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Mitochondrial protein cyclophilin-D-mediated programmed necrosis attributes to berberine-induced cytotoxicity in cultured prostate cancer cells

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ABSTRACT

The prostate cancer is one of the leading causes of men's cancer mortality. The development of alternative chemotherapeutic strategies is urgent. Berberine has displayed significant anti-prostate cancer activities. The underlying mechanisms are not fully understood. In the current study, we found that berberine induced apoptosis and programmed necrosis in cultured prostate cancer cells (LNCaP and PC-82 lines), and necrosis weighted more than apoptosis in contributing berberine's cytotoxicity. We demonstrated that mitochondrial protein cyclophilin-D (Cyp-D) is required for berberine-induced programmed necrosis. Inhibition of Cyp-D by its inhibitors cyclosporin A (CSA) or sanglifehrin A (SFA), and by Cyp-D shRNA depletion alleviated berberine-induced prostate cancer cell necrosis (but not apoptosis). Our data found that in prostate cancer cells, berberine induced reactive oxygen species (ROS) production, which dictated P53 translocation to mitochondria, where it physically interacted with Cyp-D to open mitochondrial permeability transition pore (mPTP). The anti-oxidant N-acetylcysteine (NAC), the P53 inhibitor pifithrin-α (PFTα) as well as P53 siRNA knockdown suppressed berberine-induced P53 mitochondrial translocation and Cyp-D association, thus inhibiting mitochondrial membrane potential (MMP) decrease and prostate cancer cell necrosis. In summary, the results of the present study provide mechanistic evidence that both apoptosis and programmed necrosis attribute to berberine's cytotoxicity in prostate cancer cells.

1. Introduction

The prostate cancer is becoming one of the leading causes of cancer-related men mortality in the United States and around the world [1–3]. It is reported that one of nine men over the age of 65 years is diagnosed with this devastating disease in the United States alone [1]. As surgery and current chemotherapeutic options appear not enough in curing or controlling prostate cancer, especially the resistant and metastatic forms, there is an urgent need for the development of alternative chemotherapeutic strategies.

Berberine is an isoquinoline alkaloid isolated from Huanglian (Coptis chinensis), a Chinese medicinal herb. It is also seen in other medicinal herbs. It has displayed significant anti-tumor activities both *in vitro* and *in vivo*. Berberine's effect is mediated by its transcriptional and post-transcriptional regulation of certain important oncogenes or carcinogenesis-related genes, as well as by its interactions with both DNA and RNA [4,5]. Studies have investigated the potential role of berberine in prostate cancer cells, and have found that berberine could induce dramatic cytotoxicity in cultured prostate cancer cells [6,7]. However the underlying mechanisms of berberine's effect are not fully understood, although reactive oxygen species (ROS) production [6] and cell apoptosis [7] have been proposed.

Necrotic cell death (necrosis) has long been considered a passive or un-programmed mode of cell death. But recently it has become clear that necrosis, just like apoptosis, is a molecularly regulated event that is associated with pathologies of ischemia–reperfusion (IR) injury, oxidative stresses, neurodegeneration, and UV radiation [8–16]. Thus, it is termed as programmed necrosis. Mitochondria play a key role in programmed necrosis initiation through regulating the mitochondrial permeability transition pore (mPTP), which is channel complex composed of at least three primary proteins: the voltage-dependent anion channel (VDAC) in the outer membrane, the adenine nucleotide translocator (ANT) in the inner membrane, and cyclophilin D (Cyp-D) within the mitochondrial matrix [13,17]. Without stimuli, Cyp-D is located in the matrix to keep the mPTP closed [10,18,19]. It will, however,

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associate with ANT in the inner membrane when facing critical conditions to open mPTP [20,21], causing mitochondrial depolarization, mitochondria swelling, Ca^{2+} release, and eventually cell necrosis. In the current study, we found that berberine also induces programmed necrosis in cultured prostate cancer cells.

2. Material and methods

2.1. Chemical, reagents and antibodies

Berberine chloride, N-acetylcysteine (NAC), pifithrin-α (PFTα), sanglifehrin A (SFA) and cyclosporine A (CSA) were obtained from Sigma (Sigma, St. Louis, MO); Z-VAD-fmk and necrostatin-1 were purchase from Calbiochem (Shanghai, China). Anti-β-actin, Cyp-D, VDAC-1 and rabbit/mouse IgG-horseradish peroxidase (IgG-HRP) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The antibody recognizing P53 was purchased from Cell Signaling Technology (Denver MA). In all treatments, berberine and various inhibitors was dissolved initially in a small amount of dimethyl sulfoxide (DMSO). The sub-confluent cells were treated with varying concentrations of berberine (or with inhibitors) in complete cell culture medium and cells treated only with vehicle (DMSO, 0.2% in media) served as control.

