RESEARCH ARTICLE

Curcumin provides potential protection against the activation of hypoxia and prolyl 4-hydroxylase inhibitors on prostate-specific antigen expression in human prostate carcinoma cells

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Scope: Prostate-specific antigen (PSA) is a well-known marker for diagnosing and monitoring prostate cancer. Curcumin, a yellow curry pigment, has been reported to enhance androgen receptor (AR) degradation. We examined the effects of curcumin on increasing PSA expression by hypoxia and prolyl hydroxylase inhibitors, L-mimosine and dimethyloxalylglycine (DMOG), in human prostate carcinoma LNCaP cells.

Methods and results: The ^{3}H-thymidine incorporation assay revealed that either L-mimosine or DMOG treatments attenuated cell proliferation. Immunoblot and enzyme-linked immunosorbent assays (ELISA) indicated that both L-mimosine and DMOG have an effect similar to hypoxia, which stabilized hypoxia-inducible factor-1α (HIF-1α) and induced PSA gene expression. The results of the immunoblot and transient gene expression assays indicated that induction of the PSA expression by hypoxia is both HIF-1α- and AR-dependent. Immunoblot assays revealed that a curcumin treatment (10 μM) decreased the protein abundance of AR but did not significantly affect the protein levels of HIF-1α and vascular endothelial growth factor, which were induced by hypoxia. ELISA and transient gene expression assays indicated that curcumin blocked the activation of L-mimosine or DMOG treatment on PSA expression.

Conclusions: These results indicate that curcumin blocked the enhanced effect of PSA expression by L-mimosine and DMOG that induce hypoxia condition.

Keywords:
Androgen receptor / Dimethyloxalylglycine / Hypoxia-inducible factor-1α / Hypoxia / Mimosine

1 Introduction

Curcumin, the principal curcuminoid from the rhizome of Curcuma longa Linn., is regarded to be a multifunctional effector with antioxidative activity and as a promising anticancer agent due to its antiproliferative and anti-angiogenic properties in several cancer cells including prostate cancer [1]. In vitro and in vivo studies have revealed that curcumin provides a potential protection inhibiting hypoxia-induced gene expression [2–4]. In the prostate, one of the major pharmacologic effects of curcumin is the enhancement of androgen receptor (AR) degradation [5]. Our previous study indicated that curcumin inhibits R1881-

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Abbreviations: AR, androgen receptor; **DMOG,** dimethyloxalylglycine; **FAC,** ferric ammonium citrate; **FCS,** fetal calf serum; **HIF-1α,** hypoxia-inducible factor-1α; **PSA,** prostate-specific antigen; **VEGF,** vascular endothelial growth factor

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and IL-6-mediated prostate-specific antigen (PSA) gene expression in LNCaP cells through the down-regulation of the expression and activity of AR [6]. A human clinical study also indicated that curcumin blocks PSA expression [7].

PSA, a 30–33 kDa glycoprotein expressed in all stages of prostate cancer and primarily regulated by androgen, is produced almost exclusively by the luminal epithelial cells of the human prostate. Serum PSA is a well-known biomarker for diagnosing and evaluating the status of prostate cancer [8, 9]. Although the pathophysiologic roles of PSA remain unclear, in vitro and in vivo studies have reported that PSA may facilitate refractory prostate tumor progression and may be involved in the invasion of prostate cancer [10, 11].

The results of an in vivo study revealed that mountain hypoxia did not affect the mean serum PSA values [12]. Another immunohistochemistry study did find a positive correlation between the expression of hypoxia-inducible factor-1α (HIF-1α) in radical prostatectomy specimens and the serum PSA levels [13]. An in vitro study using prostate carcinoma LNCaP cells found that hypoxia not only increases AR activity but is also synergistic with the AR to induce PSA expression [14, 15]. However, the effect of hypoxia or the prolyl hydroxylase inhibitors that induced mimetic hypoxia on the PSA expression is still not well-known.

