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ORIGINAL ARTICLES

Curcumin suppresses PPARδ expression and related genes in HT-29 cells

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Abstract

AIM: To investigate the effects of curcumin on the expression of peroxisome proliferator-activated receptorδ (PPARδ) and related genes in HT-29 cells.

METHODS: HT-29 cells were treated with curcumin (0-80 μmol/L) for 24 h. The effects of curcumin on the morphology of HT-29 cells were studied by Hoechst 33342 staining. The activity of caspase-3 was determined using DEVD-*p*NA as substrate. The levels of peroxisome PPARδ, 14-3-3ε and vascular endothelial growth factor (VEGF) in HT-29 cells were determined by Western blotting analysis and their mRNA expression was determined by real-time quantitative RT-PCR.

RESULTS: Treatment with 10-80 μmol/L curcumin induced typical features of apoptosis and activated the caspase-3 in HT-29 cells. The expression of PPARδ, 14-3-3ε and VEGF was reduced and the activity of β-catenin/Tcf-4 signaling was inhibited by curcumin treatment.

CONCLUSION: Curcumin can induce apoptosis of HT-29 cells and down-regulate the expression of PPARδ, 14-3-3ε and VEGF in HT-29.

Key words: Curcumin; 14-3-3ε; Peroxisome proliferator-activated receptorδ; Vascular endothelial growth factor; HT-29 cells

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INTRODUCTION

Peroxisome proliferator-activated receptors (PPARs) belong to the nuclear hormone receptor superfamily that enable the cell to respond to extracellular stimuli through transcriptional regulation of gene expression[1,2]. PPARs comprise three subtypes: PPARα, PPARδ and PPARγ. Many of the functions of PPARs are associated with pathways of lipid transport and metabolism[3-5]. Moreover, PPARs play important roles in cell replication, differentiation, tumorigenesis and apoptosis. For example, the expression of PPARδ is elevated in human and rat colorectal cancer cells when compared with normal colon epithelial cells[6,7]. PPARδ has also been implicated in the growth of other human cancers, including hepatocellular carcinoma, cholangiocarcinoma, breast cancer and prostate cancer[8,9].

14-3-3 proteins are anti-apoptotic and anti-inflammatory molecules in cells, which include at least 7 isoforms (β, γ, ε, η, ζ, σ, τ/θ)[10]. 14-3-3 can bind phosphorylated Bad, sequester Bad in the cytosol, and inhibit cytochrome c release, caspase-3 activation, and the apoptosis of cells. PPARδ can induce the expression of 14-3-3ε protein. Elevated 14-3-3ε augments Bad sequestration and prevents Bad-triggered apoptosis[10]. C/EBPβ protein is a mediator of PPARδ-dependent 14-3-3ε gene regulation in human endothelial cells. PPARδ can regulate the expression of C/EBPβ protein, which can bind to the C/EBP response element located at -160/-151 of the 14-3-3ε gene[11]. PPARδ can directly bind to PPAR response elements located between -1426 and -1477 of the 14-3-3ε promoter region, thereby activating 14-3-3ε promoter activity and protein expression[10]. PPARδ can also regulate the expression of vascular endothelial growth factor (VEGF), which can promote colon tumor epithelial cell survival[12,13].

Curcumin is an important polyphenol extracted from the rhizomes of *Curcuma longa L*. Several studies have shown that curcumin exerts antioxidant, anti-inflammatory, anti-carcinogenic and chemopreventive activities on many tumor cells[14]. Curcumin can also down-regulate the activity of the β-catenin/Tcf signaling pathway[15,16]. Curcumin affects the expression of the target genes of β-catenin/Tcf signaling pathway, such as c-Myc, cyclin D1 and c-Jun. PPARδ has been identified as another β-catenin/Tcf-regulated gene[6]. However, the effect of curcumin on the expression of PPARδ remains unknown. In this study, we investigated the effects of curcumin on the expression of PPARδ and related genes such as *14-3-3ε* and *VEGF*. The results showed that curcumin could inhibit the expression of PPARδ and induce the down-regulation of the related genes, including *14-3-3ε* and *VEGF*.

