

Curcumin inhibits Akt/mammalian target of rapamycin signaling through protein phosphatase-dependent mechanism

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Abstract

Akt/mammalian target of rapamycin (mTOR) signaling plays an important role in tumorigenesis and is dysregulated in many tumors, especially metastatic prostate cancers. Curcumin has been shown to effectively prevent or inhibit prostate cancer *in vivo* and inhibit Akt/mTOR signaling *in vitro*, but the mechanism(s) remains unclear. Here, we show that curcumin concentration- and time-dependently inhibited the phosphorylation of Akt, mTOR, and their downstream substrates in human prostate cancer PC-3 cells, and this inhibitory effect acts downstream of phosphatidylinositol 3-kinase and phosphatidylinositol-dependent kinase 1. Overexpression of constitutively activated Akt or disruption of TSC1-TSC2 complex by small interfering RNA or gene knockout only partially restored curcumin-mediated inhibition of mTOR and downstream signaling, indicating that they are not the primary effectors of curcumin-mediated inhibition of Akt/mTOR signaling. Curcumin also activated 5'-AMP-activated protein kinase and mitogen-activated protein kinases; however, inhibition of these kinases failed to rescue the inhibition by curcumin. Finally, it was shown that the inhibition of Akt/mTOR signaling by curcumin is resulted from calyculin A-sensitive protein phosphatase-dependent dephosphorylation. Our study reveals the profound effects of curcumin on the Akt/mTOR signaling network in PC-3 cells and provides new mechanisms for the anticancer effects of curcumin. [Mol Cancer Ther 2008;7(9):2609–20]

Introduction

The phosphatidylinositol 3-kinase (PI3K)/Akt (also termed protein kinase B)/mammalian target of rapamycin (mTOR)

signaling axis plays a central role in regulation of multiple critical cellular functions including stress responses, cell growth and survival, and metabolism (1). Activated PI3K converts phosphatidylinositol into PtdIns(3,4)P₂ (PIP₂) and PtdIns(3,4,5)P₃ (PIP₃). Consequently, phosphatidylinositol-dependent kinase 1 (PDK1) and Akt are recruited to the cell membrane, and Akt is phosphorylated at residues Thr³⁰⁸ and Ser⁴⁷³ by PDK1 and PDK2 (presumably mTOR complex 2), respectively (2, 3). Phosphorylated and activated, Akt phosphorylates and regulates a plethora of substrates including glycogen synthase kinase 3 (GSK3), Forkhead family transcription factors, and mTOR (4). On the other hand, the phosphatase and tensin homologue deleted on chromosome 10 (PTEN) counteracts PI3K activity by dephosphorylating PIP₂ and PIP₃ (5).

Specifically, mTOR is a key mediator of Akt signaling, especially in oncogenic transformation. mTOR forms two functional complexes, C1 and C2, and integrates signals from nutrients, growth factors, and cellular energy status to control cell growth and proliferation by regulating protein synthesis (6). Phosphorylation of mTOR at Ser²⁴⁴⁸ by Akt (7) or S6K1 (8) and at Ser²⁴⁸¹ by autophosphorylation is important for its activity (9). The activity of mTOR is negatively regulated by tuberous sclerosis complex 1 and 2 (TSC1 and TSC2, also known as hamartin and tuberin). TSC1/TSC2 complex dissociates Ras homologue enriched in brain from mTOR and thus inhibits mTOR activation. Akt phosphorylates TSC2 and disrupts the TSC1/TSC2 complex, leading to activation of mTOR (10). On the other hand, 5'-AMP-activated protein kinase (AMPK), which is activated by increased AMP/ATP ratio and/or tumor suppressor LKB1, inhibits mTOR activation by activating TSC1/TSC2 (11). Activated mTOR C1 phosphorylates the translation inhibitor 4E-BP1 and the ribosomal protein S6 kinase (S6K) and results in initiation of protein translation (6). p70 S6K also phosphorylates and inhibits insulin receptor substrate-1, forms a negative feed back regulation of PI3K/Akt signaling (12).

The PI3K/Akt/mTOR pathway is also controlled by serine/threonine protein phosphatases. Two major classes of serine/threonine protein kinases, PP2A and PP1, are extensively involved in many signaling pathways. It has been well documented that PP2A interacts with and dephosphorylates Akt *in vitro* and *in vivo* (13–15). PP2A has also been reported to dephosphorylate S6K in response to different stimuli (16, 17). Likewise, 4E-BP1 has been identified as a substrate of PP2A *in vivo* and *in vitro* (18, 19). Currently, no direct evidence proves that mTOR is dephosphorylated by PP2A. However, study using adenovirus implied that mTOR activity is regulated by PP2A (20), and mTOR is also involved in the regulation of PP2A

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activity (21). Compared with PP2A, PP1 is less involved in Akt/mTOR signaling possibly due to the absence of PP1 recognition sequences and docking motifs in the major components of Akt/mTOR signaling (22). Besides PP1 and PP2A, PH domain leucine-rich repeat protein phosphatase 1 and 2 have been identified as specific Akt Ser⁴⁷³ phosphatases (23).

In many human tumors, particularly prostate cancers, PI3K/Akt/mTOR signaling is dysregulated by various oncogenic events (24). The hormone-refractory prostate cancers are frequently characterized by inactivation of PTEN and activation of Akt/mTOR signaling. Akt activity is an important determinant of the sensitivity of prostate cancer cells to therapies (25). Thus, inhibition of PI3K/Akt/mTOR signaling provides promising strategies of prevention and therapies for prostate cancer (26, 27).

Curcumin (diferuloylmethane), a major chemical component of turmeric (*Curcuma longa*), possess a broad spectrum of chemopreventive and therapeutic properties against various tumors in both *in vitro* and *in vivo* models and clinical trials (28, 29). Curcumin has been shown to inhibit cell proliferation, induce apoptosis, suppress inflammation, and sensitize tumor cells to cancer therapies (30–32). The mechanism(s) underlying the anticancer activity of curcumin has been extensively investigated, and several signaling pathways including nuclear factor- κ B, activator protein-1, mitogen-activated protein kinases (MAPK), and cell cycle machinery have been suggested as the targets of curcumin (31). Recently, it has been reported that curcumin inhibits Akt/mTOR signaling in various tumor cells including prostate cancer cells (33–36); however, the molecular mechanism by which curcumin inhibits Akt/mTOR signaling remains unclear.

In the present study, we investigated the molecular mechanism by which curcumin inhibits Akt/mTOR signaling in the androgen-independent and PTEN-null PC-3 prostate cancer cells. Our results show that curcumin concentration- and time-dependently inhibits Akt/mTOR signaling, and this inhibitory effect is primarily mediated by curcumin-activated PP2A and/or unspecified calyculin A-sensitive protein phosphatase. At the same time, curcumin also activates AMPK and MAPKs, but these kinases are less involved in curcumin-mediated inhibition of Akt/mTOR signaling.