L-mimosine, a plant amino acid, acts as an iron chelator and prolyl hydroxylase inhibitor, which reversibly blocks proliferation at the late G1 phase of several cancer cells including prostate cancer [16, 17]. L-mimosine demonstrates high antiproliferation activity by increasing the expression of growth inhibiting and metastasis-suppressing genes mediated by HIF-1α-dependent and -independent mechanisms [18]. Dimethyloxalylglycine (DMOG), a pan-prolyl hydroxylase inhibitor stabilizing the HIF-1α, was used to mimic the effects of hypoxia in vivo and in vitro [19, 20]. Except for antitumor treatment, the prolyl hydroxylase inhibitors have been used in therapeutic applications including myocardial ischemia, cerebral ischemia, anemia and kidney disease [21].

The objectives of this study were to determine the effect of L-mimosine and DMOG on HIF-1α expression and its association with the gene expression of PSA in the human prostate carcinoma LNCaP cells. We evaluated the mechanisms for blocking the activation of prolyl 4-hydroxylase inhibitors on PSA expression using curcumin.

2 Materials and methods

2.1 Cell culture and chemicals

LNCaP cells were obtained from the American Type Culture Collection (Manassas, VA, USA). PCJ cells, a subculture cell line of prostate carcinoma PC-3 cells were cloned in our laboratory [22]. L-mimosine, chetomin, DMOG, curcumin and ferric ammonium citrate (FAC) were purchased from Sigma-Aldrich (St. Louis, MO, USA) and the bicinchoninic acid protein assay kit was purchased from Pierce Protein Research (Rockford, IL, USA). The fetal calf serum (FCS) was purchased from HyClone (Logan, Utah, USA), and the RPMI 1640 medium was purchased from GIBCO, Invitrogen Corporation (Grand Island, NY, USA). Cells were incubated under normoxia using a standard CO_2 incubator at 37°C in a humidified atmosphere with 5% CO_2 and 95% room air (21% O_2) until the cells grew to 70–80% confluence in RPMI-1640 medium with 10% FCS. On the day of hypoxic treatment (1% O_2, 5% CO_2 and 94% N_2), the growth medium was switched to fresh medium with 10% FCS that had been pre-equilibrated in the hypoxic incubator (APM-30D, Astec, Fukuoka, Japan). Cells were then incubated in the hypoxic incubator for another 24 h.

2.2 Cell proliferation assay with ^{3}H-thymidine incorporation

Cell proliferation in response to the prolyl 4-hydroxylase inhibitors was measured using a ^{3}H-thymidine incorporation assay. In this assay, 1×10^4 cells were cultured in each well of a 12-well plate in RPMI 1640 medium with 10% FCS and different concentrations of prolyl 4-hydroxylase inhibitors as indicated. After 24 h incubation period, 0.5 μCi/mL of ^{3}H-thymidine was added to each well of the 12-well plate. The cells were then incubated at 37°C in a humidified 5% CO_2 atmosphere for 4 h. Cells were then washed twice with cold phosphate-buffered saline (PBS) and then with cold 5% trichloroacetic acid. Cells were lysed by adding 0.5 mL of 0.5 N NaOH. Then, 400 μL of the solubilized cell solution was mixed with 4 mL of scintillation cocktail and counted in a liquid scintillation analyzer (Packard BioScience, Meriden, CT, USA). Each sample was tested in quadruplicate.

2.3 Immunoblot assays

Equal quantities of cell extract (40 μg) were separated on a 10% SDS-polyacrylamide gel, transferred and analyzed by the Western Lightning Plus-Enhanced Chemiluminescence detection system (Perkin Elmer, Waltham, MA, USA) as described by the manufacturer. The Western blot membranes were probed with 1:500 diluted polyclonal PSA antiserum (A0562, DakoCytomation, Glostrup, Denmark), 1:500 HIF-1α (610958, BD Biosciences, San Jose, CA, USA), 1:100 diluted AR antiserum (N-20 Santa Cruz Biotechnology, Santa Cruz, CA, USA), 1:3000 diluted β-actin antiserum (I-19, Santa Cruz Biotechnology) and 1:100 diluted vascular endothelial growth factor (VEGF; A-20, Santa Cruz Biotechnology). The images of immunoblot were viewed using the ChemiGenius image capture system (Syngene, Cambridge, UK) and the intensities of the different bands were analyzed using the GeneTools program of ChemiGenius (Syngene).

