Cyclosporin A suppresses prostate cancer cell growth through CaMKKβ/AMPK-mediated inhibition of mTORC1 signaling

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A B S T R A C T
Cyclosporin A (CsA) has antitumor effects on various cancers including prostate cancer. However, its antitumor mechanism is poorly understood. In this study, we showed that AMP-activated protein kinase (AMPK) mediates the antitumor effect of CsA on prostate cancer cells. CsA attenuated cell growth by inducing a G1 arrest through the inhibition of mTOR complex 1 (mTORC1) signaling. In this context, Akt was paradoxically activated downstream of the EGF receptor (EGFR)-mediated increase in phosphatidylinositol 3,4,5-trisphosphate (PIP3) production. However, CsA also caused a Ca2+/calmodulin-dependent protein kinase β (CaMKKβ)-dependent activation of AMPK, which inhibits mTORC1 signaling; this led to ineffective Akt signaling. An EGFR or Akt inhibitor increased the growth suppressive activity of CsA, whereas the combination of an AMPK inhibitor and CsA markedly rescued cells from the G1 arrest and increased cell growth. These results provide novel insights into the molecular mechanisms of CsA on cancer signaling pathways.

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1. Introduction
AMP-activated protein kinase (AMPK) is an energy sensor that controls the cellular metabolic balance in response to an increased AMP:ATP ratio in an LKB1-dependent manner [1]. Recently, the LKB1/AMPK signaling pathway has emerged as a metabolic tumor suppressor axis, linking cellular metabolism to cancer biology [2]. In particular, LKB1 deficiency causes prostate neoplasia in mice [3]. The use of metformin, an AMPK activator, is associated with a significant decrease in the relative risk of prostate cancer [4]. Furthermore, inhibition of AMPK accelerates cell proliferation and promotes malignant behavior [5]. These findings suggest that the LKB1/AMPK pathway is a promising target for prostate cancer therapy. In addition to LKB1, Ca2+/calmodulin-dependent protein kinase β (CaMKKβ) activates AMPK in response to alternative signals such as intracellular Ca2+ levels [6]. However, the role of the CaMKKβ/AMPK pathway in cancer biology is not well understood.

AMPK interferes with Akt/mTOR complex 1 (mTORC1) signaling by phosphorylating tuberous sclerosis complex 2 (TSC2), an inhibitor of mTORC1, and/or Raptor, a component of mTORC1 [7,8]. Akt/mTORC1 signaling plays a crucial role in the survival and progression of prostate cancer under androgen-depleted conditions [9]. In addition, loss of the tumor suppressor PTEN is observed in approximately 70% of metastatic prostate cancer samples [10,11]. PTEN loss leads to an increase in phosphoinositide-3 kinase (PI3K)-catalyzed phosphatidylinositol 3,4,5-trisphosphate (PIP3) production and aberrant activation of the Akt/mTORC1 signaling pathway [12]. These results suggest that AMPK could be an attractive therapeutic target for the treatment of PTEN-mutated prostate cancer [13].

Cyclosporin A (CsA) has the ability to suppress prostate cancer cell growth [14,15]. However, the effect of CsA on cancer signaling pathways is not well known. In this study, we investigated the signaling mechanisms of CsA-induced growth inhibition in prostate cancer cells. We found that CsA inhibited mTORC1 signaling by activating the CaMKKβ/AMPK pathway. These results provide novel insights into the molecular mechanisms of CsA action on cancer signaling pathways and may aid in the
development of novel therapeutic strategies against prostate cancer.

2. Materials and methods

2.1. Cell culture and chemicals

PC-3 and DU-145 cells were purchased from ATCC and cultured according to ATCC instructions. CsA and STO-609-acetic acid were purchased from Sigma–Aldrich (St. Louis, MO). Gefinitib was obtained from Selleck Chemicals (Houston, TX). AKT1-1/2, MG132, BAPTA-AM, and compound C were purchased from Roche Applied Sciences (Mannheim, Germany).

2.2. Cell cycle and growth assays

Flow cytometric assay was used to analyze cell cycle profiles. MTT assay was employed to assess cell growth. MTT reagent was purchased from Amresco (Solon, OH). These assays were performed as previously described [16].

2.3. Western blot analysis

Antibodies against pAKTSer473, pAKTThr308, AKT, pGSK3βSer9, pTSC2Thr1462, TSC2, pS6KThr389, S6K, p4EBPThr37/46, 4EBP, pPDK1Ser241, PDK1, pEGFRtyr1173, EGFR, LKB1, pAMPKThr172, AMPK, pACCSer79, ACC, pRaptorSer792, Raptor, pRbSer780, Rb, CDK1, cyclin D1, cyclin E, and p15 were obtained from Cell Signaling Technology (Beverly, MA). Antibodies against GSK3β, CDK2, CDK4, CDK6, cyclin A, cyclin B1, p21, p27 and GAPDH were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The anti-CaMKKβ antibody was obtained from BD Biosciences (Heidelberg, Germany). The anti-LC3 antibody was purchased from Medical and Biological Laboratories (Woburn, MA). The crude extracts were resolved on 6–15% SDS-PAGE gels and were probed with the indicated antibodies. The data were representative of at least three independent experiments.