Materials and Methods

Reagents, Plasmids, and Cell Culture

Curcumin, PI3K inhibitor Ly294002, MEK1 inhibitor PD98059, c-Jun NH₂-terminal kinase inhibitor II, and p38 inhibitor SB238004 were purchased from Sigma. L- α -phosphatidylinositol-3,4,5-trisphosphate, compound C, and tautomycin were purchased from EMD Biosciences. Akt1/protein kinase B α protein, active PDK1 protein, Serine/Threonine Phosphatase Assay Kit, and okadaic acid sodium salt were purchased from Upstate. MTS assay kit was obtained from Promega. [6]thymidine and L-[3,4,5]leucine were obtained from Perkin-Elmer. Calyculin A, small interfering RNA (siRNA) against tuberin/TSC2, control

scrambled siRNA, cell lysis buffer (10 \times), and antibodies against p-PI3K p85 (T458)/p55 (T199), p-PDK1 (S241), p-Akt (T308), p-Akt (S473), Akt, p-FoxO1 (S256), p-GSK3 β (S9), p-mTOR (S2448), p-mTOR (S2481), mTOR, p-p70 S6K (T389), p-S6 ribosomal protein (S235/236), p-4E-BP1 (T37/46), p-eIF4G (S1108), tuberin/TSC2, p-tuberin/TSC2 (T1462), p-AMPK α (T172), p-acetyl-CoA carboxylase (ACC; S79), and methylated and nonmethylated PP2A catalytic subunit were purchased from Cell Signaling Technology. Antibodies against HA tag, PDK1 (protein kinase B kinase), β -actin, cyclin D1, and horseradish peroxidase-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology. LipofectAMINE 2000, recombinant protein G-conjugated agarose, and all cell culture materials were purchased from Invitrogen. All the other chemicals were of the highest grade available.

HA-tagged Akt and AMPK α 1-expressing plasmids were gifts from Dr. Kun-liang Guan (University of Michigan); the constitutively activated Akt-expressing plasmid (myr-HA-Akt) was a gift from Dr. Cory Abate-Shen (University of Medicine and Dentistry, New Jersey-Robert Wood Johnson Medical School). The dominant-negative AMPK α 1 was constructed by mutation of Thr¹⁷² to alanine using QuickChange site-directed mutagenesis kit (Stratagene) and the mutation was confirmed by sequencing. Human prostate cancer PC-3 cells (American Type Culture Collection) were cultured in MEM supplemented with 10% fetal bovine serum. TSC1 (-/-) and wild-type mouse embryonic fibroblasts (MEF) were gifts from Dr. David J. Kwiatkowski (Harvard Medical School) and Dr. Shengkan Victor Jin (University of Medicine and Dentistry, New Jersey-Robert Wood Johnson Medical School) and maintained in DMEM supplemented with 10% fetal bovine serum and 3.7 mg/mL sodium bicarbonate in a humidified 5% CO₂ atmosphere at 37°C.

Cellular DNA Synthesis, Protein Synthesis, and Proliferation Evaluations

For evaluation of DNA or protein synthesis, PC-3 cells were cultured in 24-well plates and treated with various concentrations of curcumin in fetal bovine serum-free MEM medium for the indicated time. Then, 1 μ Ci/well [6]thymidine or L-[3,4,5]leucine were added into the cultures and incubated for 2 h. The cells were fixed in 10% TCA at room temperature for 15 min and washed twice with 5% TCA. The acid-insoluble material was dissolved in 2 mol/L NaOH overnight, and aliquots were used to determine the radioactivity using a liquid scintillation counter. For MTS cell proliferation assays, PC-3 cells were seeded in 96-well plates at a density of 5 \times 10³ per well and treated with various concentrations of curcumin for 24 h, and 20 μ L MTS reagent was added into each well and incubated for further 2 h. The absorbance at 490 nm was read immediately using a μ Quant microplate reader (Bio-Tek Instruments).

Transient Transfection and Western Blotting

Transient transfection was done according to the protocol provided by the manufacturer, and all experiments were done 24 h (48 h for siRNA experiments) after transfection.

The cells as indicated were cultured in six-well plates for 24 h followed by serum deprivation for 12 h and treated with various concentrations of curcumin or chemicals in serum-free medium for the indicated time. After treatment, the cells were washed with cold PBS and harvested in 1× cell lysis buffer supplemented with protease inhibitor cocktail (Roche). Cell lysates were centrifuged at 4°C, 13,000 × g for 10 min, and the protein concentrations in supernatants were determined by BCA protein assay (Pierce Biotechnology). Aliquots of lysates each containing 30 µg protein were boiled in 1× SDS loading buffer and resolved by 4% to 15% SDS-PAGE. Proteins in gel were electrotransferred to polyvinylidene difluoride membrane using a semidry transfer system. The membranes were blocked with 5% fat-free milk in PBS-0.1% Tween 20 at room temperature for 2 h and probed with specified primary antibodies (1:1,000) in 3% bovine serum albumin in PBS-0.1% Tween 20 overnight at 4°C. Then, the blots were washed with PBS-0.1% Tween 20 for 10 min three times and incubated with corresponding horseradish peroxidase-conjugated second antibodies (1:5,000) at room temperature for 1 h. The blots were washed again in PBS-0.1% Tween 20 for 10 min three times and visualized by enhanced chemiluminescence and scanned using a Gel Documentation 2000 system (Bio-Rad). Actin was blotted for each sample as loading control.

In vitro Kinase Assay

In vitro kinase assays were done using either purified active PDK1 without first 52 amino acids (PDK1Δ52) or immunoprecipitated PDK1 from lysates of PC-3 cells. PC-3 cells were cultured in 10-cm dishes, treated with the indicated concentrations of curcumin for 10 min, washed, and harvested in cell lysis buffer as described above. Aliquots of lysates each containing 500 µg proteins were precleared by incubating with protein G-conjugated agarose at 4°C with agitation for 1 h and incubated with anti-PDK1 antibody and protein G-conjugated agarose at 4°C overnight with agitation. The immunoprecipitated pellets were collected by centrifugation, washed three times with the lysis buffer, and washed twice with kinase assay buffer [25 mmol/L Tris-HCl (pH 7.5), 5 mmol/L glycerol phosphate, 2 mmol/L DTT, 0.1 mmol/L Na₃VO₄, and 10 mmol/L MgCl₂] before using. Purified Akt protein (1 µg) was incubated with either 50 ng PDK1Δ52 in the presence of the indicated concentrations of curcumin or immunoprecipitated pellets in kinase assay buffer with 1 mmol/L ATP at 30°C for 20 min with agitation. The samples were boiled in 1× SDS sample loading buffer and immunoblotted against p-Akt (T308) or PDK1.

Protein Phosphatase Assay

Serine/threonine phosphatase activity was determined using Malachite Green Phosphatase assay. PC-3 cells were cultured in six-well plates and treated with various concentrations of curcumin for 10 min, and the cells were scraped into phosphatase lysis buffer [20 mmol/L HEPES (pH 7.4), 0.1% NP-40, 1 mmol/L EGTA, 30 mmol/L β-mercaptoethanol, 0.1 mmol/L MgCl₂, 1 mmol/L phenylmethylsulfonyl fluoride, and protease inhibitor cocktail] and sonicated on ice for three 10-s pulses. The cell lysates

were centrifuged at 2,000 × g at 4°C for 5 min, and aliquots of the supernatants were used for phosphatase assay. Cell lysate (5 µL each) was diluted in 20 µL phosphatase assay buffer [50 mmol/L Tris-HCl (pH 7.0) and 0.1 mmol/L EDTA], and phosphopeptide substrate K-R-pT-I-R-R was added into the mixture to a final concentration of 200 µmol/L and incubated for 5 min. The reaction was terminated by adding 100 µL Malachite Green detection solution; 15 min later, the absorbance at 620 nm was measured and corrected by subtracting the readings of the blank without cell lysate.