2.4 HIF-1α expression vector

The human HIF-1α cDNA (MGC:10483) vector was purchased from Invitrogen Corporation. Human HIF-1α cDNA was linearized by cutting with *Bam*HI and *Xba*I and ligation with the overexpression vector pcDNA3 (Invitrogen), resulting in the insertion of HIF-1α cDNA in the polyadenylate region controlled by the cytomegalovirus promoter (pcDNA3-HIF-1α). Proper ligation and orientation were confirmed by extensive restriction mapping and sequencing.

2.5 Reporter vectors and transient gene expression assay

The reporter vectors (pPSABHE, −4801 to −3933 and −41 to −589; pPSABH, −41 to −589) containing the 5′-flanking region of the human PSA gene were cloned by 5′-deletion or PCR as previously described [23]. LNCaP cells (1×10^4 cells/well) were plated onto 24-well plates 1 day before transfection. Cells were transiently transfected using TransFast transfection reagent as previously described [24]. For the transient cotransfection of the HIF-1α experiments, cells were transfected with the same amount of plasmid in each well by adding pcDNA3 vector to eliminate the variable degrees of efficiency of the reporter activities. Reporter vector-transfected LNCaP cells were then treated with or without treatments in RPMI 1640 medium with 10% FCS for an additional 24 h. The luciferase activity was adjusted for transfection efficiency using the normalization control plasmid pCMVSPORTβgal.

2.6 PSA enzyme-linked immunosorbent assay

LNCaP cells were incubated with different concentrations of L-mimosine, DMOG and/or curcumin, as indicated, in 0.5 mL of RPMI medium with 10% FCS in a 24-well plate (1×10^5 cells/well) for 24 h. Following incubation, the supernatants of the conditioned medium from each well were collected for PSA assays using enzyme-linked immunosorbent assay (ELISA) as previously described [23]. The PSA level in each sample was adjusted by the concentration of protein in the whole cell extract, which was measured using a bicinchoninic acid protein assay.

2.7 Statistical analysis

Results are expressed as the mean ± SE of at least three independent replications of each experiment. Statistical significance was determined using Student's paired *t*-test and one-way ANOVA using the SigmaStat program for Windows, version 2.03 (SPSS, Chicago, IL, USA).

3 Results

3.1 Effect of hypoxia on gene expression of PSA in LNCaP cells

The immunoblot assays revealed that hypoxia, as compared to normoxia, significantly upregulated the protein level of HIF-1α in the LNCaP cells (Fig. 1A)., Hypoxia stabilized the HIF-1α and upregulated the gene expression of VEGF and PSA in the LNCaP cells (Fig. 1A). The PSA protein level was upregulated 2.2-fold after 24 h of hypoxic culturing as compared to the LNCaP cells incubated under normoxia (Fig. 1B). The ELISA results revealed a PSA secretion of LNCaP cells upregulated to 1.4-fold under hypoxia (Fig. 1C).

3.2 Effect of L-mimosine on prostate cancer cell growth and PSA gene expression

The ^{3}H-thymidine incorporation assay revealed that the inhibition of LNCaP cell growth occurred initially at 100 μM

