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Curcumin Exerts Antidifferentiation Effect through AMPKα-PPAR-γ in 3T3-L1 Adipocytes and Antiproliferatory Effect through AMPKα-COX-2 in Cancer Cells

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Curcumin has been reported to have the potential to prevent obesity as well as cancers. The downstream targets regulated by AMP-activated protein kinase (AMPK) for inhibiting adipocyte differentiation or cancer cell proliferation of curcumin were investigated. The activation of AMPK by curcumin was crucial for the inhibition of differentiation or growth in both adipocytes and cancer cells. Stimulation of AMPK by curcumin resulted in the down-regulation of PPAR (peroxisome proliferator-activated receptor)-γ in 3T3-L1 adipocytes and the decrease in COX-2 in MCF-7 cells. Application of a synthetic AMPK activator also supported the evidence that AMPK acts as an upstream signal of PPAR-γ in 3T3-L1 adipocytes. In cancer cells, AMPK was found to act as a regulator of ERK1/2, p38, and COX-2. Regulation of AMPK and its downstream targets such as PPAR-γ, Mapkinases, and COX-2 by curcumin appears to be important in controlling adipocytes and cancerous cells.

KEYWORDS: AMP-activated protein kinase; apoptosis of cancer cells; curcumin; peroxisome proliferator-activated receptor-γ; adipocyte differentiation

INTRODUCTION

Curcumin, a well-known component of turmeric (*Curcuma longa*), has been reported to prevent various diseases such as diabetes, obesity, and cancer. Accumulating evidence suggests that curcumin may exert its physiological activities through actions at different signaling targets (*1, 2*). Aggarwal et al. suggested that curcumin has the potential to exert therapeutic effects by modulation of multiple cellular targets (*3*). Identifying the molecular events that control the growth and proliferation of adipocytes or cancer cells have profound implications for therapeutic development in degenerative diseases.

The modulation of AMP-activated kinase (AMPK) has emerged as an important target for the prevention and treatment of cancers and atherosclerosis and as a potential obesity therapy (*4, 5*). AMPK is a sensor of cellular energy status and is known to be involved in cancer cell apoptosis (*6*) and apoptotic events in adipocytes (*7*). It has been reported that AMPK is regulated by cytokines in the control of whole-body energy balance, by antidiabetic drugs, and by natural plant products such as berberine, resveratrol, and green tea catechin epigallocatechin-3-gallate (EGCG) in various cell types (*8*). Recently, the control of obesity and diabetes through the modulation of AMPK has received considerable attention since it has been shown that the major metabolic responses to exercise are mediated through AMPK (*9*). Furthermore, in cancerous cells, AMPK has been considered a primary anticarcinogenic target due to its relationship with the tumor suppressor genes, *LKB* and *TSC2* (tuberous sclerosis complex 2) (*10*−*12*).

Peroxisome-proliferator-activated receptor (PPAR)-γ promotes adipocyte differentiation and is regarded as one of the key molecules in the development of insulin resistance and obesity (*13*). In contrast, it exhibits antiproliferatory effects on solid cancer or leukemia (*14*). Therefore, PPAR-γ agonists, including antidiabetic drugs, are claimed to be efficacious chemopreventive and chemotherapeutic agents for cancers (*15*).

Curcumin has the potential for the prevention and therapy of various degenerative diseases in various model systems (*16*). In our current experiments, we investigated the regulatory potential of curcumin in adipocytes or cancer cells through the

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activation of AMPK and PPAR-γ. Pathways linked to AMPK in the modulation of adipocyte differentiation or cancer cell apoptosis have not been well elucidated under phytochemical treatments. We examined the possibility of PPAR-γ as a downstreaming target signal of AMPK in both adipocytes and cancerous cells, and Mapkinases and cycolooxygense (COX)-2 as target molecules of AMPK in breast cancer cells.