2.4. siRNA transfection

The cells were transfected with 100 nM siRNA against CaMKKβ (siCaMKKβ) for 48 h [17], 50 nM siLKB1 [18] for 48 h, 100 nM siAMPKα1 + 100 nM siAMPKα2 [19] for 24 h using Lipofectamine RNAiMAX reagent (Invitrogen, Karlsruhe, Germany). siRNAs were purchased from Qiagen (Hilden, Germany).

2.5. ATP quantification

Intracellular ATP concentrations were quantified using the ATP Bioluminescence Assay Kit HS II according to the manufacturer’s instructions (Roche Applied Sciences, Mannheim, Germany).

2.6. Assessment of mitochondrial membrane potential

Mitochondrial membrane depolarization was determined as previously described [20]. JC-1 fluorescence probe was obtained from Molecular Probes (Eugene, OR).

2.7. FRET imaging

The cells were transfected with the FRET-based Pi(3,4,5)P3 indicator (Pippi-PiP3). A plasmid for Pippi-PiP3 was kindly provided by Prof. Michiyuki Matsuda in Kyoto University [21]. Pippi-PiP3 (FRET indicator)-expressing cells were treated with CsA at the indicated times. FRET images were captured by a Nikon Ti-E inverted microscope equipped with CoolSNAP HQ camera, excitation, and emission filter wheels. All systems were controlled by MetaMorph software (Molecular Devices, Downington, PA). The CFP (excitation 440 nm/emission 480 nm) and FRET (excitation 440 nm/emission 530 nm) images were obtained every 2 min using a time-lapse epifluorescent microscope. The filter sets and ND filters were purchased from Semrock Inc (Rochester, NY). The images were acquired with the 4 × 4 binning mode and a 200-ms exposure time. The ratio image of FRET/CFP was created with MetaMorph software. The emission ratio values were normalized to those of the starting time.

2.8. Statistical analysis

The comparison of the means among experimental groups was performed using ANOVA followed by a post hoc test. p < 0.05 was considered statistically significant.

3. Results

3.1. CsA induces a G1 arrest via inhibition of mTORC1 signaling

We first assessed the antitumor activity of CsA against PTEN-negative PC-3 cells. CsA attenuated cell growth, particularly at concentrations greater than 10 μM (Fig. 1A), and increased the percentage of G1-phase cells in a time- and concentration-dependent manner (Fig. 1B and C). CsA-induced growth inhibition and G1 arrest was also observed in DU-145 cells (Fig. 1D and E), which express functional PTEN [22]. At the molecular level, CsA decreased the expression levels of cyclin D1, but not cyclin E, and reduced the phosphorylation levels of the tumor suppressor Rb in PC-3 cells (Fig. 1F). We also found that CsA affected the expression levels of cell cycle inhibitors (p21 and p27) and activators (CDK1, CDK6, and cyclin B1) (Fig. 1G and H). These results indicate that CsA suppresses cell growth by inducing a G1 arrest in prostate cancer cells, which is irrespective of PTEN status.

Although CsA decreased the protein levels of cyclin D1, it did not affect cyclin D1 mRNA levels in PC-3 cells (Fig. 2A). In addition, the proteosome inhibitor MG132 failed to rescue the protein levels of cyclin D1 in CsA-treated cells (Fig. 2B). We therefore hypothesized that CsA decreases cyclin D1 expression through regulation of mTORC1 signaling based on three facts: (1) mTORC1 facilitates translation initiation by phosphorylating S6 kinase (S6K) or 4E-binding protein 1 (4EBP1) [23]; (2) mTORC1 increases cyclin D1 expression [24]; and (3) inhibition of mTORC1 induces a G1 arrest [25]. We found that CsA decreased phospho-S6K and 4EBP levels in a time- and concentration-dependent manner in PC-3 cells (Fig. 2C and D), supporting our hypothesis. The levels of phospho-S6K and 4EBP were also reduced in CsA-treated DU-145 cells (Fig. 2E). Because mTORC1 suppresses autophagy [26], if our hypothesis is correct, CsA would be capable of inducing autophagy. CsA-mediated inhibition of mTORC1 was further confirmed by our finding that CsA induced autophagy in PC-3 cells (Fig. 2F–I). CsA markedly increased the number of GFP-LC3 puncta and the levels of LC3-II, which are autophagy markers. Altogether, our findings indicate that CsA induces a G1 arrest by inhibiting mTORC1 signaling in prostate cancer cells.