MATERIALS AND METHODS

Reagents

RPMI-1640, fetal bovine serum (FBS), penicillin, streptomycin, and trypsin were purchased from GIBCO. Curcumin, sodium dodecylsulfate (SDS), phenylmethylsulfonylfluoride (PMSF), DNaseI and bovine serum albumin (BSA) were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Anti-VEGF, anti-Tcf-4 and horseradish peroxidase-conjugated goat anti-rabbit antibodies were obtained from Epitomics. Anti-14-3-3ε, anti-PPARδ antibody and protein A/G plus-agarose were provided by Santa Cruz Biotechnology. Nitrocellulose membrane and the enhanced chemiluminescence (ECL) detection system were purchased from Amersham (USA). PrimeScript 1st strand cDNA Synthesis Kit and PCR Kit were from Takara, Japan. Caspase-3 assay kit and nuclear and cytoplasmic protein extraction kit were purchased from Beyotime Biotech, China. Other reagents used were of analytical grade and procured locally.

Cell culture and treatment

The human colon cancer cell line HT-29 was obtained from the American Type Culture Collection (Manassas, VA, USA) and maintained in RPMI 1640, supplemented with 10% FBS, 100 U/mL penicillin and 100 μg/mL streptomycin at 37℃ in a humidified atmosphere of 5% CO_2 and 95% air. Upon reaching 70%-80% confluence, the cells were exposed to 0-80 μmol/L curcumin for 24 h.

Measurement of caspase-3 activities

Caspase-3 activities were measured as previously described[17]. Briefly, cells were lysed in a buffer containing 5 mmol/L Tris (pH 8), 20 mmol/L EDTA, and 0.5% Triton-X 100. Reaction mixture contained 20 mmol/L HEPES (pH 7.0), 10% glycerol, 2 mmol/L dithiothreitol, 50 μg protein per condition, and 200 μmol/L DEVD-*p*NA as substrate. After incubation for 24 h at 37℃, the absorbance in each well was measured at 405 nm with a microplate ELISA reader.

Nuclear staining with Hoechst 33342

Chromatin condensation was detected by nuclear staining with Hoechst 33342[18]. After treatment with 0-80 μmol/L curcumin for 24 h, cells were harvested and washed with PBS three times. Then, the cells were stained with 1 μL of Hoechst 33342 (5 mg/mL, Sigma) in 1 mL basal medium and incubated at room temperature in the dark for 15 min. Stained cells were imaged under a fluorescent microscope using 350 nm stimulation and 460 nm emission.

RNA extraction and real-time quantitative RT-PCR analysis

Total RNA was isolated using Trizol Isolation Reagent (Invitrogen, USA). RNA integrity was confirmed by denaturing agarose gel electrophoresis, and the concentration was quantified by measuring the optical density (OD) at 260 nm. One microgram total RNA was used for DNaseI treatment (Sigma) and subsequent cDNA synthesis. Reverse-transcription was performed with PrimeScript 1st Strand cDNA Synthesis Kit (Takara, Japan) according to the manufacturer's instructions. Real-time qPCR was performed on the ABI 7500 Real Time PCR System using SYBR Premix Ex Taq Ⅱ (Takara, Japan) for analyzing expression of genes. Table 1 shows the primers used for real-time quantitative RT-PCR. The amplification reactions were performed under the following PCR conditions: one cycle at 95℃ for 10 min, followed by 40 cycles of 95℃ for 15 s, 59℃ for 15 s and 72℃ for 30 s. mRNA fold changes in target genes relative to the endogenous GAPDH control were calculated as suggested by Schmittgen *et al*[19]. Each reaction was performed in triplicate.

Western blotting

Western blotting analysis was done as previously described with minor modifications to detect the expressions of PPARδ, 14-3-3ε, and VEGF protein[20]. The total cellular protein and the nuclear protein were extracted according to the instructions of nuclear and cytoplasmic extraction reagents kit (Beyotime, Haimen, China). The lysates were used to estimate their protein content with BCA protein assay. Fifty micro-grams of protein from each sample was subjected to SDS-PAGE. After electrophoresis, proteins were electroblotted to a Hybond-C Extra nitrocellulose membrane (Amersham, USA). The membrane was blocked at room temperature with 5% non-fat dry milk in TBS containing 0.3% Tween (TBS-T). The membrane was washed three times with TBS-T and incubated overnight at 4℃ with the primary antibody, anti-PPARδ (1:500), anti-14-3-3ε (1:2000), and anti-VEGF (1:1000), followed by 1 h incubation with a 1:5000 dilution of the appropriate horseradish-peroxidase-conjugated secondary antibody. After incubation, the membrane was washed with TBS-T for three times, the antigen-antibody complexes were visualized by enhanced chemiluminescence and exposure to X-ray film for 0.5 up to 30 min.