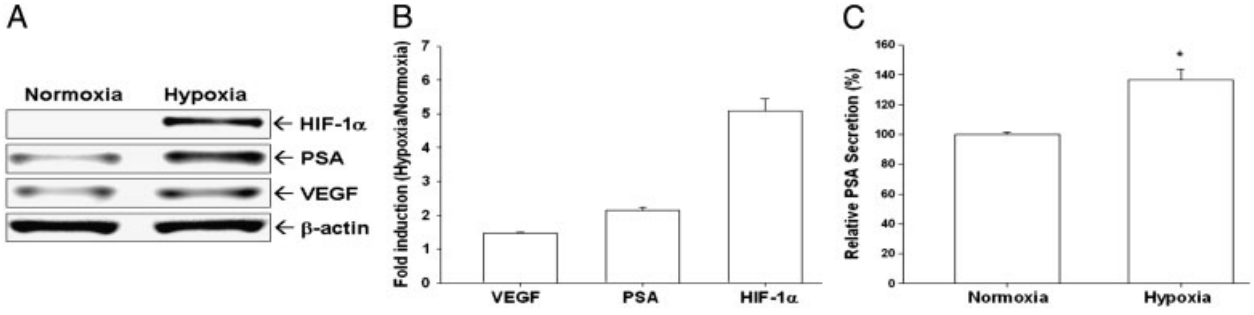

Figure 1. Hypoxia modulates PSA gene expression of LNCaP. (A) Cells were cultured under hypoxia (1% O_2) or normoxia (21% O_2) for 24 h. The cells were then lysed and the expressions of HIF-1α, PSA, VEGF and β-actin were determined by immunoblot assay. (B) The quantitative analysis was done by determining the intensity of each band for target genes and β-actin from three independent experiments. Data are presented as the fold-induction (±SE; $n = 3$) of the relative density of the PSA/β-actin or VEGF/β-actin (±SE) of the hypoxia treatment in relation to the normoxia treatment. (C) Cells were cultured under hypoxia (1% O_2) or normoxia (21% O_2) for 24 h. The conditioned media were collected for PSA assay. Data are expressed as the mean percent ± SE of six preparations relative to the control solvent-treated group ($^*p < 0.01$).

of L-mimosine, increasing as the dose was increased. The 800 μM of L-mimosine significantly blocked 39% of the ^{3}H-thymidine incorporation in the LNCaP cells after treatment with L-mimosine for 24 h (Fig. 2A). Immunoblot assays indicated that the L-mimosine not only stabilized the HIF-1α but also induced PSA expression in the LNCaP cells. The PSA protein level was upregulated by 24 h of L-mimosine treatments, which increased in a dose-dependent manner (Fig. 2B). The quantitative analysis was done by determining the intensity of each band for the different genes and β-actin from three independent experiments; the data is presented in Fig. 2C. The PSA protein level was upregulated 1.4-fold after being treated with 400 μM of L-mimosine for 24 h. The ELISA results also indicated that the L-mimosine treatment for 24 h induced PSA secretion in a dose-dependent manner. The PSA secretion of LNCaP cells upregulated to 2.2-fold under the treatment of 800 μM of L-mimosine (Fig. 2D).

3.3 Effect of DMOG on prostate cancer cell growth and PSA gene expression

We continued our work to evaluate the effect of DMOG, the pan-prolyl hydroxylase inhibitor, on cell proliferation and PSA expression. The ^{3}H-thymidine incorporation assay revealed that the inhibition of LNCaP cell growth occurred initially at 125 μM of DMOG. The 1000 μM dose of DMOG significantly blocked 25% of the ^{3}H-thymidine incorporation in the LNCaP cells after treatment for 24 h (Fig. 3A). The immunoblot assays showed that HIF-1α stabilization was induced in cells cultured in 21% oxygen by treatment with DMOG. DMOG treatments upregulated the PSA protein levels in the LNCaP cells (Fig. 3B). The quantitative analysis indicated that the PSA protein level was upregulated 2.0-fold after being treated with 500 μM of DMOG for 24 h (Fig. 3C). The results of the ELISA revealed that the PSA secretion of LNCaP cells was upregulated to 2.3-fold following treatment with 500 μM of DMOG (Fig. 3D).

3.4 Upregulation of L-mimosine on the gene expression of PSA is due to iron chelation

Since L-mimosine is an iron chelator, in addition, we proceeded to determine whether iron blocked the inducing effect of L-mimosine on PSA gene expression. The immunoblot assay revealed that the L-mimosine-induced increase in PSA gene expression was blocked after cotreatment with