3.2. CsA activates Akt signaling by increasing PIP2 levels via EGFR activation

Because Akt activates mTORC1 signaling, we examined whether CsA inhibits Akt activity. Contrary to our expectations, CsA elevated the levels of phospho-Akt rather than reduced them in PC-3 cells (Fig. 3A and B). In addition, CsA increased the levels of phospho-GSK3β and -TSC2 (on Thr-1462), which are Akt substrates (Fig. 3A and B). The increased phospho-Akt and -GSK3β
Fig. 1. CsA attenuates cell growth by inducing a G1 arrest. PC-3 (A–D and F–H) or DU-145 (D and E) cells were treated with CsA at the indicated concentrations or with 10 μM (if not specified) for the indicated times or 24 h (if not specified) prior to each analysis. (A) Cell growth was assessed using MTT assays and was expressed as the relative absorbance compared to vehicle-treated cells harvested at time zero, which was set to 100%. (B, C and E) The cell fraction was expressed as the percentage of each cell cycle phase. (D) The cell extracts were treated with CsA for 72 h prior to MTT assays. (F–H) The cell extracts were probed using the indicated antibodies. The data are expressed as the mean ± SEM (n = 3–6). *p < 0.05, ***p < 0.005.

Levels were also observed in CsA-treated DU145 cells (Fig. 3C). Under the same conditions, the total expression levels of Akt were not affected by CsA. These results indicate that CsA activates Akt rather than inhibits it. To clarify the paradoxical Akt activation in CsA-treated cells, we investigated whether CsA increases the level of PIP3, a key activator of Akt. Time-lapse FRET imaging analysis showed that PIP3 levels increased in CsA-treated PC-3 cells, reaching a maximum level within 4–5 h and decreasing thereafter (Fig. 3D), indicating that CsA increases the PI3K/Akt pathway. Based on two facts that the EGF receptor (EGFR) activates PI3K, and CsA
activates the EGFR [27], we questioned whether CsA might activate Akt through EGFR activation. The results indicate that CsA temporally increased phospho-EGFR levels (Fig. 4A), and the EGFR inhibitor gefitinib reversed phospho-Akt levels in CsA-treated PC-3 cells (Fig. 4B). These results indicate that CsA activates Akt signaling downstream of the EGFR-mediated increase in PIP3 production in PC-3 cells. However, mTORC1 inhibition-induced activation of Akt still remains to be tested. Interestingly, the EGFR inhibitor gefitinib or the Akt inhibitor AKTI-1/2 markedly increased the antitumor activity of CsA in PC-3 cells (Fig. 4C), suggesting a potential role of the EGFR/Akt pathway in tumor cell resistance to CsA and the potential usefulness of a combination therapy composed of CsA with EGFR/Akt inhibitors. The potential of combination strategy was ascertained using DU-145 cells (Fig. 4D). Altogether, we clarify that CsA inhibits mTORC1 signaling, but paradoxically activates Akt.

3.3. AMPK mediates CsA-induced phenotypes

Because AMPK inhibits mTORC1 signaling by phosphorylating TSC2 (on Thr-1227 and Ser-1345) and/or Raptor [7,8], we hypothesized that AMPK may explain the paradoxical molecular events that we observed. Indeed, a recent paper reported that CsA activates AMPK in the rat hippocampus [28]. These results led us to study the potential role of AMPK in the antitumor action of CsA on prostate cancer. In our study, we found that CsA activated AMPK and its substrate acetyl-CoA carboxylase (ACC), and increased phospho-Raptor levels in a time- and concentration-dependent manner in PC-3 cells (Fig. 5A and B). We also observed that CsA elevated phospho-AMPK, -ACC, and -Raptor levels in DU-145 cells (Fig. 5 and D). Therefore, our results demonstrate that CsA concurrently activates two opposing signals, Akt and AMPK, but net functional outcome is inhibition of mTORC1 signaling (summarized in Fig. 5E and F which are based on the data from PC-3 cells), indicating that AMPK leads to ineffective Akt signaling in CsA-treated cells.

