Regulation of mutual inhibitory activities between AMPK and Akt with quercetin in MCF-7 breast cancer cells

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Abstract. In lieu of elucidating bidirectional connecting mechanism between AMP-activated protein kinase (AMPK) and survival signal Akt we applied MCF-7 breast cancer cells to determine whether AMPK modulation alters Akt signals and vice versa. Suppression of Akt activities with a synthetic Akt inhibitor alleviated AMPK activities suggesting that Akt is capable of inhibiting AMPK. Also the activation of AMPK with quercetin strongly abrogated Akt activities. Treating cancer cells with AMPK siRNA or Compound C resulted in marked increment of Akt dephosphorylation indicating that AMPK has antagonistic activities towards Akt. However, quercetin exerted Akt inhibitory activities in the absence of AMPK activation. Quercetin induced partial co-localization of phospho-Akt and phospho-AMPK in the nucleus even though their interaction seems to be indirect since the immunoprecipitation data indicate there was no direct binding between total Akt and AMPK. These results suggest there is a mutual suppressive interaction between AMPK and Akt. The investigation of mutual suppression between Akt and AMPK by chemo-preventive agents such as quercetin may provide a mechanistic rational for controlling breast tumor cell growth.

Introduction

Akt, a serine/threonine protein kinase, is recognized as a significant regulator of multiple cellular processes, including cell growth, survival, apoptosis and cell cycle progression (1-3). Akt is activated by phosphorylation at Ser473 or Thr308 often as a downstream sequence of phosphatidyl inositol-3 kinase (PI3K) in response to several growth factors, such as insulin-like growth factor (IGF)-1 or epidermal growth factor (EGF) (4). The aberrantly activated Akt is frequently found in many human cancers including breast cancer (5,6). A recent report suggests that Akt is a reliable biomarker for predicting the chemo-preventive effect of anti-cancer agents in breast cancer patients (7). Inhibition of apoptosis and stimulation of cell proliferation and metastasis have been shown to be carried out by the phosphorylation of Akt downstream targets including tuberous sclerosis complex 2 (TSC2)/mamalian target of rapamycin (mTOR), nuclear factor κB (NF-κB), Bad, and glycogen synthase kinase-3β (GSK-3β) (8,9). This may lead to continuous survival of cancer cells. Therefore, regulating Akt and Akt-regulated downstream pathways has emerged as a promising strategy for controlling cancer with chemo-preventive agents.

Observations that AMPK activators are capable of dephosphorylating Akt evoked the importance of the regulation of AMPK/Akt pathway with chemo-preventive agents in cancer control (10-12). Furthermore, the Akt/TSC2/mTOR signaling pathway via the activation of AMPK has been proposed as a promising approach for tumor suppression. Several researchers have highlighted the controversial regulation between Akt and AMPK in several cancer cell models. In MDA-MB-231 cells, adiponectin-activated AMPK inhibits Akt through the activation of protein phosphatase 2A (PP2A), which directly dephosphorylates Akt (11). In other cell models of MCF-7 it was shown that the activated AMPK with anti-cancer agent blocked phosphorylation of Akt (12). On the other hand, it has been shown that Akt negatively regulates AMPK by decreasing the AMP/ATP ratio (13). It has not been resolved whether direct interaction exists between Akt and AMPK, and further mutual suppression between these two proteins in a cancer cell system challenged with anti-cancer agents. In this study, we investigated the bidirectional regulatory mechanism between Akt and AMPK in quercetin-treated MCF-7 breast cancer cells. We found that Akt and AMPK do not directly bind to each other although they partially co-localized in the nucleus. We found that the activation of AMPK resulted in the suppression of Akt, and Akt was capable of inhibiting AMPK. These observations suggest that their interaction may occur with connecting signal(s) in both of positive suppression of AMPK on Akt and the negative regulation of Akt on AMPK.

Materials and methods

Cell culture and reagents. MCF-7 cells were obtained from the American Type Culture Collection (Manassas, VA) and grown in RPMI-1640 medium containing 10% fetal bovine
serum at 37°C in a 5% CO2 atmosphere. Quercetin was purchased from Sigma-Aldrich (St. Louis, MO, USA), and Compound C was obtained from Calbiochem (San Diego, CA, USA). LY294002 was purchased from Tocris (Bristol, UK). Specific antibodies that recognize AMPKα1, Akt, β-actin, and the phosphorylated forms of AMPKα1Thr172, ACCSer79 and AktSer473 were obtained from Cell Signaling Technology (Danvers, MA, USA).