MATERIALS AND METHODS

Cell Culture and Reagents. 3T3-L1 cells (ATCC, Manassas, VA) were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (Gibco, Grand Island, NY, USA) and 1% antibiotics (Gibco, Grand Island, NY, USA) at 37 °C in a 5% CO_2 atmosphere. MCF-7 (ATCC) cells and HT-29 cells (ATCC) were cultured in RPMI 1640 medium containing 10% fetal bovine serum and 1% antibiotics. Specific antibodies recognizing the phosphorylated forms of AMPK Thr^{172}, ACC Ser^{79}, PPAR-γ, phospho-ERK, p-p-38, COX-2, and β-Actin were purchased from Cell Signaling Technology (Danvers, MA). Insulin, 3-isobutyl-1-methylxanthine (IBMX), and dexamethasone were obtained from Sigma-Aldrich (St. Louis, MO). Curcumin and 5-aminoimidazole-4-carboxamide-ribose (AICAR) were also purchased from Sigma-Aldrich.

Adipocyte Differentiation. 3T3-L1- preadipocyte cells were grown in 12-well plates, and adipocyte differentiation was induced for 2 days with hormonal mixtures containing 1 μM dexamethasone, 5 μg/mL insulin, and 0.5 mM IBMX. After 2 days, the medium was replaced with a normal medium containing insulin (5 μg/mL).

Oil Red O Staining. After the completion of differentiation, the cells were fixed with 3.5% formaldehyde for 20 min, and the differentiated cells were then stained with Oil Red O dye (Sigma-Aldrich) as described by Hwang et al. (*17*).

Protein Extract and Western Blotting. The cells were washed with phosphate-buffered saline (PBS), scraped into lysis buffer (50 mM Tris-HCl [pH 7.4], 1% NP-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, phosphatase inhibitor cocktail (Sigma-Aldrich, St. Louis, MO), and 1 mM PMSF), and subjected to Western blot analysis with specific antibodies.

PPAR-γ Transcriptional Activity Assay. The cells were transiently transfected with an expression plasmid, PPAR-γ, RXR, PPAR-responsive element (PPRE)-driven luciferase reporter vector, and β-galactosidase. After 18 h, the cells were treated with rosiglitazone, a PPAR-γ agonist, in the absence or presence of curcumin for 24 h. The cells were then used for luciferase reporter gene assay. Luciferase activity was assessed by the Luciferase Assay System (Promega, Madison, WI, USA).

Chromatin Staining with Hoechst 33342 and Cell Proliferation Assessed by an MTT Assay. Cells were stained with 10 μM Hoechst 33342 dye (Sigma-Aldrich) for 30 min, and the cells were then fixed with 3.7% *para*-formaldehyde for 15 min. After PBS washing, fluorescence intensity was assessed by a fluorescence microscope. Cells seeded on 96-well microplates at a density of 4,000 cells per well were incubated with curcumin for the indicated concentrations and times. The respective medium was removed, and the cells were then incubated with 100 μL of MTT [(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (Sigma-Aldrich) solution (2 mg/mL MTT in PBS) for 4 h. Absorbance was determined using a microplate reader (Molecular Devices, Sunnyvale, CA, USA) at 599 nm.

RNA Isolation and RT-PCR. Total RNA was extracted with Tri-Zol reagent (Life Technologies, Glasgow, UK) according to the manufacturer's instructions, and cDNA synthesis was performed with 1 μg of total RNA. Synthesized cDNA was used for amplification of a specific target. The amplified products were visualized on 1% agarose gels.

Detection of Reactive Oxygen Species (ROS). 3T3-L1 cells were treated with 10 μM 2′,7′-dichlorofluorescein diacetate (DCFH-DA) (Sigma-Aldrich) for 30 min, fixed with 3.7% *para*-formaldehyde for 15 min, and washed with PBS, and the fluorescence intensity was assessed by a fluorescence microscope (Olympus Optical Co., Tokyo, Japan).

Fluorescence-Activated Cell Sorting Analysis. Total cells were harvested by trypsinization, collected by centrifugation, washed with PBS, fixed with 70% ethanol, and stained in PBS containing 10 μg/mL propidium iodide (Sigma-Aldrich) and 300 μg/mL RNase. After sorting out viable cells, the fluorescence intensity was measured by flow cytometry (Becton-Dickinson Biosciences, San Jose, CA) using excitation and emission wavelengths of 488 and 525 nm, respectively.

Statistical Analysis. Data are presented as the mean ± SD. Data were analyzed by one-way ANOVA using Statistical Package for Social Science (SPSS, Chicago, IL). Differences were considered to be significant at $p < 0.05$.