Statistical Analysis

All experiments in this study were repeated at least two times with similar results. The values and relative percentages are presented as mean ± SD of four separate samples. Statistical analysis was done by the two-tailed Student's *t* test for unpaired data, with *P* < 0.05 considered statistically significant.

Results

Curcumin Inhibited DNA/Protein Synthesis, Cell Proliferation, and Akt/mTOR Signaling in PC-3 cells

Because Akt/mTOR signaling controls protein translation and cell proliferation, we firstly determined the effects of curcumin on the DNA/protein synthesis of PC-3 cells. As indicated by [³H]TdR and [³H]Leu incorporation assays, curcumin inhibits DNA and protein synthesis in a similar concentration-dependent pattern to the inhibition of cell proliferation determined by MTS assay (Fig. 1A). Moreover, the time-course study indicates that the inhibition of protein synthesis occurred earlier than the inhibition of DNA synthesis (Fig. 1B).

Next, the effects of curcumin on the Akt/mTOR signaling were examined. PC-3 cells were treated with various concentrations of curcumin for 1 h, harvested, and analyzed by Western blotting. As shown in Fig. 1C, curcumin inhibited the phosphorylation of Akt (T308 and S473), FoxO1 (S256), GSK3β (S9), tuberin/TSC2 (T1462), mTOR (S2448/2481), p70 S6K (T389), S6 (S235/236), 4E-BP1 (S37/46), and eIF4G (S1108) in a similar concentration-dependent manner. At the same time, curcumin induced the phosphorylation of AMPKα and one of its substrates, ACC, indicating that AMPK was activated. MAPKs, including extracellular signal-regulated kinase 1/2, c-Jun NH₂-terminal kinase, and p38MAPK, were also activated by curcumin treatment (data not shown). However, the phosphorylation state of PDK1 and PKC remained unchanged.

In the following studies, we focused on the Akt/mTOR signaling axis. When PC-3 cells were treated with 40 µmol/L curcumin, the phosphorylation of Akt at Thr³⁰⁸ was promptly inhibited within 5 min followed by inhibition of phosphorylation of mTOR, Akt at Ser⁴⁷³, and the other downstream targets including 4E-BP1, eIF4G, p70 S6K, and S6 (Fig. 1D). In all experiments, the total Akt, mTOR, 4E-BP1, p70 S6K, and S6 were also blotted and showed no significant change. Moreover, the expression of cyclin D1 was also inhibited after 1 h of curcumin treatment (Fig. 1D), similar as reported in ref. 32.

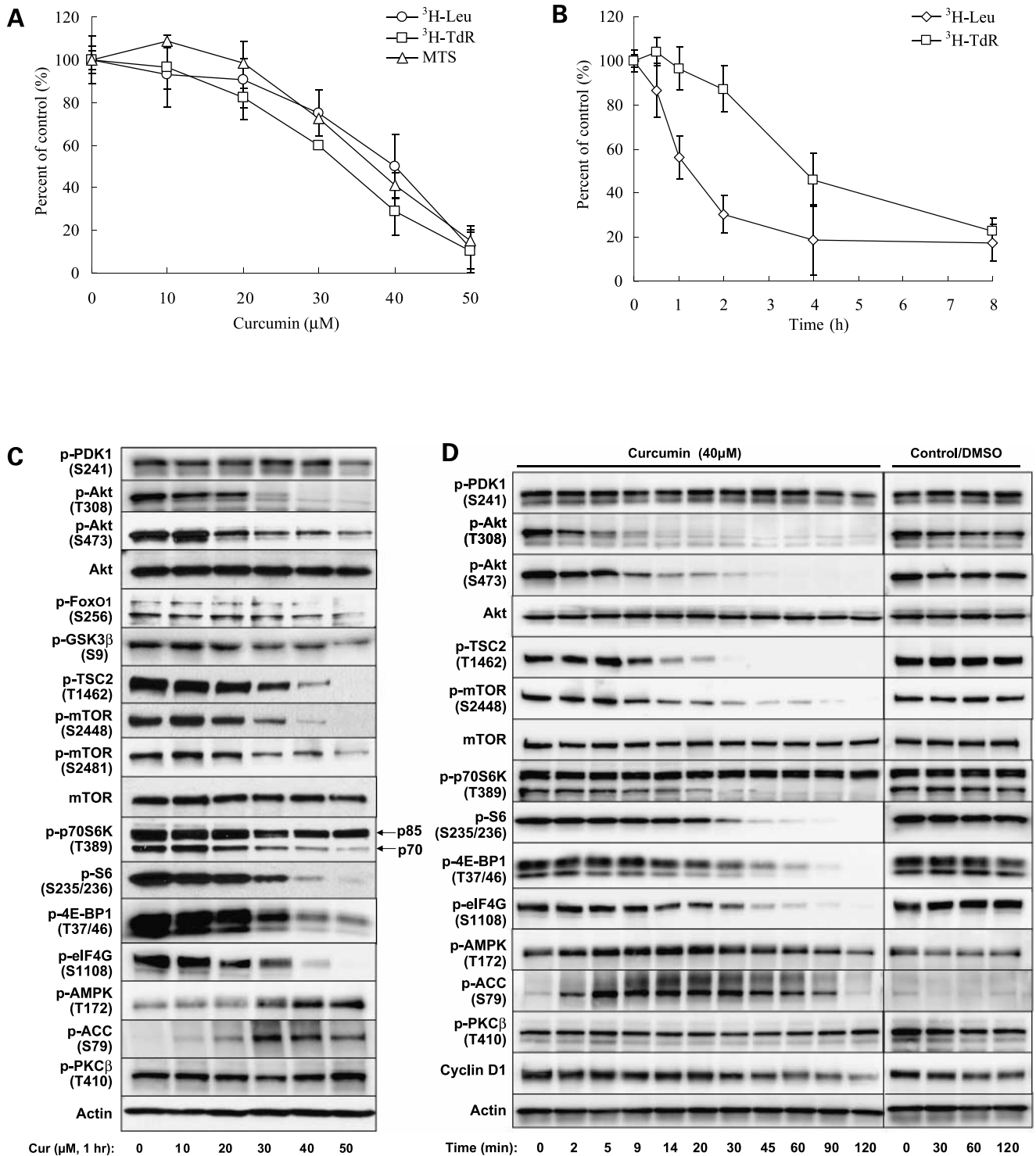


Figure 1. Curcumin concentration- and time-dependently inhibited cell proliferation, protein/DNA synthesis, and Akt/mTOR signaling in PC-3 cells. **A**, cells were treated with various concentrations of curcumin in serum-free media for certain period then the cell viability (after 24 h), protein synthesis and DNA synthesis (after 8 h) were measured by MTS assay, ³H-Leu and ³H-TdR incorporation, respectively. **B**, cells were treated with 50 μM of curcumin for indicated time, then the protein and DNA synthesis were measured by ³H-Leu and ³H-TdR incorporation, respectively. The results are presented as percentage of untreated control and each value is the mean ± SD of 4 parallel samples. **C** and **D**, cells were treated with indicated concentrations of curcumin or DMSO (control) in serum-free media for indicated time, then harvested and immuno-blotted against indicated proteins using their phosphor-specific antibodies. Actin was blotted as a loading control. Please note that two isoforms of S6K were visualized but the p85 isoform was not affected by curcumin.