Immunofluorescence staining. Cells were seeded on a 12-well plate with cover glasses. After treatment at the indicated time and dose, the cells were fixed, permeabilized, and double stained with p-Akt (green) and anti-mouse Alexa 488 secondary antibody or with p-AMPKα1 (red) and anti-rabbit Alexa 546 secondary antibody. Cell nuclei were stained with Hoechst 33342 dye (blue) and observed by confocal microscope. (B) The cells were treated with quercetin (0-200 μM) for 6 h, and total proteins were subjected to Western blot analysis using p-AMPKα1Thr172, AMPKα1, p-AktSer473 and Akt antibodies.

Figure 1. Quercetin decreases Akt activity and increases AMPKα1 activity in MCF-7 breast cancer cells. (A) The cells were treated with quercetin (0-200 μM) for 6 h. Cells were fixed, permeabilized, and double stained with p-Akt (green) and anti-mouse Alexa 488 secondary antibody or with p-AMPKα1 (red) and anti-rabbit Alexa 546 secondary antibody. Cell nuclei were stained with Hoechst 33342 dye (blue) and observed by confocal microscope. (B) The cells were treated with quercetin (0-200 μM) for 6 h, and total proteins were subjected to Western blot analysis using p-AMPKα1Thr172, AMPKα1, p-AktSer473 and Akt antibodies.
transfection, the cells were seeded on a 6-well plate (2.5x10^5 cells/ml) in antibiotic-free medium. Following an overnight incubation, the targeting siRNAs were transfected using DharmaFECT4 transfection reagent (Dharmacon) according to the manufacturer’s instructions. After 72-h incubation, the cells were analyzed by Western blot analysis.

**Co-immunoprecipitation assay.** Cells were seeded on 60-mm culture dishes and treated with quercetin. Cells were scraped into lysis buffer (1 M Tris-HCl, 0.5 M EDTA, 1 M NaCl, 1 M DTT, 10% NP-40, 10 mg/ml BSA, 0.1 M PMSF), and the lysates were precleared with protein G-beads (Zymed, San Francisco, CA, USA) for 4 h at 4°C. Proteins were then precipitated with protein G-bound primary antibody for 12 h at 4°C. After washing, protein interaction was observed by Western blot analysis.

**Results**

**Dephosphorylation of Akt and activation of AMPKα1 by quercetin, and partial co-localization of Akt and AMPKα1 in the nucleus.** To examine the effects of quercetin on Akt and AMPKα1 activities, cells were treated with different concentrations of quercetin for 6 h, and the levels of phospho-Akt and AMPKα1 were examined by immunofluorescence staining. Akt was highly activated in the membrane and cytosol of MCF-7 breast cancer cells under serum-containing conditions, but activated Akt was abolished in a dose-dependent manner following quercetin treatment. In addition, quercetin increased the levels of phospho-AMPKα1 in the cytosol and nucleus, and activated AMPKα1 appeared to translocate to the nucleus following treatment with increasing concentrations of quercetin (Fig. 1A). Interestingly, partial co-localization was observed between phospho-Akt and phospho-AMPKα1 in nucleus. This is indicated by the appearance of yellow spots at concentrations of quercetin above 100 μM. The regulatory effects of quercetin on Akt and phospho-AMPKα1 were also confirmed by Western blotting. Quercetin effectively inhibited Akt through dephosphorylation but activated AMPKα1 in a dose-dependent manner in MCF-7 breast cancer cells (Fig. 1B).

**Akt and AMPKα1 have mutual inhibitory activities in MCF-7 breast cancer cells.** To examine the regulatory mechanism between AMPKα1 and Akt, MCF-7 cells were treated with specific inhibitors of Akt and AMPKα1 (LY294002 and Compound C, respectively) and quercetin. Inhibition of AMPKα1 by Compound C increased the quercetin-induced inhibition of Akt activity compared to quercetin treatment alone, but the phospho-Akt level was lower than the control group. These results indicate that quercetin-activated AMPKα1 exerts inhibitory effects on Akt, and suggests that quercetin could inhibit Akt activity through an AMPKα1-independent pathway. In addition, treatment with LY294002 and quercetin inhibited Akt activity, but activated AMPKα1, more effectively than quercetin alone (Fig. 2A). Moreover, inhibition of Akt by LY294002 alone caused activation of AMPKα1 and phosphorylation of ACC, a substrate of AMPKα1 (Fig. 2B). These results suggest that Akt has inhibitory effects on AMPKα1 in MCF-7 breast cancer cells, and the suppression of Akt activity leads to an increase in AMPKα1 activity. To determine whether quercetin activates AMPKα1 through suppressing Akt, we examined the effects of quercetin on Akt following suppression of AMPKα1 by siRNA. Quercetin strongly inhibited Akt activity in the absence of AMPKα1 (Fig. 2C). Taken together, these results suggest that quercetin activates AMPKα1 by removing the inhibitory effects of Akt, and quercetin-activated AMPKα1 suppresses Akt.