Table 1 Primers used for real-time quantitative RT-PCR

Apoptosis modulator	Forward primer (5′→3′)	Reverse primer (5′→3′)
PPARδ	GCAGGCTCTAGAATTCCATC	GTGCAGCCTTAGTACATGTC
VEGF	AGGAGGAGGGCAGAATCATCA	CTCGATTGGATGGCAGTAGCT
14-3-3ε	GAGCGATACGACGAAATGGT	CCTTGGACTCGCCAGTGTTAG
GAPDH	GGCAAATTCAACGGCACAGT	AGATGGTGATGGGCTTCCC

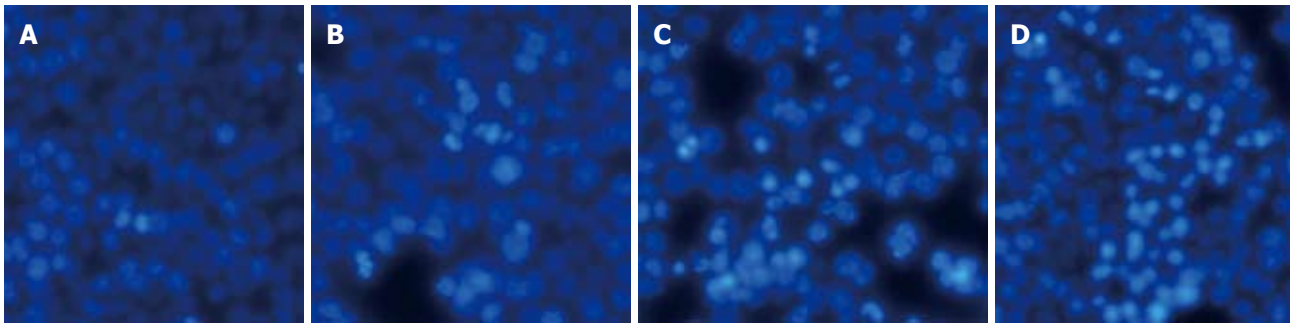

Figure 1 Induction of apoptosis in HT-29 cells by curcumin. Fluorescence images of HT-29 cells using Hoechst 33258 staining showed curcumin induced typical apoptotic morphological changes. A: Control; B: HT-29 cells incubated with 20 μmol/L curcumin; C: HT-29 cells incubated with 40 μmol/L curcumin; D: HT-29 cells incubated with 60 μmol/L curcumin.

Immunoprecipitation

The immunoprecipitation was done as previously described with minor modifications[16]. The nuclear lysates containing 500 μg protein were incubated with 5 μg primary antibody overnight at 4℃. Fifty microliters of protein A/G plus-agarose (Santa Cruz Biotechnology) was added and the complex was incubated at 4℃ overnight. The beads were washed three times with high salt buffer (1 mol/L Tris-HCl, pH 7.4, 0.50 mol/L NaCl, and 1% Nonidet P-40) and twice with lysis buffer to eliminate non-specific binding. The immunoprecipitated complexes were released with 2 × sample buffer for Western analysis.

Transfection and luciferase assay

Transient transfection was performed using Fugene 6 Transfection Reagent (Roche) in accordance with the manufacturer's instructions. Briefly, HT-29 cells were seeded at a density of 2×10^5 cells per well in six-well plates. After 24 h, cells were transfected with 0.5 μg luciferase reporter constructs (TOPflash or FOPflash, respectively) and 0.5 μg β-galactosidase gene. Three hours after transfection, the cells were treated with 0-80 μmol/L curcumin and the incubation was continued for 24 h. Then, the cells were collected and resuspended in Luciferase Reporter Lysis Buffer (Promega, USA). The cell lysates were centrifuged and aliquots (70 μL) of the supernatant were assayed for the activity of luciferase and galactosidase. Reporter activity was normalized for variations in transfection efficiency using β-galactosidase as an internal control. Experiments were performed three times independently.

