative AMPK (DN). Cells were changed to the fresh medium A supplemented with 10% FBS 24 hr after transduction. After overnight incubation, cells were treated with (-) or without (+) 50 μM resveratrol. Cell lysates were prepared and immunoblot analyses were performed as described in Fig. 1, 24 hr after treatment with resveratrol.

Second, we determined whether the changes by resveratrol could be mimicked by overexpression of AMPK in BT-474 cells, or be abolished by blocking AMPK in the presence of resveratrol. To address this issue, we transduced BT-474 cells with adenovirus expressing the constitutively active or the dominant-negative AMPK in the absence or presence of resveratrol. As shown in Fig. 4, the expression of constitutively active AMPK (CA) in BT-474 cells grown in the absence of resveratrol decreased the expression of ACACA and FASN while ACLY remained unaffected (lane 3). In addition, constitutively active AMPK caused decrease in mTOR expression and phosphorylation of S6, while Akt was not affected significantly. These changes caused by constitutively active AMPK agree with those caused by resveratrol in BT-474 cells (lane 2). The expression of a dominant-negative AMPK (DN) in BT-474 cells caused no obvious changes when the cells were grown in the absence of resveratrol (lane 4). It is supposed that the AMPK activity is very low in BT-474 cell which were grown in the absence of resveratrol, so that DN

AMPK produced no change in the amounts of ACACA and FASN. However, the effects of resveratrol in BT-474 cells were completely blocked by a DN AMPK (lane 5). These results strongly suggest that the downregulation of mTOR requires not PI3K/Akt pathways but activation of AMPK for resveratrol to suppress ACACA and FASN in BT-474 cells.

Taken together, the current study demonstrated that resveratrol downregulates ACACA and FASN driven by HER2 in BT-474 cells. It is suggested that activation of AMPK and the consequent downregulation of mTOR are the important regulators for this downregulation. However, it is suggested that PI3K/Akt pathway is not critical for the downregulation of mTOR by resveratrol. These findings are the first to show that resveratrol could downregulate mTOR signaling pathway in breast cancer cells. This information has clinical importance that resveratrol might be effective especially in HER2-overexpressing breast cancers, where the mTOR signaling pathway plays an important role on cancer cell proliferation and survival.

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