RESULTS

Inhibitory Effect of Curcumin on 3T3-L1 Adipocyte Differentiation Involves AMPK Activation and Reduction of PPAR-γ Transcription Activity. We evaluated the effects of curcumin on the inhibition of adipocyte differentiation. As shown in **Figure 1A** and **B**, curcumin markedly blocked adipocyte differentiation. We investigated the involvement of AMPKα, PPAR-γ, or ROS generation in the process of curcumin-induced inhibition of adipocyte differentiation. The activation of AMPKα following curcumin treatment (10−50 μM) was comparable to the treatment with AICAR, a synthetic AMPK activator (**Figure 1C**). Additionally, the inhibitory effect of curcumin on PPAR-γ transcription activity was observed (**Figure 1D**). We found that curcumin increased intracellular ROS levels in 3T3-L1 adipocytes. As shown in **Figure 1E**, curcumin markedly increased DCF fluorescence, indicating ROS generation during the AMPKα activation process.

Modulation of AMPKα and PPAR-γ Is Involved in the Regulation of Cancer Cell Apoptosis and Adipogenesis. To confirm the involvement of AMPKα and PPAR-γ in the regulation of adipocytes and cancer cells, we tested the effects of AMPKα activation or PPAR-γ modulation on the control of differentiation and proliferation of 3T3-L1 adipocytes and HT-29 cells. Treatment with AICAR significantly blocked the differentiation of adipocytes and growth of cancer cells (**Figure 2A**). Concomitant with growth regulation by AICAR, increased AMPKα and ACC phosphorylation and the modulation of PPAR-γ expression were observed in both types of cells. However, AICAR inhibited PPAR-γ expression in adipocytes while increasing PPAR-γ expression in colon cancer cells (**Figure 2B** and **C**). Treatment of differentiating 3T3-L1 adipocytes with several other differentiation blockers such as genistein and EGCG resulted in the abrogation of PPAR-γ expression (**Figure 2D**). These results show the possibility of AMPKα as an inhibitory molecule of adipogenesis and PPAR-γ as a downstream target of AMPKα activation. When we tested PPAR-γ as an AMPKα downstream target molecule in cancerous cells, the inhibitory regulation was not observed, in contrast to the results obtained in adipocytes. Surprisingly, AICAR treatment elevated PPAR-γ expression in AMPKα-activated colon cancer cells (**Figure 2C**).

Effects of Curcumin on the Other Molecular Events Related to Cancerous Cell Proliferation and Apoptosis. In the previous experiments, we observed that curcumin may exhibit its antiadipogenic effects through the activation of AMPKα and that AMPKα can control cancer cell death. Next, we investigated the other molecular events related to cancer cell apoptosis by treating breast cancer cells with curcumin. Curcumin treatment markedly inhibited cancer cell proliferation and arrested the cell cycle at the sub G1 phase in a dose-dependent manner (**Figure 3A** and **B**). Furthermore, the levels of the survival genes such as VEGF and apoptosis-related genes