Curcumin Acted at Downstream of PI3K/PDK1

PI3K catalyzes the production of PIP3 and thus activates downstream signaling including Akt/mTOR. The activity of PI3K is controlled by the binding of regulatory subunits (p85/p55/p101) to catalytic subunits (p110) and a series of phosphorylation events (37). In our experiments, the phosphorylated p85/p55 was barely detectable and no change in its phosphorylation state on curcumin treatment was observed (data not shown). The phosphorylation of PDK1 at Ser²⁴¹ on the activation loop, which is necessary for PDK1 activity, was also not altered by curcumin treatment at the tested concentrations and time points (Fig. 1C and D). We further checked the effect of PIP3 on curcumin-mediated inhibition. Addition of exogenous PIP3 effectively rescued the inhibitory effects of specific PI3K inhibitor LY294002 on the downstream signaling; however, it had no effect on the curcumin-induced inhibition (Fig. 2A).

Because the phosphorylation of Akt at T308, which is catalyzed by PDK1, was the first one to be inhibited, we speculated that curcumin might directly inhibit PDK1 activity toward Akt. To test this hypothesis, the effect of curcumin on PDK1 activity was examined using purified His-tagged Akt1 as substrate. Purified active PDK1 without the first 52 amino acids (PDK1 Δ 52; Fig. 2B) or endogenous PDK1 immunoprecipitated from curcumin-treated PC-3 cells (data not shown) was used for *in vitro* kinase assay. However, curcumin failed to inhibit PDK1 activity both *in vitro* and *in vivo*. Moreover, the phosphorylation of PKC, which is catalyzed by PDK1, was not significantly changed by curcumin treatment (Fig. 1C and D), indicating that PDK1 is not the direct target of curcumin.

Overexpression of Akt or Constitutively Activated Akt Only Partially Restored Curcumin-Mediated Inhibition

To assess the role of Akt in curcumin-mediated inhibition of mTOR signaling and cell proliferation, PC-3 cells were transiently transfected with plasmids encoding HA-Akt, myr-HA-Akt, or empty vector. The transfected cells were treated with various concentrations of curcumin, and the phosphorylated protein levels and cell proliferation were analyzed by Western blotting and [³H]thymidine incorporation assay. Overexpression of Akt significantly restored curcumin-mediated inhibition of Akt phosphorylation but showed less effect on the inhibition of the phosphorylation of mTOR, 4E-BP1, and S6. Overexpression of myr-HA-Akt, which is anchored at the cell membrane by the myr group and thus constitutively activated by PDK1, resulted in highly phosphorylated Akt, which could not be inhibited by curcumin, and augmented the basal phosphorylation of mTOR, 4E-BP1, and S6; surprisingly, the phosphorylation of mTOR, 4E-BP1, and S6 was still significantly inhibited by curcumin (Fig. 3A). Similarly, overexpression of HA-Akt or myr-HA-Akt partially but significantly restored cyclin D1 level and the proliferation of PC-3 cells treated with curcumin (Fig. 3B and C). These results suggest that the inhibition of Akt phosphorylation partially contributed to curcumin-mediated inhibition of mTOR signaling and cell proliferation but is unlikely to be the primary mechanism targeted by curcumin.

AMPK and MAPKs Were Activated by Curcumin but Not Responsible for Curcumin-Mediated Inhibition of Akt/mTOR Signaling

AMPK is a negative upstream regulator of mTOR (11). Indeed, we found that curcumin induced a prompt and robust phosphorylation of AMPK α at Thr¹⁷², which is required for

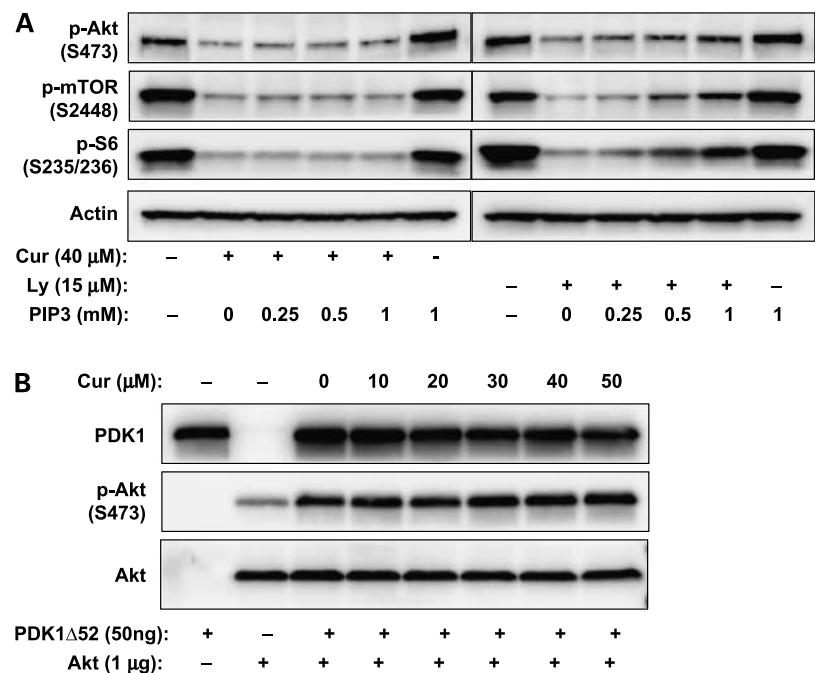


Figure 2. **A**, PIP3 rescued PI3K inhibitor LY294002-mediated but not curcumin-mediated inhibition of Akt/mTOR signaling in PC-3 cells. Cells were incubated with 40 μ M of curcumin or 15 μ M of LY294002 in the presence of increasing concentrations of PIP3 for 1 h, then harvested and the indicated proteins were blotted. **B**, curcumin did not inhibit PDK1 activity towards Akt. 1 μ g of purified His-tagged Akt1 protein was incubated with 50 ng of purified active PDK1 (PDK1 Δ 52) in the presence of indicated concentrations of curcumin in kinase assay buffer/1 mM ATP for 20 min, then phosphor-Akt T308 and PDK1 were immuno-blotted.