2.2. Cell culture

Human prostate carcinoma PC-3, PC-82 and LNCaP cell lines were obtained from the American Type Culture Collection (Manassas, VA). The tumor cells were cultured as monolayer in RPMI-1640 supplemented with 10% heat inactivated fetal bovine serum (FBS, Hyclone, Shanghai, China), 100 μg/ml penicillin–streptomycin (Invitrogen, Carlsbad, CA) and maintained in an incubator.

2.3. Cell viability assay

The methyl thiazolyl tetrazolium (MTT) assay was performed to assess the cell viability according to instructions from the manufacturer (Sigma). Briefly, cancer cells were planted into 96-well plates. At the end of each treatment, 20 μL of MTT (5 mg/ml, Sigma,) was added for 4 h. Then the medium was discarded carefully and 150 μL of DMSO was added. Absorbance was recorded at 570 nm with the Universal Microplate Reader (Bio-Tek instruments) using wells without cells as blanks. The value of treatment group was normalized to that of control group.

2.4. Trypan blue staining of "dead" cells

The number of dead prostate cells (trypan blue positive) after treatment was counted, and the percentage (%) of "dead" cells was calculated by the number of the trypan blue positive cells divided by the total number of the cells.

2.5. Cell apoptosis assay

Apoptosis in human prostate cancer cells caused was quantitatively determined by flow cytometry using the Annexin V Apoptosis Detection Kit (BD, Shanghai, China) following the manufacturer's instructions. Briefly, after treatment, cells were then harvested by brief trypsinization, washed with cold PBS, and incubated with Annexin V and propidium iodide (PI) for cellular staining in binding buffer at room temperature for 10 min in the dark. The stained cells were analyzed by FACS using a FACSCalibur instrument (BD Biosciences, San Jose, CA) equipped with CellQuest 3.3 software. Percentage of Annexin V positive cells was recorded as apoptosis percentage.

2.6. Reactive oxygen species (ROS) detection

Intracellular ROS generation was measured by flow cytometry using dichlorofluorescin (DCF) oxidation assay. DCFH-DA enters passively into the cells and is cleaved by nonspecific cellular esterases and oxidized in the presence of ROS. Briefly, $3 * 10^5$ prostate cancer cells were plated in 60-mm culture plates and allowed to attach by overnight incubation. After treatment, cells were incubated with DCFH-DA (5 μM) for 1 h at 37 °C. Thereafter, cells were washed with PBS and kept in 1 ml PBS, ROS fluorescence was analyzed using flow cytometer. The value of treatment group was normalized to that of control group.

2.7. Mitochondrial membrane potential (MMP) detection

The mitochondrial membrane potential ($\Delta\psi_m$) was determined by flow cytometry using the lipophilic cationic probe JC-1 (5,5′, 6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazol-carbocyanine iodide) Detection Kit, following the manufacturer's protocol (Molecular Probes Inc., Eugene, OR), and as described previously [22]. Loss of MMP was indicated by the increase of JC-1 intensity. The value of treatment group was normalized to that of control group.

2.8. Western Blotting

After treatment, cells were harvested by trypsinization and washed three times with PBS. Subsequently, cells were solubilized in 20 mM Tris–HCl, pH 7.6, 140 mM NaCl, 1 mM EDTA, 0.5% Triton X-100, 1 μM pepstatin A, 20 μM leupeptin, 0.2 mM 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride at 4 °C for 30 min. The cell lysates were centrifuged at 1000*g* for 5 min to separate the nuclei. The concentration of total protein was determined by the BCA assay kit (Pierce, Rockford, IL). For SDS–PAGE, 20–30 μg of total protein was loaded per lane. Interested proteins were detected with primary antibodies. Horseradish peroxidase-conjugated secondary antibodies were then used and visualized by chemiluminescence. Intact mitochondria were isolated from 1 to 2×10^7 prostate cancer cells using the "Mitochondria Isolation Kit for Cultured Cells" (Pierce, Rockford, IL) according to the manufacturer's instruction.

2.9. Mitochondrial Immunoprecipitation (Mito-IP)

Mitochondrial lysates (500–600 μg) were pre-cleared with 30 μl of protein IgA/G-beads (Sigma) for 30 min at 4 °C. After centrifugation for 10 min at 4 °C in a micro-centrifuge, the supernatant was rotated overnight with 2 μg of anti-Cyp-D overnight. The protein IgA/G-beads (35 μl) were then added to the supernatants for 4 h at 4 °C. Then the pellets were washed six times with cold PBS and 1 time with lysis buffer, resuspended in lysis buffer, and then assayed in Western blotting to detect the immuno complex.