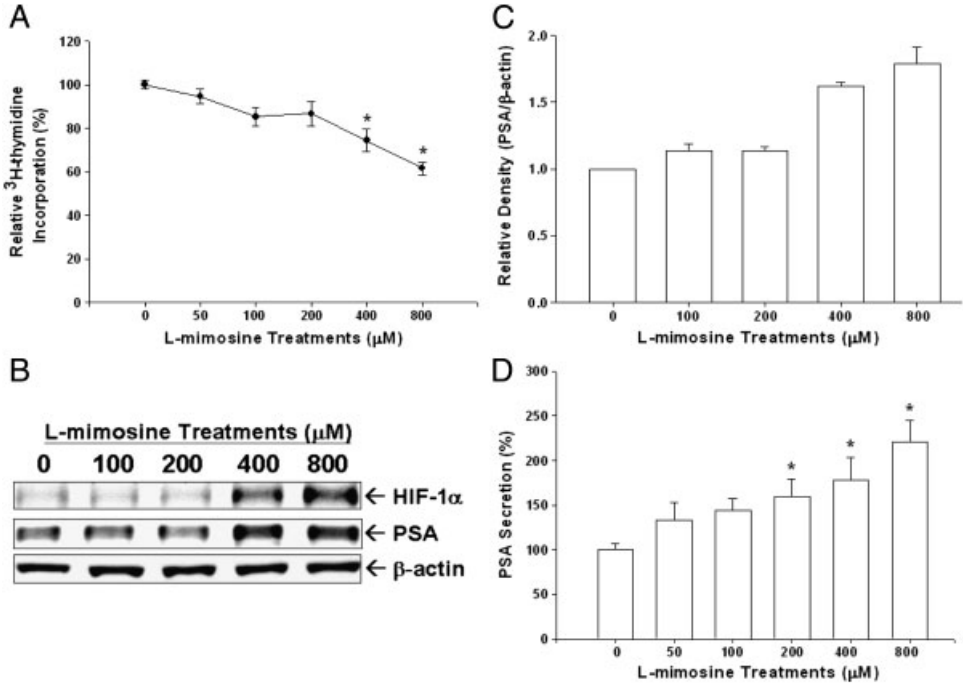

Figure 2. L-mimosine modulates the cell proliferation and gene expression of PSA in LNCaP cells. (A) Cells were treated with varying concentrations of L-mimosine as indicated for 24 h and the cell proliferation was determined by the ^{3}H-thymidine incorporation assay. Each point on the curve represents the mean percentage ± SE ($n = 4$) relative to the control solvent-treated group (0 μM of L-mimosine treatment) (*$p < 0.01$). (B) Cells were treated with varying concentrations of L-mimosine as indicated for 24 h. Cells were lysed and the expressions of HIF-1α, PSA and β-actin were determined by immunoblot assay. (C) The quantitative analysis was done by determining the intensity of each band for PSA and β-actin from three independent experiments. Data are presented as the fold-induction (± SE; $n = 3$) of the relative density of the PSA/β-actin (± SE) in relation to the control solvent-treated group. (D) Cells were treated with varying concentrations of L-mimosine as indicated for 24 h. The conditioned media were collected for PSA assay. Data are expressed as the mean percent ± SE of six preparations relative to the control solvent-treated group (*$p < 0.01$).