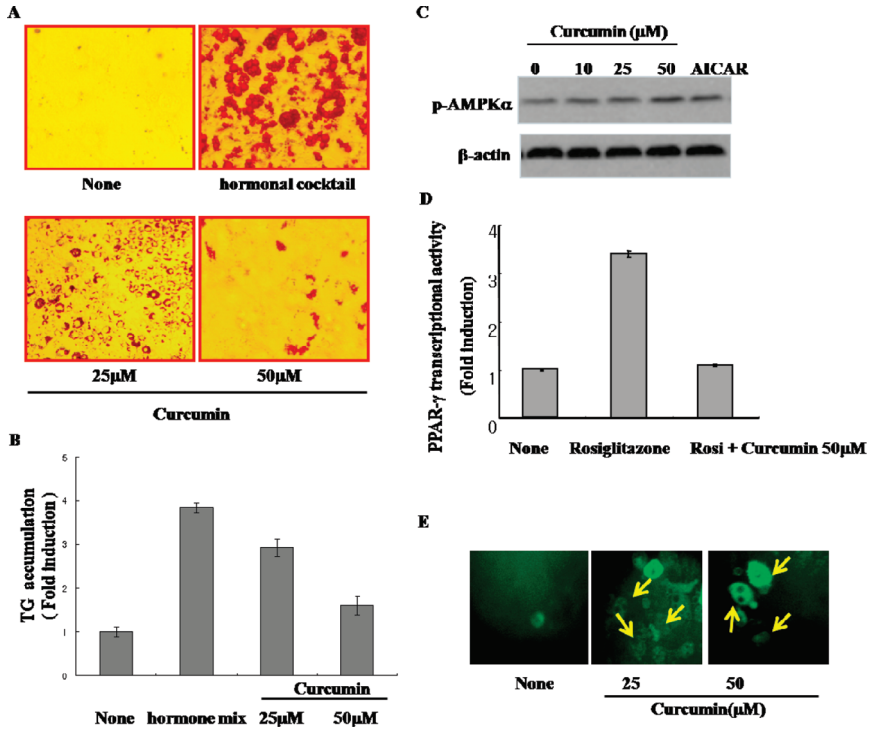

Figure 1. Effects of curcumin on 3T3-L1 adipocyte differentiation and activity of AMPKα and PPAR-γ. Postconfluent 3T3-L1 cells were differentiated in the absence or presence of curcumin for 8 days. The morphological changes associated with adipocyte differentiation were photographed after Oil Red O staining (**A**). Stained lipids were quantified by measuring absorbance (**B**). AMPKα and ACC phosphorylation levels were assessed by Western blot analysis (**C**). PPAR-γ transcription activity was determined as described in Material and Methods (**D**). Cells were incubated with 10 μM DCFH-DA, and the fluorescence intensity was assessed by a fluorescence microscope (**E**).

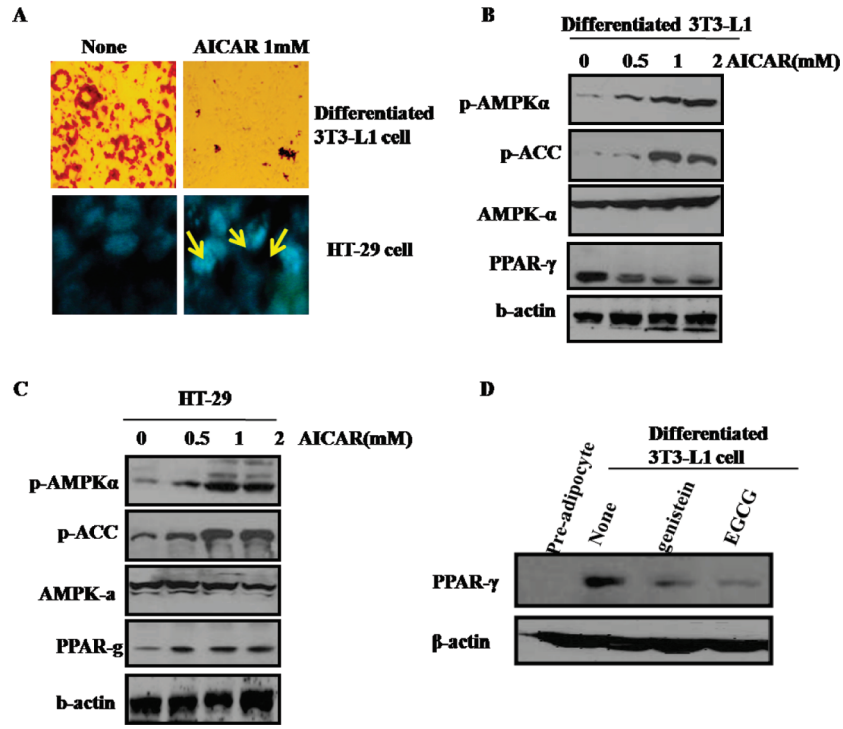

Figure 2. Role of AMPKα in differentiated adipocytes and cancer cells. Postconfluent 3T3-L1 cells were differentiated in the absence or presence of AICAR for 8 days. The morphological changes associated with adipocyte differentiation were photographed after Oil Red O staining. HT-29 cells were incubated in the presence of 10 μM Hoechst33342, and fluorescence microscopic images were obtained (**A**). The levels of AMPKα and PPAR-γ were determined by Western blot analysis in differentiated adipocytes (**B**). HT-29 cells were treated with AICAR, and the levels of AMPKα and PPAR-γ were determined (**C**). Postconfluent 3T3-L1 cells were differentiated in the absence or presence of genistein and EGCG for 8 days. The effects of genistein (100 μM) and EGCG (100 μM) on PPAR-γ expression were also determined by Western blot analysis (**D**).