2.10. SiRNA and transfection

The double strand siRNA against human P53 and the negative control siRNA were purchased from Cell Signaling Tech. We applied siRNA duplexes at a final concentration of 100 nM using Lipofectamine 2000 Reagent (Invitrogen, Carlsbad, CA, USA) according to recommended procedure. Expression of target protein (P53) was tested by Western blotting 48 h after transfection to confirm siRNA efficiency.

2.11. Cyp-D shRNA stable knockdown

The lentiviral particles with human Cyp-D shRNA (Santa Cruz Biotech) (10 μl/ml) were added to the prostate cells, after 24 h,

fresh growth medium was added, and cells were further cultured for another 24 h. Puromycin (0.25 μg/ml) was added to select resistant stable colonies. The selection took 2–3 weeks. Expression of Cyp-D in stable cells was detected by Western blotting. Control cells were infected with scramble-shRNA containing lentiviral particles (Sigma).

2.12. Statistical analysis

The results from the *in vitro* studies are representative of at least three independent experiments. Statistical differences were analyzed by one-way ANOVA followed by multiple comparisons performed with post hoc Bonferroni test (SPSS version 18). Values of $p < 0.05$ were considered statistically significant. All quantitative data were shown as mean ± standard deviation (SD).

3. Results

3.1. Berberine induces apoptosis and programmed necrosis in cultured prostate cancer cells

We first tested the effect of berberine against prostate cancer cells. The MTT cell viability assay results in Fig. 1A demonstrated that berberine dose-dependently decreased the viability of LNCaP prostate cancer cells. Further, the number of trypan blue positive cells was significantly increased after berberine stimulation (Fig. 1B). To dissect the role of apoptosis and necrosis in berberine-induced cytotoxicity, the apoptosis inhibitor (z-VAD-fmk) and the necrosis inhibitor (necrostatin-1) were applied. Results in Fig. 1C and D showed that z-VAD-fmk (ZVAD) only slightly inhibited viability loss and cell death by berberine, while necrostatin-1, the necrosis inhibitor, dramatically inhibited berberine-induced LNCaP cytotoxicity. Significantly, combination of necrostatin-1 and ZVAD almost blocked berberine-induced LNCaP cell death (Fig. 1C and D). Similar results were seen in another prostate cancer cell line (PC-82) (Fig. 1F). Results in Fig. 1E confirmed apoptosis induction by berberine in LNCaP cells, which was largely inhibited by ZVAD. Note that only a small proportion (<15%) of LNCaP cells with berberine stimulation were apoptotic (Fig. 1E). These results indicate that berberine induces both apoptotic and necrotic death in cultured prostate cancer cells, and necrosis plays a more important role in contributing berberine's cytotoxicity.

3.2. Mitochondrial Cyp-D is required for berberine-induced necrosis in cultured prostate cancer cells

As discussed, mitochondrial protein Cyp-D plays a key role in regulating mPTP opening and programmed necrosis under a number of stresses [19,23,24]. The results above have shown that necrosis is a major contributor of berberine's cytotoxicity. Thus, we tested the potential role of Cyp-D in berberine-induced cytotoxicity in cultured prostate cancer cells. Results in Fig. 2A and B showed that Cyp-D inhibitors cyclosporin A (CSA) [25] and sanglifehrin A (SFA) [26] largely inhibited berberine-induced viability decrease and cell death in LNCaP cells. Further, shRNA-mediated stable knockdown of Cyp-D (Fig. 2C) dramatically suppressed berberine-induced cytotoxicity (Fig. 2D and E). Interestingly, berberine-induced cell apoptosis was not affected by Cyp-D inhibitors