Statistical analysis

Results are presented as mean ± SE. Comparisons between multiple groups were performed using the one-way ANOVA followed by Dunnett's test. Differences were considered to be significant at $P < 0.05$.

RESULTS

Induction of HT-29 cell apoptosis by curcumin

Hoechst 33342 staining assay was performed to observe the effects of curcumin on cell nuclear morphology. As shown in Figure 1, the control cells displayed intact nuclear structure, while nuclei with chromatin condensation and formation of apoptotic bodies were seen in cells incubated with curcumin in a dose-dependent fashion (Figure 1A-D). Caspase-3 activation is an important marker of apoptosis, so we determined the activities of caspase-3 in HT-29 treated with curcumin for 12 or 24 h. Caspase-3 activities were highly elevated by curcumin at the concentration of 20-80 μmol/L (Figure 2).

Curcumin suppressed the expression of PPARδ

PPARδ plays important roles in the growth and proliferation of many cancer cells[8]. PPARδ has been identified as one of the down-streaming targets of the β-catenin/Tcf-4 pathway[6]. We studied whether curcumin affected the expression of PPARδ in HT-29 cells. HT-29 cells were incubated with curcumin at different concentrations. PPARδ gene expression at the mRNA level was detected by quantitative RT-PCR. The results showed that 40-80 μmol/L of curcumin could significantly reduce the level of PPARδ in HT-29 cells (Figure 3A). Whole-cell extracts were prepared and analyzed by Western blotting. Curcumin reduced the level of PPARδ protein in a dose-dependent manner (Figure 3B).

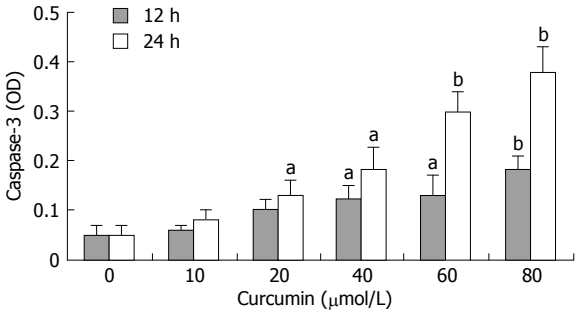

Figure 2 Caspase-3 activity was markedly increased by curcumin in a dose-dependent manner. Each bar denotes mean ± SD (n = 3). ($^aP < 0.05$ *vs* control; $^bP < 0.01$ *vs* control).

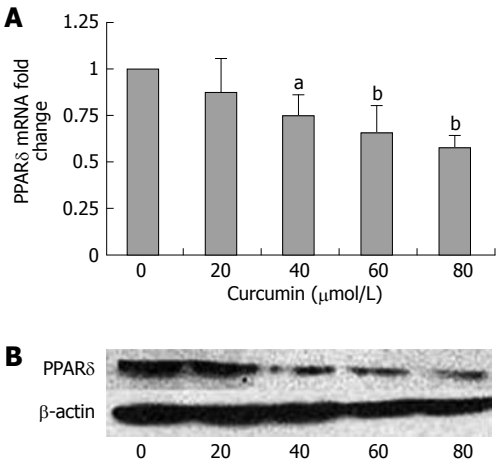

Figure 3 Curcumin inhibits the expression of PPARδ in HT-29 cells. A: Real-time quantitative RT-PCR indicated that curcumin markedly reduced the level of PPARδ mRNA. Values are % reduction in PPARδ mRNA fold changes caused by curcumin compared with cells without curcumin treatment. Values are mean ± SD from three samples per group. GAPDH was used as an internal control; B: The effects of curcumin on the level of PPARδ protein were determined by Western blotting. β-actin was used as an internal marker. ($^aP < 0.05$ *vs* control; $^bP < 0.01$ *vs* control).