such as p53 and p21 were altered (**Figure 3C**). Curcumin exposure induced the increase in chromatin condensation representing apoptotic cell death (**Figure 3D**). These antiproliferatory and apoptotic effects were indicated maximally at 80

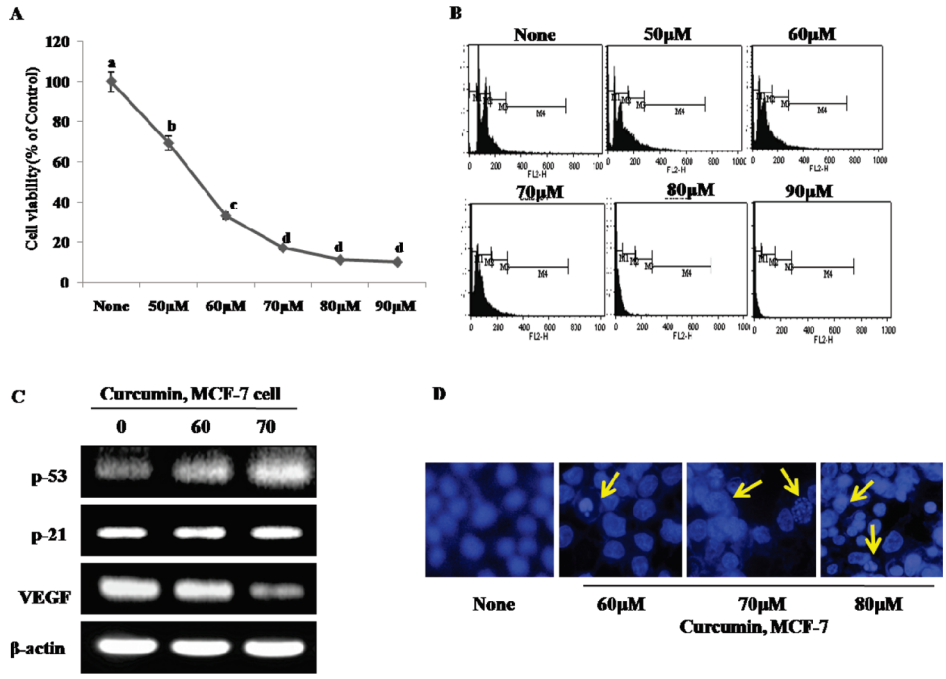

Figure 3. Curcumin exerts apoptotic effects on MCF-7 breast cancer cells. MCF-7 breast cancer cells were treated with different concentrations of curcumin for 12 h, and cell viability was examined by the MTT assay. Means with different superscripts are significantly different at $p < 0.05$ (**A**). The cells were treated with different concentrations of curcumin for 24 h and stained with 10 μg/mL propidium iodide, and the cell cycle was then evaluated by FACS analysis (**B**). Cells were treated with curcumin for 6 h, RNA was extracted with a Trizol reagent, and RT-PCR was performed using specific primers (**C**). Under the same conditions, the cells were incubated for 30 min in the presence of 10 μM Hoechst33342 and examined using a fluorescence microscope (**D**).