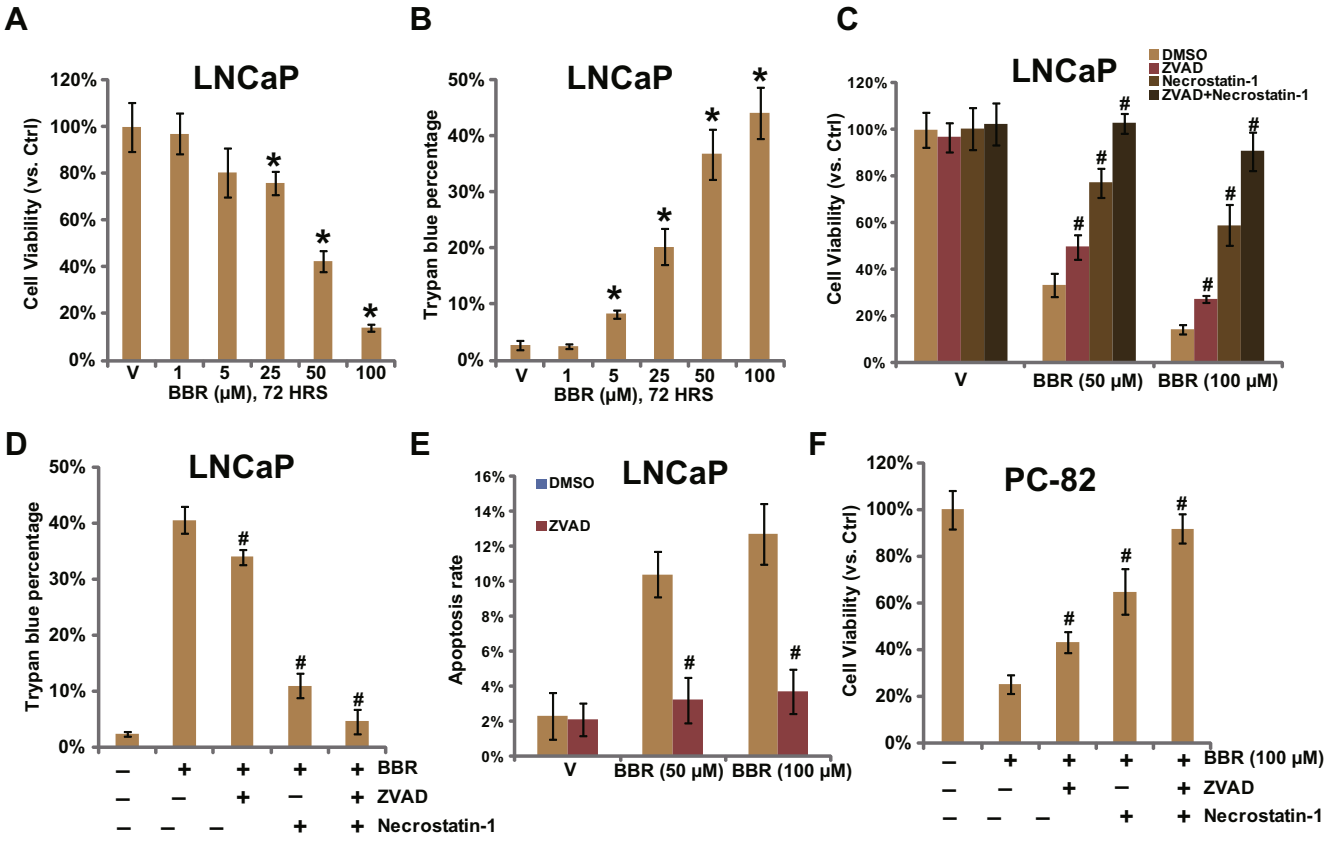

Fig. 1. Berberine induces apoptosis and programmed necrosis in cultured prostate cancer cells. Prostate cancer LNCaP cells were either treated with vehicle (0.2% DMSO, "V") or stimulated with increasing concentrations of berberine (BBR, 1–100 μM), cells were further cultured for 72 h, cell viability and cell death were tested by MTT assay (A) and trypan blue staining (B), respectively. LNCaP or PC-82 cells were pre-treated with zVADfmk (ZVAD, 50 μM), necrostatin-1(10 μM), or both for 1 h, followed by indicated BBR stimulation, cell viability (C and F) and cell death (D) were tested. The effect of ZVAD on BBR (100 μM, 72 h)-induced apoptosis was tested by Annexin V FACS (E). $^{*}P < 0.05$ vs. "V" group (A and B). $^{\#}P < 0.05$ vs. BBR group (C–F).