Curcumin decreased the expression of 14-3-3ε and VEGF

14-3-3ε plays important roles in protecting cells from apoptosis[21]. It harbors three contiguous PPAR response elements (PPREs), which are the responsive promoter region of 14-3-3ε activation. Deletion of PPREs abrogated PPARδ-mediated 14-3-3ε to PPARδ up-regulation[10]. On the basis of this study, we hypothesized that curcumin could inhibit the expression of 14-3-3ε in HT-29 cells. The real-time quantitative PCR showed that treatment with 60 and 80 μmol/L curcumin for 24 h could markedly reduce the 14-3-3ε mRNA level in HT-29 cells (Figure 4A). The Western blotting showed that curcumin treatment also inhibited the 14-3-3ε protein expression at the concentration of 20-80 μmol/L (Figure 4C).

VEGF can stimulate endothelial cell proliferation and prevents apoptosis in the endothelial cells of newly formed vessels[22]. VEGF has been identified as one of the potential targets of PPARδ in colorectal cancer (CRC) cells[13]. We hypothesized that curcumin can decrease the expression of VEGF. The real-time quantitative RT-PCR showed that 40-80 μmol/L of curcumin could significantly down-regulate the expression of VEGF (Figure 4B). Consistent with the VEGF mRNA level, VEGF protein was also decreased by curcumin at the concentrations of 20-80 μmol/L (Figure 4D).

Curcumin down-regulated β-catenin/Tcf signaling pathway and inhibited β-catenin associated with Tcf-4 in nuclei of HT-29 cells

The association of β-catenin with Tcf-4 is required for activation of β-catenin/Tcf signaling, so we determined the level of the β-catenin/Tcf-4 complex in the nucleus of HT-29 cells. We used anti-Tcf-4 antibody to co-immunoprecipitate the complex of Tcf-4 and β-catenin from nuclear extracts and determined the amount of β-catenin by immunobloting. Curcumin markedly dereased the level of β-catenin/Tcf-4 complex (Figure 5A). However, the level of total β-catenin in the nucleus was not significantly affected by incubation of curcumin (Figure 5A). These results suggested that curcumin could inhibit β-catenin associated with Tcf-4 in the nucleus. Because PPARδ is a target gene of β-catenin/Tcf-4, curcumin may decrease the expression of PPARδ through blocking the β-catenin/Tcf-4 signaling pathway.

PPARδ has been identified as a target of the β-catenin/Tcf signaling pathway[6]. We hypothesized that curcumin can inhibit the expression *via* the β-catenin/Tcf signaling pathway. We transfected HT-29 cells with either TOPflash or FOPflash. The cells were incubated with 0-80 μmol/L curcumin for 24 h and the luciferase activity was determined. As a result, curcumin could decrease the transcriptional activity of β-catenin/Tcf-4 signaling pathway in HT-29 cells (Figure 5B).

DISCUSSION

Curcumin can induce apoptosis of many cell lines. However, the mechanism is still unclear. In this study, we demonstrated that curcumin could induce the apoptosis of HT-29 cells and down-regulate the expression of PPARδ, 14-3-3ε and VEGF, which suggested that curcumin-induced apoptosis was attributed to the inhibition of the expression of PPARδ, 14-3-3ε or VEGF.

The PPARs are ligand-activated transcription factors that are members of the nuclear hormone receptor superfamily. PPARs form heterodimers with the retinoic X receptor and bind to DNA in correspondence to specific PPRE located in the promoter of target genes[23]. The PPAR subfamily includes PPARα, PPARδ (or β) and PPARγ, which share extensive structural homology[23]. Studies have shown that PPARα and PPARγ play important roles in such physiological processes as fatty acid metabolism, glucose metabolism, immunity, and cellular differentiation[24,25]. However, the physiological role of PPARδ is less studied. Recently, PPARδ has been found to be related to carcinogenesis[26,27]. Curcumin has been proved to inhibit the growth and differentiation of many cancer cell lines[28,29]. Administration of 30 mg/kg curcumin by intraperitoneal injection for 2 wk could