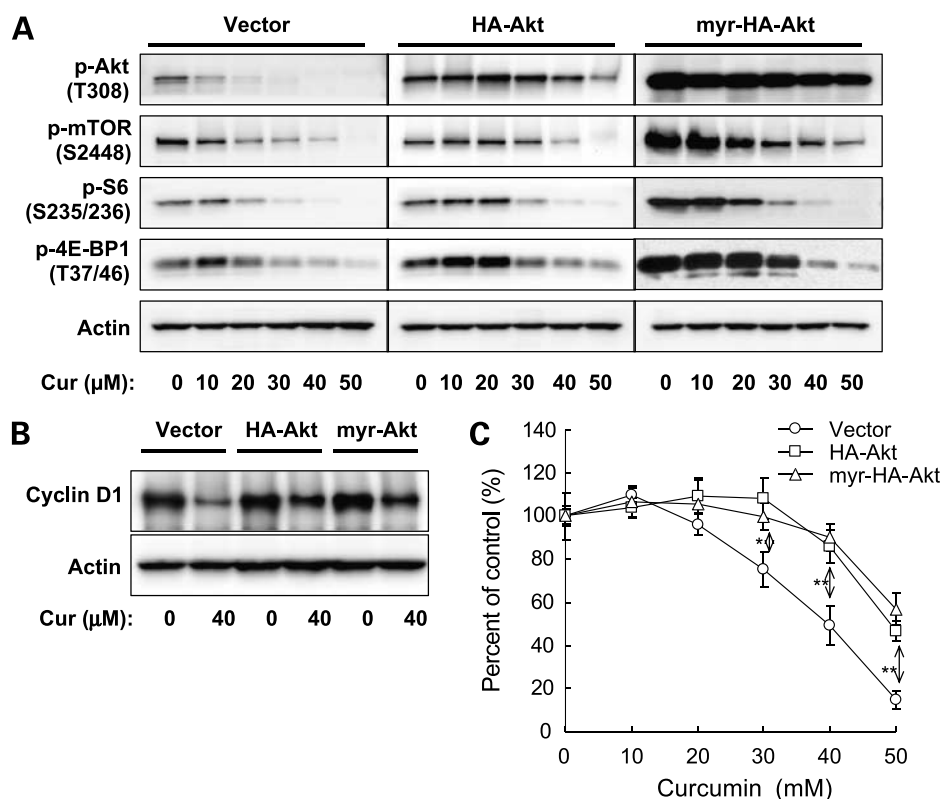


Figure 3. Overexpression of HA-Akt or myr-HA-Akt only partially rescued curcumin-mediated inhibition in PC-3 cells. **A**, cells were transfected with equal amount of indicated plasmids for 24 h, serum-starved for 12 h, then treated with various concentrations of curcumin in serum-free media for 1 h, and harvested and blotted against indicated proteins. **B**, cells were transfected with equal amount of indicated plasmids for 24 h, then treated with 40 μ M of curcumin in serum-free media for 4 h, and harvested and blotted against cyclin D1 or actin. **C**, cells were transfected with equal amount of indicated plasmids for 24 h, and then treated with various concentrations of curcumin in serum-free media for 24 h, and the cell viability was determined by MTS assay. The results are presented as percentage of untreated control and each value is the mean \pm SD of 4 parallel samples. The difference between vector and HA-Akt or myr-HA-Akt is verified by *t* test. *, *P* < 0.05; **, *P* < 0.01.

AMPK activation. Concurrently, ACC, a substrate of AMPK, was also phosphorylated on curcumin treatment (Fig. 1C and D). To assess the involvement of AMPK in curcumin-mediated inhibition of mTOR signaling, we firstly tested the effect of an AMPK inhibitor, compound C. As shown in Fig. 4A, pretreating the cells with compound C inhibited the phosphorylation of ACC and AMPK; however, it showed no effect on curcumin-mediated inhibition of mTOR signaling. The Thr¹⁷² of AMPK α 1 was mutated to alanine to construct a dominant-negative form of AMPK (38), and the inhibition of cellular AMPK activity by overexpression of the AMPK α 1/T172A in PC-3 cells was confirmed by inhibition of the phosphorylation of ACC (Fig. 4B). Overexpression of AMPK α 1 slightly potentiated the inhibitory effect of curcumin on mTOR signaling as indicated by decreased phosphorylation of mTOR, 4E-BP1, and S6. Nevertheless, curcumin-mediated inhibition remained unaffected (Fig. 4C). These results indicate that activation of AMPK by curcumin is not the main reason for curcumin-mediated inhibition of mTOR signaling.

Curcumin also activated extracellular signal-regulated kinase 1/2, c-Jun NH₂-terminal kinase, and p38 in PC-3 cells. Yet again, specific inhibitors against the activated MAPK pathways had no effect on curcumin-mediated inhibition of mTOR signaling (data not shown).

Disruption of TSC1/TSC2 Complex Only Marginally Restored Curcumin-Mediated Inhibition of mTOR Signaling

Both Akt and AMPK regulate mTOR signaling through TSC1-TSC2 complex (39). Here, we checked the possible

role of TSC1-TSC2 in curcumin-mediated inhibition by using TSC1 knockout MEFs or siRNA against TSC2/tuberin. TSC1 (-/-) MEFs displayed remarkably elevated phosphorylation of mTOR, p70 S6K, S6, and 4E-BP1 compared with wild-type MEFs. However, incubation of TSC1 (-/-) MEFs with curcumin still effectively inhibited the phosphorylation of mTOR, p70 S6K, S6, and 4E-BP1 although to a less extent due to higher basal levels (Fig. 5A). Moreover, transfection of TSC2/tuberin siRNA into PC-3 cells inhibited the expression of tuberlin, mildly reduced the basal phosphorylation level, and only marginally counteracted curcumin-mediated inhibition (Fig. 5B) but showed no effect on the basal level or curcumin-mediated inhibition of the phosphorylation of Akt. These results suggest the existence of inhibitory mechanism(s) of mTOR signaling independent of tuberin/hamartin complex, it is to say, independent of the inhibition of Akt or the activation of AMPK.

Curcumin-Mediated Inhibition of Akt/mTOR Signaling Is Dependent on Calyculin A-Sensitive Protein Phosphatase Activity

To explore the involvement of protein phosphatases in curcumin-mediated inhibition of Akt/mTOR signaling, we used three pharmacologic inhibitors to inhibit different phosphatases. Calyculin A is a potent protein serine/threonine phosphatase inhibitor that inhibits both PP1 and PP2A, whereas okadaic acid potently inhibits PP2A but have less effect on PP1, and tautomycin preferentially inhibits PP1 activity. Treatment of PC-3 cells with calyculin

A or okadaic acid induced a slight increase of basal phosphorylation level. Notably, pretreatment with calyculin A concentration-dependently reversed curcumin-mediated inhibition of the phosphorylation of Akt, mTOR, S6, and 4E-BP1, with 100 nmol/L calyculin A completely blocked curcumin-mediated inhibition. Okadaic acid showed a similar but much weaker effect compared with calyculin A. On the other hand, tautomycin had no effect on curcumin-mediated inhibition of Akt/mTOR signaling even at a concentration of 1 μ mol/L (Fig. 6A). The effects of calyculin A on curcumin-mediated inhibition of cyclin D1 and cell proliferation were also determined. As shown in Fig. 6B, calyculin A fully reversed the inhibition of cyclin D1 expression by curcumin. [3 H]leucine incorporation assay was used for proliferation assay because MTS or [3 H]TdR assays require longer treatment but prolonged incubation with calyculin A leads to cell detachment and death. Although 100 nmol/L calyculin A itself slightly inhibited [3 H]leucine incorporation, pretreatment with calyculin A remarkably reversed curcumin-mediated inhibition (Fig. 6C). The data suggest that curcumin inhibits Akt/mTOR signaling through calyculin A-sensitive protein phosphatase(s), and restoration of Akt/mTOR phosphorylation by calyculin A reversed the antiproliferative effects of curcumin.

PP2A core enzyme consists of catalytic C and regulatory A subunits, and the C subunit is targeted to reversible methylation that regulates PP2A activity (40). However, incubation of PC-3 cells with curcumin changed neither the protein level nor the methylation state of PP2A catalytic subunit (data not shown). Next, the cellular protein phosphatase activity on curcumin treatment was determined by Malachite Green Phosphatase assay. As shown in Fig. 6D, incubation of PC-3 cells with curcumin for 10 min concentration-dependently increased the protein phosphatase activity in the cell extract, and this curcumin-stimulated activity could be inhibited by calyculin A. Taken together, these data indicate that incubation with curcumin activated PP2A and/or unspecified calyculin A-sensitive protein phosphatase(s) and led to dephosphorylation of Akt, mTOR, and their downstream substrates.