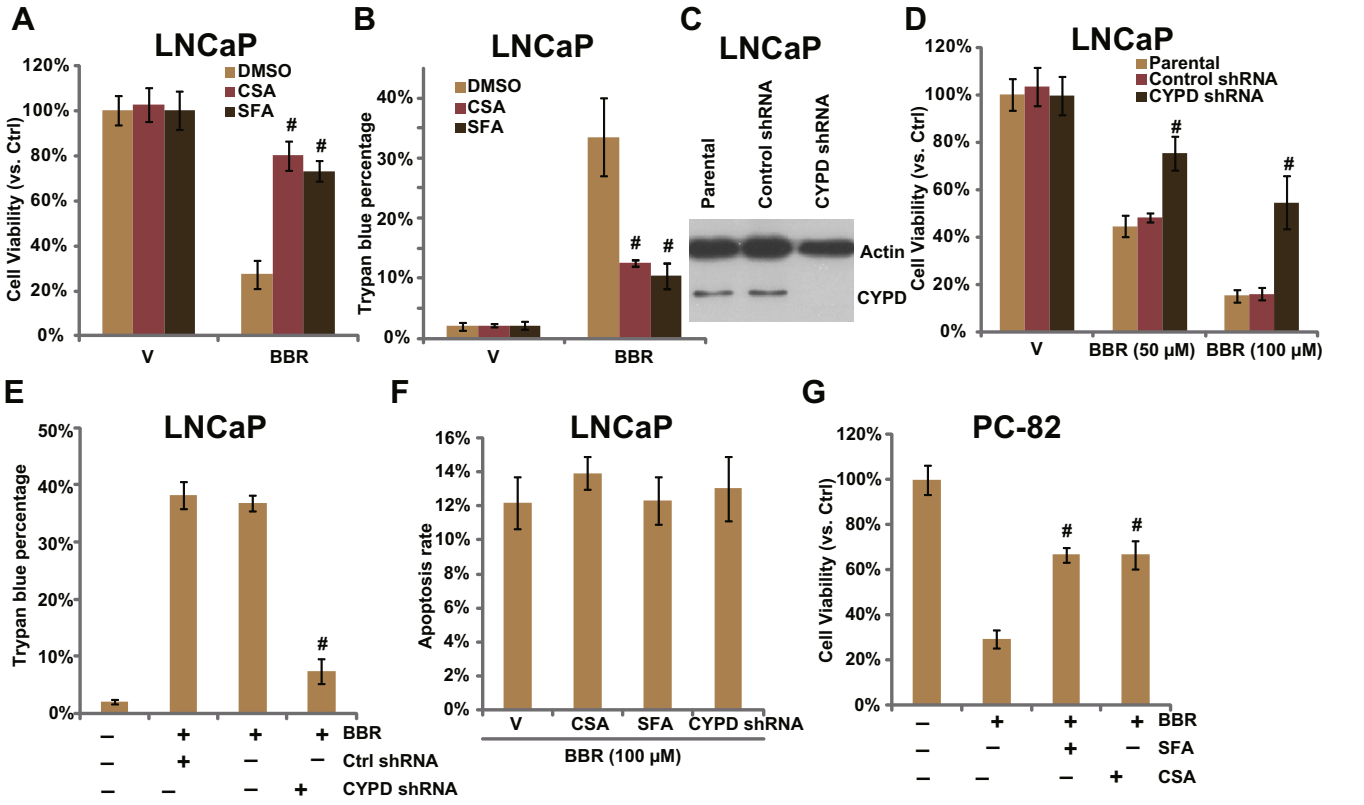

Fig. 2. Mitochondrial Cyp-D is required for berberine-induced necrosis in cultured prostate cancer cells. LNCaP or PC-82 cells were pretreated with cyclosporine A (CSA, 0.2 μM) or sanglifehrin A (SFA, 10 μM) for 1 h, followed by indicated berberine (BBR) stimulation, cell viability (A and G) and death were tested (B). Stable LNCaP cells transfected with scramble control shRNA (Ctrl shRNA) or Cyp-D shRNA, as well as their parental cells were treated with indicated BBR, expression of Cyp-D and β-actin was tested by Western blotting (C), cell viability (D) and cell death (E) were also tested. The effect of CSA (0.2 μM) or SFA (10 μM) or Cyp-D shRNA on BBR (100 μM, 72 h)-induced LNCaP cell apoptosis was also tested (F). $^{\#}P < 0.05$ vs. BBR only group (A, B, D, E and G). "V" stands for vehicle (0.2% DMSO) group.

or depletion (Fig. 2F). In PC-82 prostate cancer cells, berberine-induced viability decrease was also inhibited by Cyp-D inhibitors (CSA and SFA) (Fig. 2G). Together, these results suggest that mitochondrial Cyp-D is required for berberine-induced necrosis (but not apoptosis) in cultured prostate cancer cells.

3.3. Berberine induces P53 mitochondrial translocation and Cyp-D association in cultured prostate cancer cells

A recent study by Vaseva et al., demonstrated that P53 travels to mitochondria to regulate mPTP opening and programmed necrosis [14]. According to that study, P53 forms a complex with Cyp-D in mitochondria, serving as the key initial step for necrosis [14]. Following that, multiple groups have shown that certain anti-cancer agents induce mitochondrial Cyp-D-P53-dependent cancer cell necrosis [9,27]. Here, we tested whether the similar scenario also happened in berberine-treated prostate cancer cells. Results in Fig. 3A showed berberine induced P53 mitochondrial translocation in LNCaP cells. Further, the mito-IP data in Fig. 3B confirmed that berberine-activated P53 formed a complex with mitochondrial protein Cyp-D, and the effect of berberine was dose-dependent. Berberine-induced P53 translocation and mitochondrial P53/Cyp-D association was also observed in PC-82 prostate cancer cells (Fig. 3C). These results show that berberine induces P53 translocation to mitochondria, where it associates Cyp-D in prostate cancer cells.