We then examined the causal relationship between AMPK activation and mTORC1 inhibition in CsA-treated PC-3 cells. The AMPK inhibitor known as compound C (CC) decreased phospho-Raptor levels and restored phospho-S6K and -4EBP levels in CsA-treated cells (Fig. 5G). The siRNA against AMPK (siAMPK) also rescued mTORC1 signaling in CsA-treated cells (Fig. 5H), confirming that CsA inhibits mTORC1 by activating AMPK. Because an antibody against phospho-TSC2Thr-1227/Ser-1345 is not commercially available, we could not determine the AMPK-catalyzed phosphorylation of TSC2. We then investigated the causal role of AMPK in the CsA-induced G1 arrest. AMPK inhibition by CC markedly restored the G1 arrest in CsA-treated cells (Fig. 5I), and siAMPK also rescued cells from the G1 arrest (Fig. 5J). At the molecular level, AMPK knockdown recovered cyclin D1 expression and phospho-Rb levels in CsA-treated cells (Fig. 5K). Moreover, CC or siAMPK relieved growth inhibition by CsA (Fig. 5L). Altogether, these results indicate that CsA-induced activation of AMPK induces a G1 arrest by inhibiting mTORC1 signaling in prostate cancer cells.

3.4. CaMKKβ mediates CsA-induced activation of AMPK

Because AMPK is activated by an increased AMP:ATP ratio [29], we examined the effects of CsA on mitochondrial function in PC-3 cells. CsA did not affect cellular ATP levels (Fig. 6A) or
mitochondrial membrane potential compared to H2O2 as a positive control (Fig. 6B), indicating that CsA did not cause apparent mitochondrial dysfunction. Moreover, LKB1 expression was not affected by CsA (Fig. 5A and B), and LKB1 knockdown failed to suppress phospho-AMPK levels in CsA-treated cells (Fig. 6C).

Because AMPK is also activated by CaMKKβ, which is independent of changes in the AMP:ATP ratio [30,31], we investigated whether CaMKKβ mediates CsA-induced activation of AMPK in PC-3 cells. The CaMKK inhibitor STO-609 abolished the CsA effect on AMPK activation (Fig. 6D). Similar results were obtained from experiments using the Ca2+ chelator BAPTA-AM or siRNA against CaMKK (Fig. 6E and F). These results demonstrated that CaMKKβ, but not LKB1, is crucial for the CsA-induced activation of AMPK in prostate cancer cells.

4. Discussion

In this study, we describe the following results: (1) CsA attenuates cell growth by inducing a G1 arrest; (2) CsA inhibits mTORC1 signaling, but paradoxically activates Akt signaling through the EGFR pathway; (3) the AMPK activated by CsA inhibits mTORC1 signaling, and this leads to ineffective Akt signaling; and (4) CaMKKβ, but not LKB1, is crucial for AMPK activation by CsA. These novel results demonstrate that CsA inhibits mTORC1 signaling through a CaMKKβ-mediated activation of AMPK in prostate cancer cells.

Androgen deprivation therapy is initially effective in treatment of metastatic prostate cancer. However, most metastatic prostate cancers relapse and progress into CRPC that is essentially untreatable [32]. Therapeutic agents for the management of CRPC show an improvement in overall survival by approximately 3–4 months [33,34]. Small cell carcinoma of prostate typically lacks androgen receptor and prostate-specific antigen, which makes the tumor cells unresponsive to hormonal therapy [35]. In these regards, our results suggest that therapeutic use of CsA might have a survival benefit in treatment of CRPC or small cell carcinoma of prostate. In addition, considering that rapamycin and its analogs are immunosuppressants with antitumor properties [36], the suppressive effect of CsA on anti-tumor immune responses is not likely to limit its clinical use. Furthermore, our results show that...
combination of CsA with EGFR or AKT inhibitors is more effective in cancer growth inhibition than either alone, providing an important clue to consider the possible clinical application.

We revealed that CsA concurrently activates the EGFR/P38K/Akt and the CaMKβ/AMPK pathways, but the latter effectively suppresses the oncogenic signaling of the former, suggesting that the CaMKβ/AMPK signaling pathway might be a promising target for cancer therapy, particularly against cancer types with deregulated activity of the EGFR/P38K/Akt pathway. Because CsA simultaneously activates both oncogenic and tumor suppressive signals, the balance between these signals might be crucial for determining the pharmacological action of CsA. Therefore, our study could provide a conceptual framework for the development of novel strategies directed toward combination therapy targeting the Akt/mTORC1 and the CaMKβ/AMPK pathways.

In addition to antitumor activity of CsA, it has cancer-promoting capabilities depending on the cell/tissue types [37–39]. Indeed, CsA enhances cell proliferation in skin keratinocytes [40]. These results suggest that cell context-specific signaling accounts for the determination of complex phenotypic outcomes after CsA treatment. As mentioned before, the balance between oncogenic and tumor suppressive signals might be crucial for determining CsA-induced complex phenotypic outcomes. Therefore, our results may provide a basis for future investigations aimed at understanding these complex phenotypic outcomes.

Conflict of interest

The authors declare no conflicts of interest.

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