Discussion

Curcumin has been shown to inhibit the phosphorylation and activation of Akt in PC-3 cells (41); however, the effects of curcumin on the downstream signaling of Akt have not been explored. In the present study, we firstly showed that curcumin also inhibited the phosphorylation of Akt

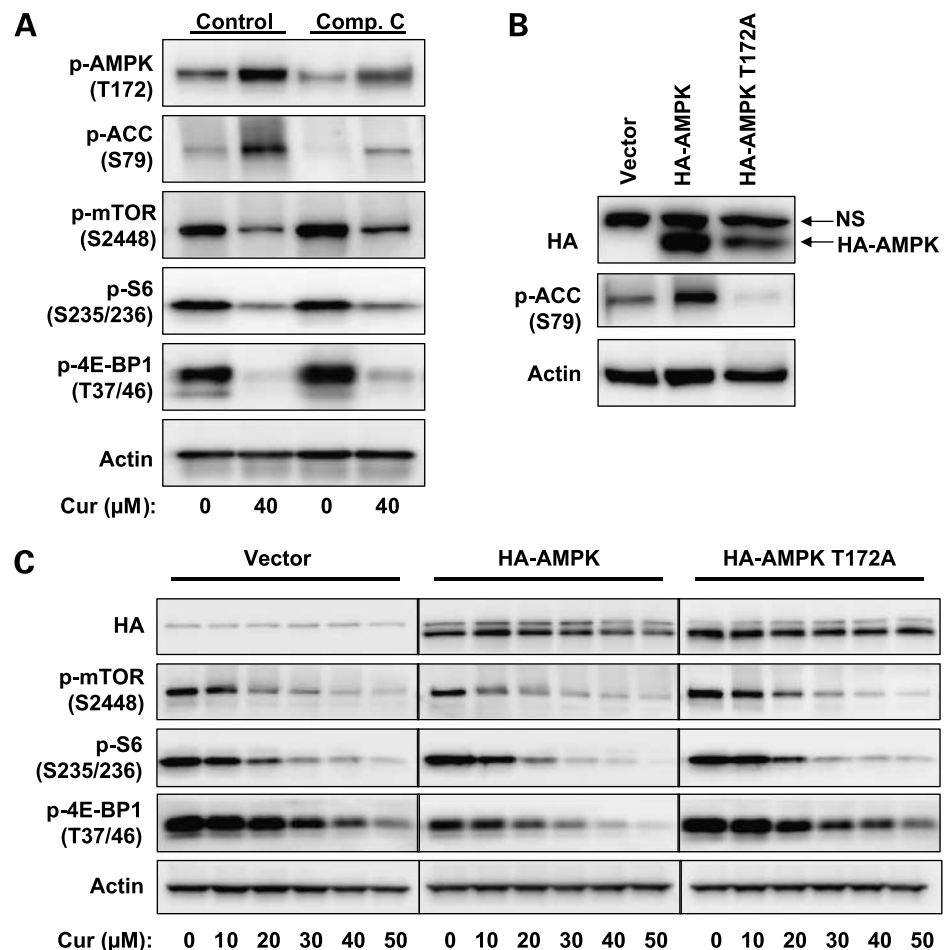


Figure 4. Activation of AMPK is not the major reason for curcumin-mediated inhibition of mTOR signaling in PC-3 cells. **A**, cells were pretreated with 10 μ M of compound C for 15 min then treated with 40 μ M of curcumin in the presence of compound C for 1 h, then harvested and blotted against indicated proteins. **B**, cells were transfected with indicated plasmids for 24 h, then harvested and blotted against HA, phospho-ACC and actin. NS: Non-specific band. **C**, cells were transfected with indicated plasmids for 24 h, serum-starved for 12 h and then treated with various concentrations of curcumin in serum-free media for 1 h, and harvested and blotted against indicated proteins.

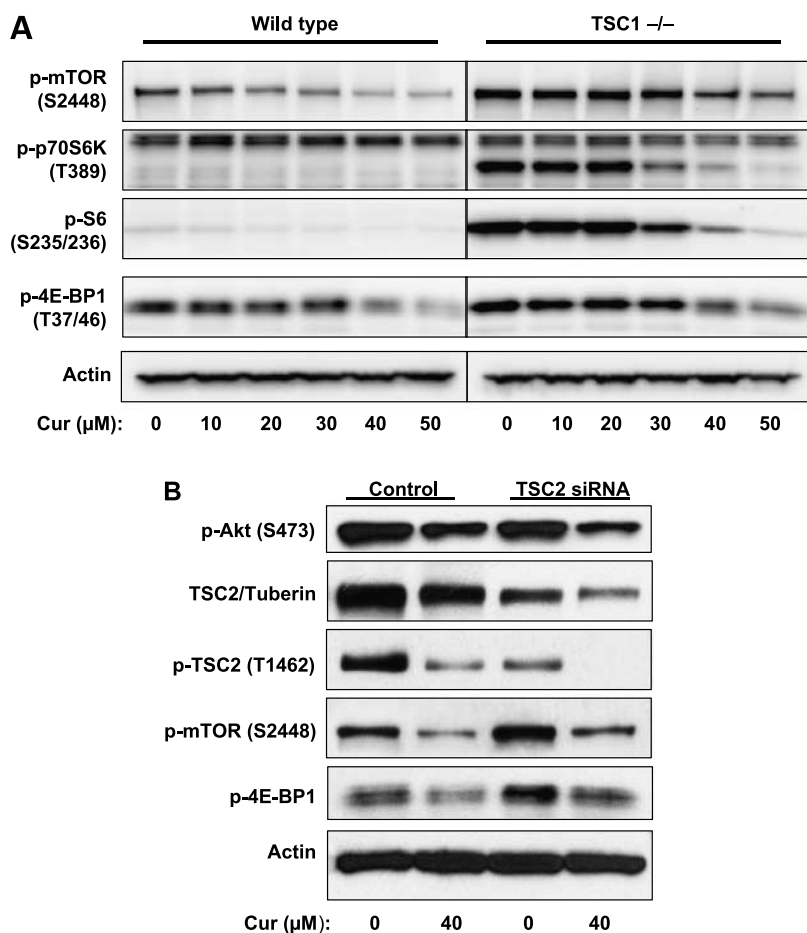


Figure 5. Disruption of TSC1-TSC2 complex failed to rescue curcumin-mediated inhibition of mTOR signaling. **A**, wild type or TSC1^{-/-} MEFs were serum-starved for 12 h then incubated with various concentrations of curcumin in serum-free media for 1 h, and harvested and blotted against indicated proteins. **B**, PC-3 cells were transfected with siRNA against TSC2/tuberin or scrambled control RNA for 48 h, serum-starved for 12 h, then treated with 40 μM of curcumin in serum-free media for 1 h, and harvested and blotted against proteins indicated.

substrates GSK3, FKHR1, TSC2, and mTOR as well as mTOR downstream targets 4E-BP1, eIF4G, p70 S6K, and S6 in a similar concentration-dependent manner as with Akt (Fig. 1C). In support of the role of Akt/mTOR signaling in the control of protein synthesis, curcumin inhibited protein synthesis and DNA synthesis in PC-3 cells (Fig. 1A and B), and these inhibitions could be partially but significantly rescued by overexpression of Akt or by restoration of Akt/mTOR signaling by calyculin A (Figs. 3B and 6C). Cyclin D1, which is critical for cell proliferation, has been reported to be regulated by Akt/mTOR post-transcriptionally (32). In PC-3 cells, the expression of cyclin D1 was also inhibited by curcumin and can be restored by overexpression of Akt or by calyculin A (Figs. 1D, 3A, and 6B). These results are consistent with the important roles of Akt/mTOR signaling in cell survival and proliferation.