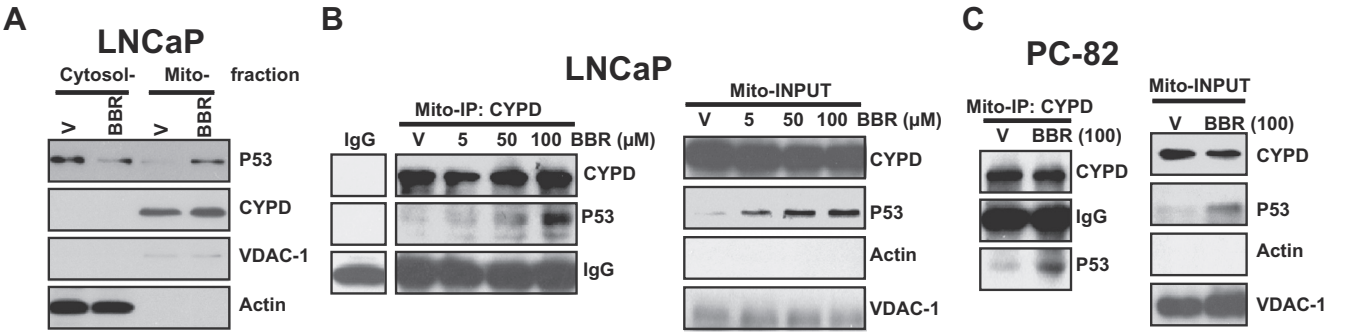

Fig. 3. Berberine induces P53 mitochondrial translocation and Cyp-D association in cultured prostate cancer cells. LNCaP cells were treated with berberine (BBR, 100 μM) for 6 h, the mitochondrial and cytosol fractions were isolated, and expression of Cyp-D, VDAC-1, P53 and β-actin was tested by Western blotting (A). LNCaP or PC-82 cells were treated with indicated BBR for 6 h, the association between P53 and Cyp-D was tested by mito-IP, expression of P53, Cyp-D, VDAC-1 and β-actin in "INPUT" was also tested (B and C). "V" stands for vehicle (0.2% DMSO) group.

3.4. ROS production is required for berberine-induced P53–Cyp-D mitochondrial association and mPTP opening

Next, we studied the upstream signaling for berberine-induced P53–Cyp-D mitochondrial association. Since a recent study showed that ROS production is the key mediator of berberine-induced prostate cancer cell death, we thus tested the role of ROS in berberine's effect. Results in Fig. 4A demonstrated that anti-oxidant N-acetylcysteine (NAC) significantly inhibited berberine-induced P53–Cyp-D mitochondrial association. Further, Cyp-D inhibitor CSA and P53 siRNA also suppressed mitochondrial P53–Cyp-D complexation by berberine (Fig. 4A). However, NAC, but not CSA, inhibited berberine-induced P53 mitochondrial translocation (Fig. 4A, INPUT). These results indicated that CSA directly interfered P53–Cyp-D association, while NAC likely blocked P53 mitochondrial translocation. JC-1 results in Fig. 4B demonstrated that NAC, CSA and P53 siRNA largely inhibited berberine-induced MMP loss, indicating that mitochondrial P53–Cyp-D complexation might be required for berberine-induced mPTP opening. Further, it was found that NAC, the P53 inhibitor pifithrin-α (PFTα) or P53