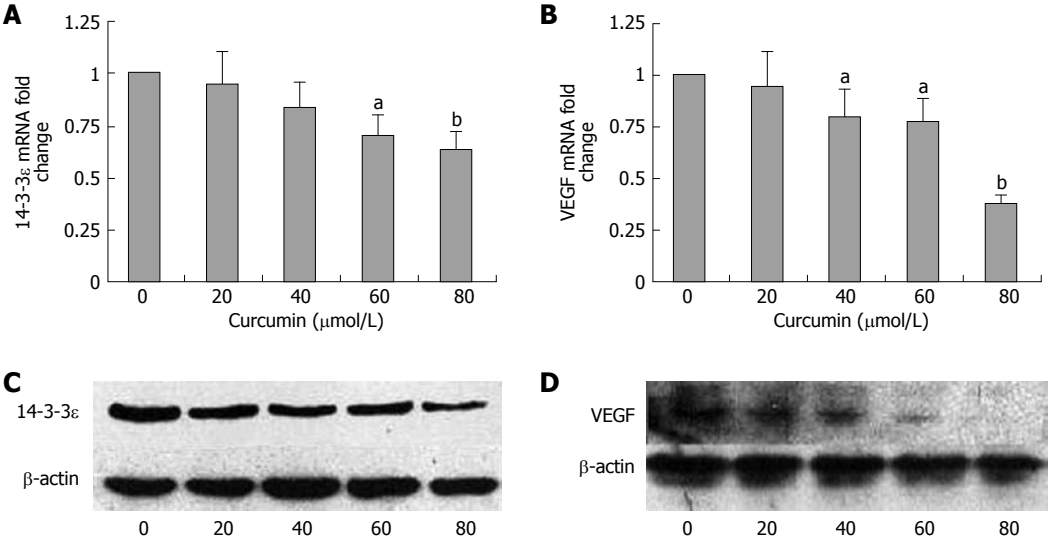

Figure 4 The expression of 14-3-3ε and VEGF is down-regulated by curcumin. Real-time quantitative RT-PCR showed that curcumin decreased the mRNA levels of 14-3-3ε (A) and VEGF (B). Values are means ± SD from three samples per group. GAPDH was used as an internal control for quantitative RT-PCR. Western blotting was performed to determine the effects of curcumin on the expression of 14-3-3ε (C) and VEGF (D). β-actin was used as internal marker for Western blotting. ($^aP < 0.05$ *vs* control; $^bP < 0.01$ *vs* control).

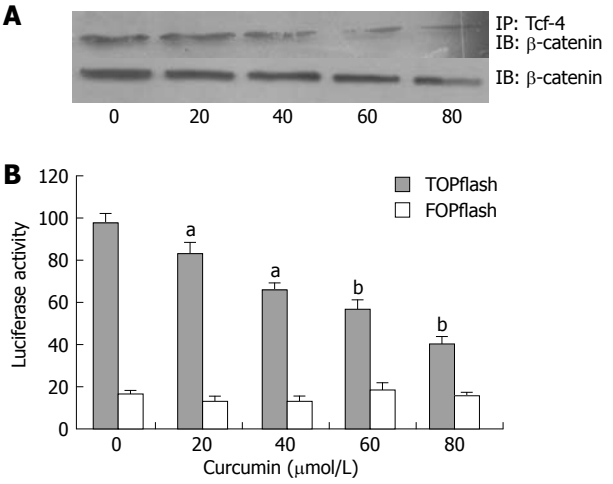

Figure 5 Curcumin inhibited β-catenin association with Tcf-4 and down-regulated the β-catenin/Tcf reporter activities in HT-29 cells. A: Immunoprecipitation and immunoblotting results showed that curcumin inhibited β-catenin associated with Tcf-4 but had no effects on the level of β-catenin in nucleus; B: Curcumin significantly reduced TOPflash luciferase activity but had no effect on the activity of FOPflash. Values represent means ± SE of three independent experiments. ($^aP < 0.05$ *vs* control; $^bP < 0.01$ *vs* control).

significantly improve the expression of PPARγ in colonic tissues of rats[30]. To the best of our knowledge, the effects of curcumin on PPARδ have not been investigated. In this study, we found that curcumin could inhibit the expression of PPARδ in HT-29 cells. We hypothesize that there are two possible reasons. First, He *et al*[6] have identified PPARδ as a β-catenin/Tcf-regulated gene. In this study, curcumin could inhibit the β-catenin associated with Tcf-4 and down-regulate the activity of the β-catenin/Tcf-4 signaling pathway, which is in agreement with the studies of Jaiswal *et al* and Park *et al*[15,16]. These studies indicate that curcumin may reduce the expression of PPARδ by blocking the β-catenin/Tcf-4 signaling pathway. Second, PPARδ can also be up-regulated by oncogenic K-Ras[31], suggesting that curcumin may reduce the expression of PPARδ by other signaling pathways. We will further study the mechanism that curcumin inhibits the expression of PPARδ.