Curcumin has been reported to inhibit Akt/mTOR signaling in other cancer cells (33), but the underlying mechanism remains unknown. One major objective of this study is to delineate the molecular mechanism by which curcumin inhibits Akt/mTOR signaling. Firstly, we examined the effect of curcumin on the p85 subunit of PI3K. The phosphorylation of p85 in PC-3 cells is barely detectable and was not affected by curcumin treatment (data not shown). LY294002, a specific PI3K inhibitor, inhibited the phosphor-

ylation of Akt and mTOR, and this inhibition could be restored by addition of exogenous PIP3. In contrast, exogenous PIP3 failed to restore curcumin-mediated inhibition (Fig. 2A). Moreover, it has been well documented that in many cancer cells, including PC-3 cells, the activation of Akt/mTOR signaling axis is less dependent on upstream signals due to loss of PTEN function (25). Actually, as reported by others (33, 42) and confirmed in our laboratory, curcumin also inhibited Akt/mTOR signaling and proliferation in DU145 prostate cancer cells, which carry wild-type PTEN. Taken together, these evidences suggest that curcumin inhibits Akt/mTOR signaling at downstream of PI3K.

As shown in Fig. 1D, the phosphorylation of Akt at Thr³⁰⁸ was the first to be inhibited. This led to the hypothesis that curcumin could directly inhibit PDK1-mediated phosphorylation of Akt and led to the inhibition of downstream signaling. Phosphorylation of PDK1 at Ser²⁴¹ is necessary for its activity but may not be the major regulatory factor (43). However, curcumin did not inhibit the phosphorylation of PDK1 S241 (Fig. 1C and D). Moreover, curcumin failed to inhibit the kinase activity of PDK1 to Akt both *in vitro* and *in vivo* (Fig. 2B; data not shown), suggesting that PDK1 is not the direct target of curcumin. Similar observations have been reported that Akt/mTOR signaling can be inhibited independent of PI3K/PDK1 (19).

Next, we examined the role of Akt in curcumin-mediated inhibition. Overexpression of Akt or the constitutively activated myr-Akt increased the basal level of phosphorylated Akt, mTOR, and downstream molecules. However, curcumin still effectively inhibited mTOR and downstream signaling, although to a less extent, which is possibly due to the increased basal phosphorylation level (Fig. 3A). These results, especially curcumin inhibited Akt downstream signaling, although the phosphorylation of myr-Akt was not inhibited at all, strongly suggest the existence of inhibitory mechanism, which is independent of inhibition of Akt.

Coincidentally, AMPK was activated by curcumin in a time course comparable with the inhibition of Akt phosphorylation (Fig. 1C and D). Overexpression of AMPK in PC-3 cells slightly potentiated the inhibition of mTOR signaling by curcumin, but neither pharmacologic inhibitor nor dominant-negative overexpression showed significant restoration of curcumin-mediated inhibition (Fig. 4). Although curcumin-activated AMPK is not the major

reason for curcumin-mediated inhibition of Akt/mTOR signaling, how curcumin activates AMPK and its physiologic significance deserves further investigation in the future.

TSC1/TSC2 complex inhibits mTOR activity by activating the GTPase activity of Ras homologue enriched in brain, and both Akt and AMPK converged at TSC1/TSC2 to regulate mTOR activity (39). Consistent with the incompetence of constitutive activation of Akt or inhibition of AMPK to rescue mTOR signaling, disruption of the function of TSC1/TSC2 complex only marginally rescued curcumin-mediated inhibition (Fig. 5). Knockout of TSC1 in MEFs led to hyperphosphorylation of mTOR, 4E-BP1, p70 S6K, and S6; nonetheless, curcumin effectively inhibited the phosphorylation with a similar concentration dependency to that in wild-type MEFs (Fig. 5A). It is notable that curcumin effectively inhibited mTOR signaling in the noncancerous MEFs, although to a less extent than in PC-3 cells, suggesting that curcumin-mediated inhibition of Akt/mTOR signaling is independent on PTEN status.

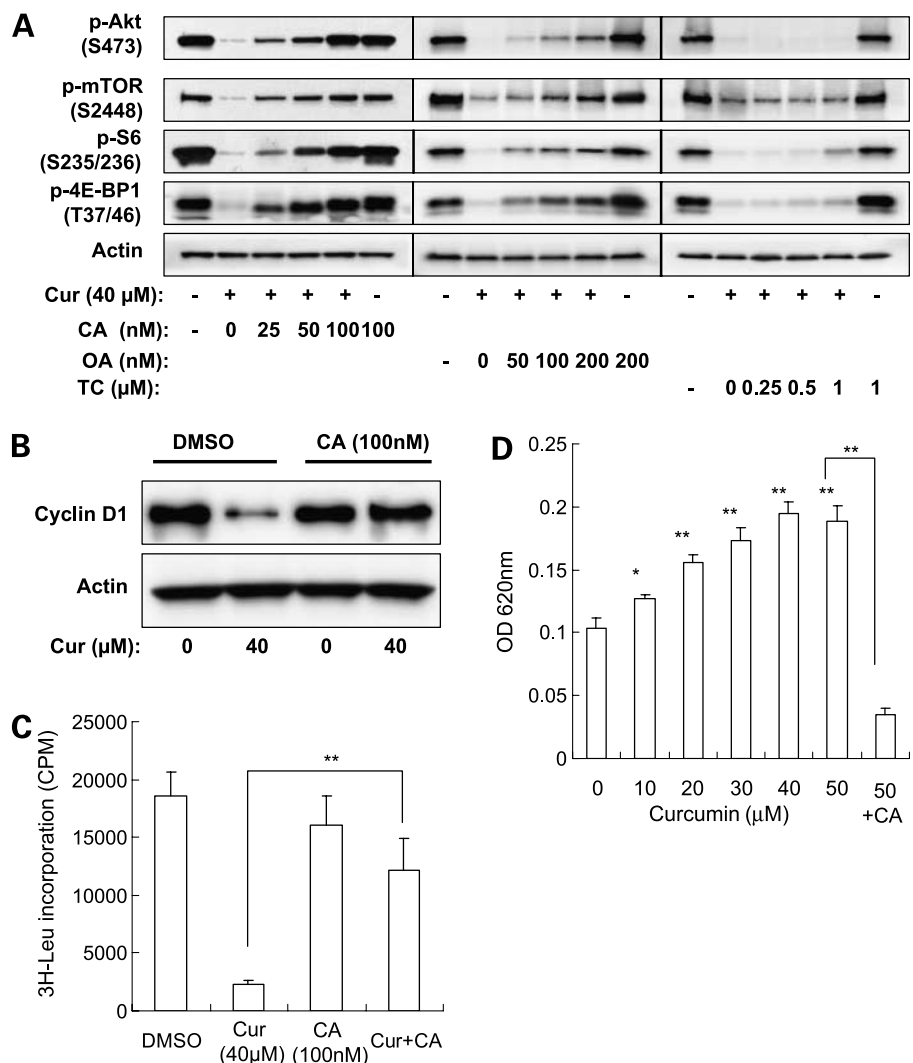


Figure 6. Curcumin-mediated inhibition of Akt/mTOR signaling in PC-3 cells is dependent on PP2A and/or unspecified calyculin A-sensitive protein phosphatase activity. **A**, cells were pretreated with indicated concentrations of protein phosphatase inhibitors for 15 min, then incubated with 40 μM of curcumin in the presence of protein phosphatase inhibitors in serum-free media for 1 h, and harvested and blotted against indicated proteins. **B** and **C**, cells were pretreated with 100 nM of calyculin A for 15 min, then incubated with 40 μM of curcumin in the presence of calyculin A for 4 h, and then **(B)** cells were harvested and blotted against cyclin D1 and actin or **(C)** ³H-Leu incorporation was determined as described in Material and Methods. **D**, cells were treated with indicated concentrations of curcumin for 10 min, then harvested in phosphatase lysis buffer and the protein phosphatase activities in lysates were determined as described in Material and Methods. The results are expressed as mean ± SD of 4 parallel samples. *, *P* < 0.05; **, *P* < 0.01.