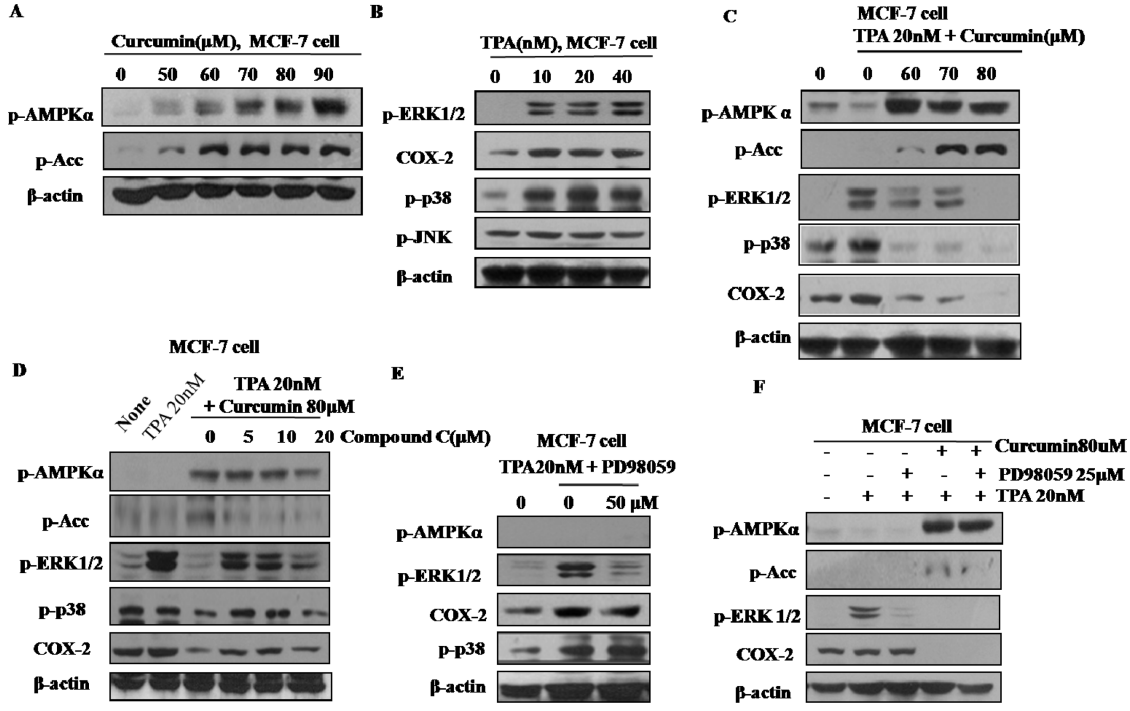

Figure 4. Curcumin inhibits MAPK pathways through AMPKα activation. MCF-7 cells were exposed to different concentrations of curcumin for 6 h, and AMPKα and ACC phosphorylation was assessed (**A**). Cells were treated with TPA for 6 h, and Western blot analysis of the designated proteins was carried out (**B**). The cells were pretreated with 20 nM TPA for 30 min and then exposed to curcumin (60−80 μM) for 6 h (**C**). Prior to treatment with TPA, MCF-7cells were pretreated with compound C for 30 min and then exposed to curcumin (**D**); additionally, the cells were pretreated with 20 nM TPA for 30 min and exposed to PD98059(50 μM) for 6 h (**E**). Cells were also pretreated with 20nM TPA for 30 min and then cotreated with PD98059 (25 μM) and curcumin (80 μM) (**F**).

μM. As shown in **Figure 4A**, curcumin can stimulate AMPKα phosphorylation in MCF-7 breast cancer cells. Next, we examined whether AMPKα activation by curcumin can down-regulate COX-2 and MAPKs in the presence of 12-*O*-tetradecanoylphorbol-13-acetate (TPA). Our results indicated that TPA treatment increased COX-2 expression and ERK1/2, and p-38 phosphorylation (**Figure 4B**), and that these actions were abrogated by the combined treatment of TPA and curcumin

(**Figure 4C**). To confirm the role of AMPKα and the possible downstream MAPK signals, we used compound C, a synthetic AMPKα inhibitor. Cotreatment of the cells with curcumin and compound C (5−20 μM) resulted in a decrease in AMPKα and ACC activities and stimulation of COX-2, p-ERK, and p-p38 MAPKs in a dose-dependent manner (**Figure 4D**). The ERK1/2 inhibitor PD98059 reduced COX-2 expression (**Figure 4E**), but the suppression of COX-2 and ERK1/2 by PD98059 did not affect AMPKα expression (**Figure 4F**). These results strongly suggest that AMPKα is an upstream signal of ERK1/2-COX-2 expression in breast cancer cells cotreated with TPA and curcumin and that AMPKα is a negative regulator of cancer-promoting proteins such as ERK1/2, p38, and COX-2 in phytochemical-treated cancer cells.