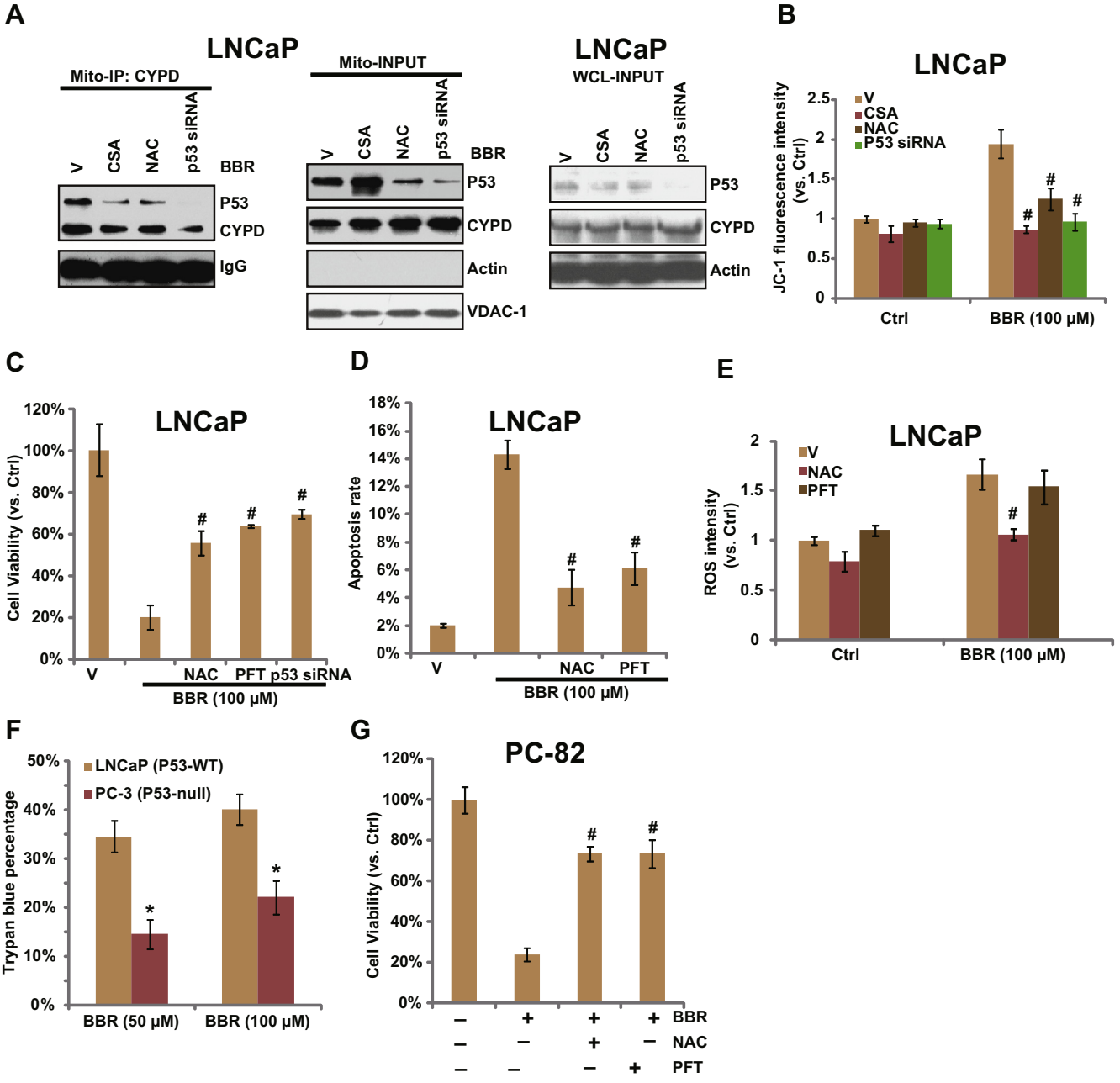

Fig. 4. ROS production is required for berberine-induced P53–Cyp-D mitochondrial association and mPTP opening. LNCaP cells were pretreated with N-acetylcysteine (NAC, 0.5 mM) or CSA (0.2 μM) for 1 h, or transfected with P53 siRNA (100 nM, 48 h), cells were then stimulated with berberine (BBR, 100 μM) for 6 h, mitochondrial association between P53 and Cyp-D was tested by mito-IP (A, left), expression of P53, VDAC-1, β-actin and Cyp-D in mitochondrial fraction or the whole cell lysates (WCL) was tested by Western blotting (A, middle and right), note P53 knockdown by the target siRNA (A, right), JC-1 fluorescence intensity, an indicator of MMP loss, was also tested (B). The effect of NAC (0.5 mM), pifithrin-α (PFT, 5 μM) or P53 siRNA (100 nM, 48 h) on berberine (BBR, 100 μM, 72 h) induced viability change and apoptosis in LNCaP/PC-82 cells was tested by MTT assay (C and G) and Annexin V FACS (D), respectively. LNCaP cells were pretreated with NAC (0.5 mM) or PFT (5 μM) for 1 h, cells were then stimulated with BBR (100 μM) for 3 h, ROS level was tested (E). Same number of LNCaP and PC-3 cells were treated with indicated BBR for 72 h, cell viability was tested (F). $^{\#}P < 0.05$ vs. BBR only group (B–E, G), $^{*}P < 0.05$ vs. LNCaP cells (F). "V" stands for vehicle (0.2% DMSO) group.