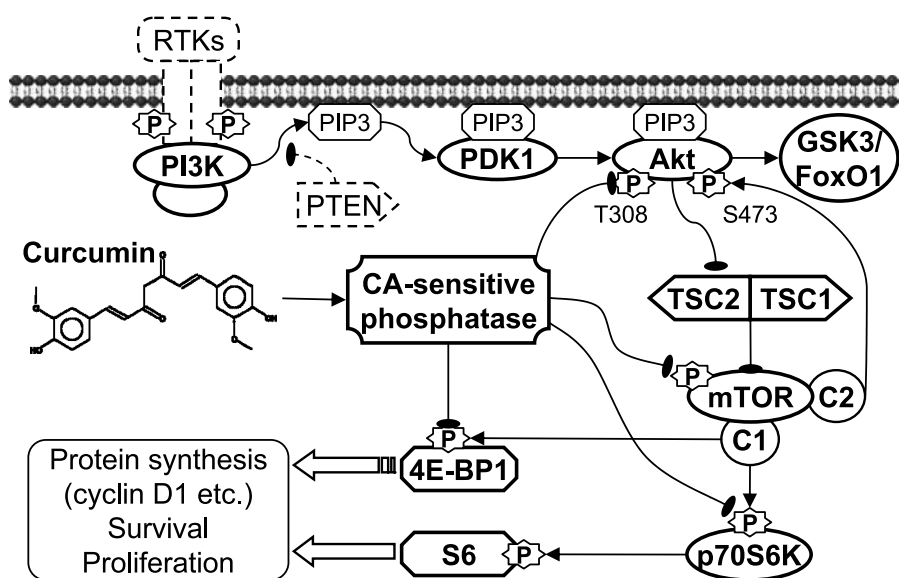


Figure 7. Summary of the mechanisms by which curcumin inhibits Akt/mTOR signaling and cell survival/proliferation in PC-3 prostate cancer cells. Curcumin activated PP2A and/or unspecified calyculin A-sensitive protein phosphatase activities towards Akt, mTOR, and their downstream substrates GSK3, FoxO1, p70S6K and 4E-BP1, and finally inhibited the expression of proteins that are essential for cell survival and proliferation. Curcumin also activated MAPKs and AMPK; however these kinases did not play important roles in the curcumin-mediated inhibition of Akt/mTOR signaling and cell proliferation.

Likewise, knockdown of TSC2 in PC-3 cells by siRNA mildly increased the basal phosphorylation level of mTOR and 4E-BP1, but the phosphorylation could still be inhibited by curcumin (Fig. 5B).

Multiple feedback loops exist in the regulation of Akt/mTOR signaling. Importantly, p70 S6K phosphorylates and inhibits insulin receptor substrate-1, resulting in a negative feedback to Akt/mTOR signaling (12). By this mechanism, inhibition of mTOR signaling often leads to activation of Akt and tumor cells could gain resistance to mTOR inhibitors (44). However, in PC-3 cells, curcumin inhibited both Akt and mTOR similarly (Fig. 1C). Furthermore, the inhibition of Akt phosphorylation at Thr³⁰⁸ occurred much earlier than the inhibition of phosphorylation of Akt at Ser⁴⁷³, mTOR, and other downstream components (Fig. 1D). Based on these observations, it is unlikely that curcumin inhibited Akt/mTOR axis by directly inhibiting mTOR.

MAPKs, especially p38, have been reported to be involved in the inhibition of Akt signaling (13). Curcumin activated extracellular signal-regulated kinase 1/2, c-Jun NH₂-terminal kinase, and p38 in PC-3 cells, but the involvement of MAPKs in the inhibition of Akt/mTOR signaling by curcumin was ruled out by the failure of specific inhibitors to restore Akt/mTOR phosphorylation (data not shown).

Having excluded the inhibition/activation of upstream kinases from the major inhibitory mechanism, we turned to explore the possible involvement of protein phosphatases, specifically serine/threonine protein phosphatase, because the phosphorylation and dephosphorylation that regulate the components of Akt/mTOR signaling pathway mainly occur at threonine or serine. PP1 and PP2A account for the majority of serine/threonine protein phosphatase activity in most cells (45). The PP1 inhibitor tautomycin exhibited only a very weak restoration of Akt/mTOR phosphorylation at a concentration much higher than that required for

inhibition of PP1 (Fig. 6A). On the other hand, calyculin A fully reversed curcumin-mediated dephosphorylation of Akt, mTOR, S6, and 4E-BP1. Similar result was observed for the expression of cyclin D1 (Fig. 6B). Moreover, calyculin A effectively rescued the curcumin-mediated inhibition of [³H]leucine incorporation in PC-3 cells (Fig. 6C). The effect of okadaic acid was less potent but still significant, suggesting that curcumin-mediated inhibition of Akt/mTOR signaling and cell proliferation is dependent on PP2A and/or unspecified calyculin A-sensitive protein phosphatases.

Curcumin has been found to activate Src homology 2 domain-containing tyrosine phosphatase 2 in brain microglia (46). In another study, curcumin was shown to up-regulate MKP5 to repress inflammatory responses in prostate cells (47). Here, we found that curcumin also activated serine/threonine protein phosphatase activity in PC-3 cells (Fig. 6D). The activities of protein phosphatases are subjected to multiple levels of regulation; however, the exact mechanisms are still largely unknown (48). As an example, PP2A holoenzyme, which has a diversity of substrates, is composed of a core heterodimer of catalytic (C) and scaffold (A) subunits and a wide variety of regulatory (B) subunits. The specific activities against certain substrates are regulated by different combinations of subunits and their phosphorylation or methylation status (14, 49). Curcumin showed no significant effect on the methylation status of C subunit (data not shown); however, it did activate serine/threonine protein phosphatase activity in PC-3 cells.

Contrasting to more than 300 serine/threonine kinases in the human genome, only less than 30 serine/threonine phosphatases were identified to the date (48), and new protein phosphatases are being identified (23). Our experimental results support the involvement of PP2A and/or unspecified calyculin A-sensitive protein phosphatases in curcumin-mediated inhibition of Akt/mTOR

signaling and proliferation; however, further investigation is required to identify the specific phosphatases activated by curcumin.

As summarized in Fig. 7, curcumin activated PP2A or unspecified calyculin A-sensitive protein phosphatase activity toward Akt, mTOR, and possibly their downstream molecules, leading to the inhibition of Akt/mTOR signaling, and the expression of proliferation-essential proteins, such as cyclin D1, finally inhibited the cell survival and proliferation. Our study systematically dissected the effects of curcumin on the Akt/mTOR signaling in PC-3 cells, revealed the importance of Akt/mTOR inhibition for the antiproliferative activity of curcumin, and shed new light on the mechanisms of anticancer activities of curcumin.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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