**Direct binding between Akt and AMPKα1 proteins was not observed.** To investigate whether the regulatory effects that occur between AMPKα1 and Akt are due to their direct binding, we performed a co-immunoprecipitation assay using Akt or AMPKα1 specific antibodies. We found that Akt
and AMPKα1 do not bind directly (Fig. 3A and B), but that quercetin effectively inhibited Akt and activated AMPKα1 in a time-dependent manner (Fig. 3C). We propose that the highly activated Akt signals observed in normal MCF-7 breast cancer cells may indicate inhibitory activity on AMPKα1 (Fig. 4A). In addition, quercetin could activate AMPKα1 by suppressing Akt activity or other Akt-independent pathways. Additionally, activated AMPKα1 is also involved in suppressing Akt activity, which may contribute to the apoptotic effects of quercetin in breast cancer (Fig. 4B).

Discussion

Quercetin, a well-investigated flavonoid, effectively inhibits cell proliferation and cell cycle progression and induces apoptosis in various cancer cells (14-17). We previously reported that quercetin inhibits cell growth and induces apoptosis in MCF-7 cells through strong activation of AMPK that suppresses the expression of the anti-apoptotic molecule Cox-2 (18). Quercetin also induces apoptosis in several types of cancer cells by suppressing Akt-mediated anti-apoptotic activity, cell cycle progression, and cell survival-related pathways (15,19). In this study, we investigated the regulatory mechanism by which quercetin alters Akt and AMPK, two important apoptosis-regulating molecules, and the mutual inhibitory mechanism between Akt and AMPK signals in quercetin-treated MCF-7 breast cancer cells, and our data indicated that quercetin was capable of activating AMPK by removing inhibitory activity of Akt on AMPK, and by direct activation on AMPK. These observations imply that Akt and AMPK have mutual inhibitory effects, and quercetin modulates both of these molecules through up- or down-regulating ability in controlling cell growth or apoptosis.

Recent reports have shown that suppression of Akt by AMPK is mediated through the activation of phosphatase and tensin homologue (PTEN) or PP2A, and the involvement of IRS-1 between Akt and AMPK was suggested (11,12,20). On the other hand, the inhibitory effect of Akt on AMPK...
was explained by either alteration of intracellular ATP by Akt or the regulatory activities of Akt on AMPK upstream kinases or phosphatases (13,21). Our results confirmed that Akt and AMPK signals have mutual inhibitory activities in MCF-7 cell system. Treating MCF-7 cells with Akt inhibitor LY294002 or quercetin activated AMPK. The combination of quercetin and LY294002 inhibited Akt while activating AMPK more strongly than quercetin treatment alone. The activated AMPK by quercetin exerted inhibitory effects on Akt as observed by elevated phosphorylation of Akt following inhibition of AMPK by Compound C.

Because of the bidirectional relationship that occurs between Akt and AMPK following quercetin treatment, we hypothesized that quercetin may cause these two proteins to bind to each other. Immunofluorescence staining showed slight co-localization of Akt and AMPK bind to each other. Hypothesized that quercetin may cause these two proteins to bind to each other. Immunofluorescence staining showed slight co-localization of Akt and AMPKα1 in the nucleus of quercetin-treated MCF-7 cells. However, co-immunoprecipitation revealed that Akt and AMPK do not bind to each other. These results suggest that there might exist another signaling molecule such as phosphatase down-regulating Akt and up-regulating AMPK activities by quercetin.

In conclusion, we demonstrated that Akt and AMPK have mutual inhibitory activities, and quercetin regulated these signals through suppressing or stimulating their actions in MCF-7 breast cancer cells. These findings imply that quercetin strongly inhibits Akt activity, possibly through activating AMPK as well as through directing its suppressive effects against Akt. These observations suggest that anticancer agents, such as quercetin, have regulatory effects on both Akt and AMPK, and this could provide an effective strategy for preventing different types of cancers, including breast cancer.

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References