14-3-3 are cytosolic proteins serving as a scaffold to interact with a large number of proteins[32]. They may protect cells from apoptosis through their binding and sequestering phosphorylated Bad in cytosol[21]. Seven isoforms of 14-3-3 proteins have been identified in mammalian cells[10]. It has been indicated that 14-3-3ε is a target gene of PPARδ, which can bind to the PPRE upstream of 14-3-3ε promotor region[10]. We found that curcumin decreased the expression of 14-3-3ε in HT-29 cells. To the best of our knowledge, curcumin-induced down-regulation of 14-3-3ε has not reported previously.

VEGF is also up-regulated by the activation of PPARδ in colon carcinoma cells[12]. Several studies have indicated that curcumin can down-regulate the expression of VEGF in different cell lines[33,34]. In the present study, we found that curcumin could inhibit the expression of VEGF in HT-29 cells. VEGF plays important roles in tumor angiogenisis[35]. Our results suggest curcumin may inhibit the angiogenisis of colorectal tumors.

In summary, our study shows that curcumin can suppress the expression of PPARδ and the related genes such as *14-3-3ε* and *VEGF* in HT-29 cells. The reasons why curcumin inhibits the expression of PPARδ and related protein are still unclear. Curcumin can down-regulate the activity of β-catenin/Tcf-4 signaling pathway, which suggests that curcumin may decrease the expression of PPARδ by the inhibition of β-catenin/Tcf-4 signaling activity in HT-29 cells.

COMMENTS

Background

Colorectal cancer is one of the leading causes of death and is a major public health problem in Western countries. Peroxisome proliferator-activated

receptors (PPARs) play important roles in cell replication, differentiation, tumorigenesis, and apoptosis. The expression of PPARδ is elevated in human and rat colorectal cancer cells when compared with normal colon epithelial cells.

Research frontiers

Curcumin can affect the expression of the target genes of β-catenin/Tcf signaling pathway, such as c-Myc, cyclin D1, c-Jun, *etc*. PPARδ has been identified as another β-catenin/Tcf-regulated gene. However, the effect of curcumin on the expression of PPARδ remains unknown. In this study, the authors demonstrate that curcumin could affect the expression of PPARδ and related genes such as 14-3-3ε and vascular endothelial growth factor (VEGF).

Innovations and breakthroughs

Recent studies have suggested the PPARδ play important roles in colorectal carcinogenesis. It activates the expression of 14-3-3ε, which can sequester the pro-apoptotic protein, Bad, to inhibit the apoptosis of cancer cells. It is the first report showing that the curcumin down-regulates the expression of PPARδ and 14-3-3ε.

Applications

PPARδ is found as a new target of curcumin to induce the apoptosis of HT-29 cells. New curcumin derivatives can be developed to efficiently inhibit the growth and differentiation of colorectal cancer cells by down-regulating the expression of PPARδ.

Terminology

Curcumin is an important polyphenol extracted from the rhizomes of *Curcuma longa L*. PPARs belong to the nuclear hormone receptor superfamily that enable the cell to respond to extracellular stimuli through transcriptional regulation of gene expression, including PPARα, PPARδ, and PPARγ. 14-3-3 proteins are anti-apoptotic and anti-inflammatory molecules in cells, which include at least seven isoforms (β, γ, ε, η, ζ, σ, τ/θ). Elevated 14-3-3ε augments Bad sequestration and prevents Bad-triggered apoptosis.

Peer review

Curcumin can induce apoptosis of many cell lines. However, the mechanism is still unclear. In this study, the authors demonstrated that curcumin could induce the apoptosis of HT-29 cells and down-regulate the expression of PPARδ, 14-3-3ε and VEGF. More importantly, the authors showed that curcumin could markedly lower the level of β-catenin/Tcf-4 complex without affecting the level of total β-catenin in nucleus after incubation of curcumin. These results suggested that curcumin could inhibit β-catenin associated with Tcf-4 in nucleus.

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