DISCUSSION

In this study, the possible roles of treatment with curcumin on adipocyte and cancer cell control were elucidated. The results revealed that curcumin inhibited adipocyte differentiation, activated AMPK, and increased intracellular ROS generation. Also, the confirmation of the involvement of AMPK and PPAR-γ in the regulation of adipocytes was carried out, and the treatment with AMPK activator, AICAR, and curcumin resulted in the significant abrogation of adipocyte differentiation and down-regulation of PPAR-γ transcription. In curcumin-treated breast cancer cells, an activation of AMPK was observed with the concomitant growth inhibition and cell cycle arrest at the sub G_1 phase possibly via the alteration of the VEGF survival gene or p53 and p21 apoptosis-related genes. In TPA-challenged breast cancer cells, the curcumin treatment resulted in an activation of AMPK, with a significant decrease in the expressions of ERK1/2, p38, and COX-2, which could be responsible for the antiproliferatory effect of curcumin.

Accumulating evidence indicates that curcumin exerts its effects on a wide range of molecular targets controlling antiobesity or anticancer activities (*18*−*20*). The mechanism by which curcumin exerts its effects on various target molecules can render it as a promising candidate for the prevention and/or treatment of obesity and cancers. In the current study, the molecular events involving AMPK, an emerging target molecule of both obesity and cancer, were investigated in adipocytes and cancer cells. AMPK plays an essential role in energy homeostasis of various cell types (*21, 22*). PPAR-γ was reported as the key regulatory component in the control of obesity, diabetes, and cancer (*23*−*25*). Here, the possible link between AMPK and PPAR-γ in adipocytes and cancer cells was investigated to provide the molecular basis of AMPK and PPAR-γ involvement in obesity or cancer. We showed that AMPK in 3T3-L1 adipocytes was rapidly activated after treatment with curcumin or AICAR. Surprisingly, AMPK activation led to dual responses of PPAR-γ in 3T3-L1 and HT-29 cells. Curcumin significantly induced PPAR-γ expression in cancer cells, whereas it abrogated the expression of PPAR-γ in 3T3-L1 adipocytes. Despite these contrasting results, curcumin exerted antiproliferatory responses in both of these types of cells. In many tumor types, the decreased expression of PPAR-γ and high levels of COX-2 are observed (*26*−*28*). Therefore, the stimulation of PPAR-γ expression by curcumin in the cancer cells examined seems to be beneficial to tumor control. However, it is quite difficult to explain the basis of the dual actions of AMPK on PPAR-γ expression in either adipocytes or cancer cells. One of the possible answers is that there exist different second regulators between AMPK and PPAR-γ in adipocytes and cancer cells, and thereby they deliver the AMPK signaling message differentially.

We also determined the role of curcumin in regulating apoptosis through the signaling pathways of AMPK/MAPKs/COX-2 in cancer cells. We observed that curcumin functions as an AMPK activator, similar to the synthetic AMPK activator AICAR. Furthermore, it has been shown that the activation of AMPK by curcumin in cancer cells is closely related to the modulation of MAPKs and COX-2. We observed that curcumin down-regulates TPA-induced pERK1/2 and p38 activities, and COX-2 and that these suppressive activities of curcumin were abrogated by cotreatment with compound C, an AMPK inhibitor. Recently, it has been proposed that the MEK → ERK1/2 pathway determines the localization of AMPK either in the cytoplasm or nucleus of cancer cells (*29*). Therefore, the interactions between AMPK and Makinases appeared to be interconnected to determine the fate of the others. By challenging the curcumin-treated MCF-7 cells with the ERK1/2 inhibitor, we found that the inhibition of ERK1/2 is mediated by the upstream regulator AMPK in cancer cells.

We have shown that curcumin inhibits the differentiation process of adipocytes by the activation of AMPK via the downstream of PPAR-γ and the growth of cancer cells by the modulation of AMPK/MAPKs/COX-2. Since AMPK is considered one of the preventive or therapeutic targets for metabolic diseases such as diabetes, obesity, and cancers, the effective modulation of AMPK by curcumin could promise to be an effective approach for treating these diseases.

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