siRNA largely inhibited berberine-induced viability decrease in prostate cancer cells (Fig. 4C and G). Importantly; berberine-induced LNCaP cell apoptosis was also inhibited by NAC and PFTα (Fig. 4D). Thus, we propose that ROS production and P53 activation are not only required for berberine-induced cell apoptosis, they are also important for berberine-activated P53–Cyp-D mitochondrial association, mPTP opening and programmed necrosis. Results in Fig. 4E demonstrated that NAC, but not PFTα, suppressed berberine-induced ROS production in LNCaP cells. MTT results in Fig. 3F showed LNCaP cells (expressing WT-P53) were more sensitive to berberine than PC-3 cells (expressing mutated P53) [28], further supporting the role of P53 in berberine-mediated cytotoxicity. Together, these results suggest that ROS production might be the upstream signal for berberine-induced P53–Cyp-D mitochondrial association and mPTP opening.

4. Discussions

The incidence of prostate cancer is increasing at an alarming rate in the United States and around the world. Currently, there has been no effective treatment for the metastatic prostate cancer, especially those with hormone ablation therapy failed [2,3,29]. Different groups have showed the anti-prostate cancer ability of berberine [6,7]. In the current study, we found that berberine induced apoptosis and programmed necrosis in cultured prostate cancer cells, and necrosis weighted more than apoptosis in contributing berberine's cytotoxicity. Mitochondrial Cyp-D plays an important role in berberine-induced programmed necrosis. Inhibition of Cyp-D by its inhibitors (CSA or SFA), or by Cyp-D shRNA depletion alleviated berberine-induced cell necrosis (but not apoptosis). Berberine induced ROS-dependent P53 translocation to mitochondria, where it physically interacted with Cyp-D. The anti-oxidant NAC, the P53 inhibitor (PFTα) as well as P53 siRNA depletion suppressed berberine-induced P53 mitochondrial translocation and Cyp-D association, thus inhibiting subsequent mPTP opening and subsequent cell death. Significantly, ROS production and P53 activation were also required for berberine-induced apoptosis, as NAC or PFTα decreased berberine-induced prostate cancer cell apoptosis. Thus, both apoptosis and programmed necrosis contributed to berberine's cytotoxicity in cultured prostate cancer cells.

Using strategies such as gene knockout, siRNA knockdown, mutation/over-expression and pharmacological inhibition, recent *in vitro* and *in vivo* studies have confirmed the critical role of mitochondrial protein Cyp-D in certain stresses-induced programmed necrosis. Cyp-D translocates to the IMM and forms a complex with ANT, causing mPTP opening and eventual cell necrosis. To date, the most specific inhibitor of Cyp-D is cyclosporin A (CSA), which blocks the PPIase activity of Cyp-D and stops Cyp-D-ANT-1 association, thus shutting down mPTP [25]. Sanglifehrin A (SFA) is another potent inhibitor of Cyp-D, which also inhibits Cyp-D's PPIase activity, thus preventing it from binding to ANT [26]. Here we found that these Cyp-D inhibitors or Cyp-D shRNA depletion dramatically inhibited berberine-induced MPP loss and suppressed cell necrosis. Berberine-induced cell apoptosis was not affected by Cyp-D inhibition. Thus, we conclude that berberine induces Cyp-D-regulated necrosis in cultured prostate cancer cells.

Recent studies have shown that berberine-induced anti-cancer ability is associated with P53 activation [30,31]. But how activated P53 causes cancer cell death is still not fully understood. Here, we propose that berberine-activated P53 could travel to mitochondria, opening mPTP and promoting cell necrosis. Inhibition of P53 by its inhibitor or by siRNA suppressed berberine-induced MPP loss, cell necrosis and apoptosis. Studies also suggest that berberine induces the ROS generation in both androgen-independent (PC-3) and androgen-dependent (LNCaP) prostate cancer cells, which is required for cell apoptosis [6]. NAC, the anti-oxidant, inhibited berberine-induced ROS production and cancer cell apoptosis [6]. In consistent with these studies, we also observed ROS production in berberine-treated LNCaP cells, and NAC inhibited berberine-caused ROS production and cell apoptosis. Significantly, NAC also inhibited P53 mitochondrial translocation, subsequent Cyp-D association and MPP loss. These results indicate that berberine-induced ROS production might be the upstream signal for both cell apoptosis and necrosis.

In conclusion, the *in vitro* results of this study suggest that both apoptosis and programmed necrosis contributed to berberine-induced cytotoxicity in cultured prostate cancer cells.

Conflict of interests

The authors have no conflict of interests.

Acknowledgments

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