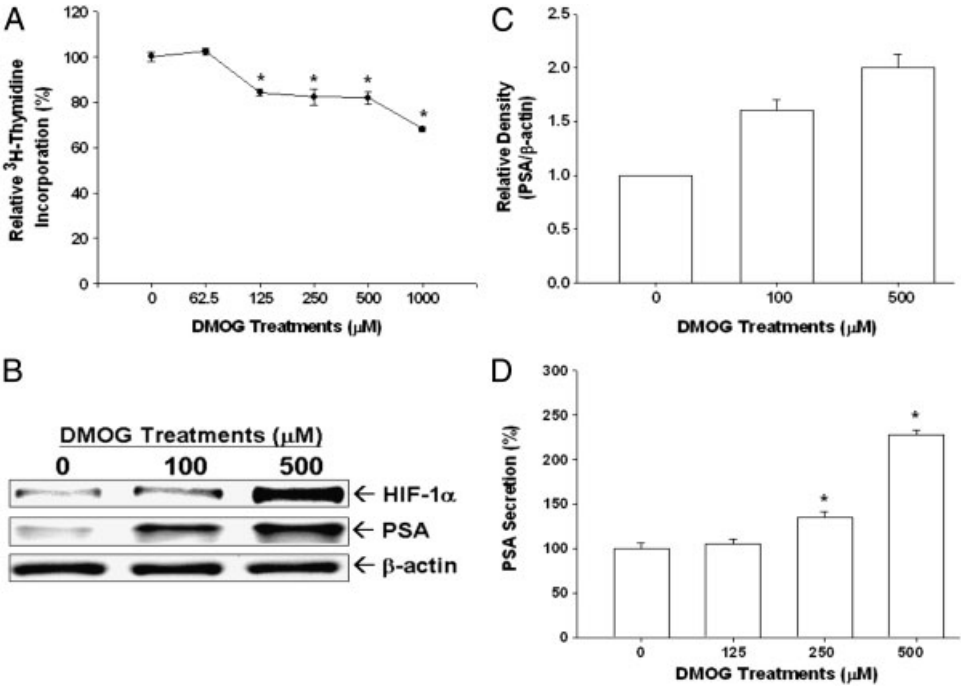

Figure 3. DMOG modulates the cell proliferation and gene expression of PSA in LNCaP cells. (A) Cells were treated with varying concentrations of DMOG as indicated for 24 h and the cell proliferation was determined by the ^{3}H-thymidine incorporation assay. Each point on the curve represents the mean percentage ± SE ($n = 4$) relative to the control solvent-treated group (0 μM of DMOG treatment) ($^*p < 0.01$). (B) Cells were treated with varying concentrations of DMOG as indicated for 24 h. Cells were lysed and the expressions of HIF-1α, PSA and β-actin were determined by immunoblot assay. (C) The quantitative analysis was done by determining the intensity of each band for PSA and β-actin from three independent experiments. Data are presented as the fold-induction (± SE; $n = 3$) of the relative density of the PSA/β-actin (± SE) in relation to the control solvent-treated group. (D) Cells were treated with varying concentrations of DMOG as indicated for 24 h. The conditioned media were collected for PSA assay. Data are expressed as mean percent ± SE of four preparations relative to the control solvent-treated group ($^*p < 0.01$).

FAC, a cell-permeable iron donor (Fig. 4A). The results of the quantitative analysis are presented in Fig. 4B. Similar results were also found in the transient gene expression (Fig. 4C) and ELISA (Fig. 4D) assays indicating that FAC blocked the inducing effect of L-mimosine on PSA gene expression.

3.5 Effect of hypoxia on PSA expression depends on the synergy of HIF-1α and AR on the enhancer region of the PSA gene

Transient gene expression assays indicated that transient overexpression of HIF-1α under normoxia induced PSA promoter activity when using the reporter vector (pPSABHE) containing the promoter (−41 to −589) and enhancer (−4801 to −3933) regions of the PSA gene. However, HIF-1α did not increase the promoter activity of PSA when cotransfected with the reporter vector (pPSABH), which only contains the promoter (−41 to −589) region (Fig. 5A). Further transient gene expression assays revealed that PSA promoter activity was upregulated 1.9- to 2-fold when the cells were treated with 200–400 μM of L-mimosine; however, these results were only found in the pPSABHE reporter vector but not in the pPSABH reporter vector (Fig. 5B). Hypoxia enhances the PSA protein level, while chetomin, a p300 inhibitor, attenuated PSA gene expression by hypoxia (Fig. 5C). The transient gene expression assay using the AR-null prostate carcinoma PCJ cells indicated that transient overexpression of HIF-1α had only a small but not significant effect on the PSA promoter activity. Transient overexpression of AR induced PSA promoter activity by 2.3-fold and the cotransfected AR and HIF-1α enhanced PSA promoter activity by 3.8-fold. AR enhancing the effect of HIF-1α on PSA promoter activity expression is also dependent on the enhancer region (−4801 to −3933) of 5′-flanking of the PSA gene. Moreover, curcumin blocked the activation of transient overexpression of AR and/or HIF-1α on the PSA promoter activity in the PCJ cells (Fig. 5D).

3.6 Curcumin blocks the increases of hypoxia and prolyl 4-hydroxylase inhibitors on PSA expression

The immunoblot assay revealed that curcumin blocks the increase of hypoxia on PSA expression (Fig. 6A, top). The

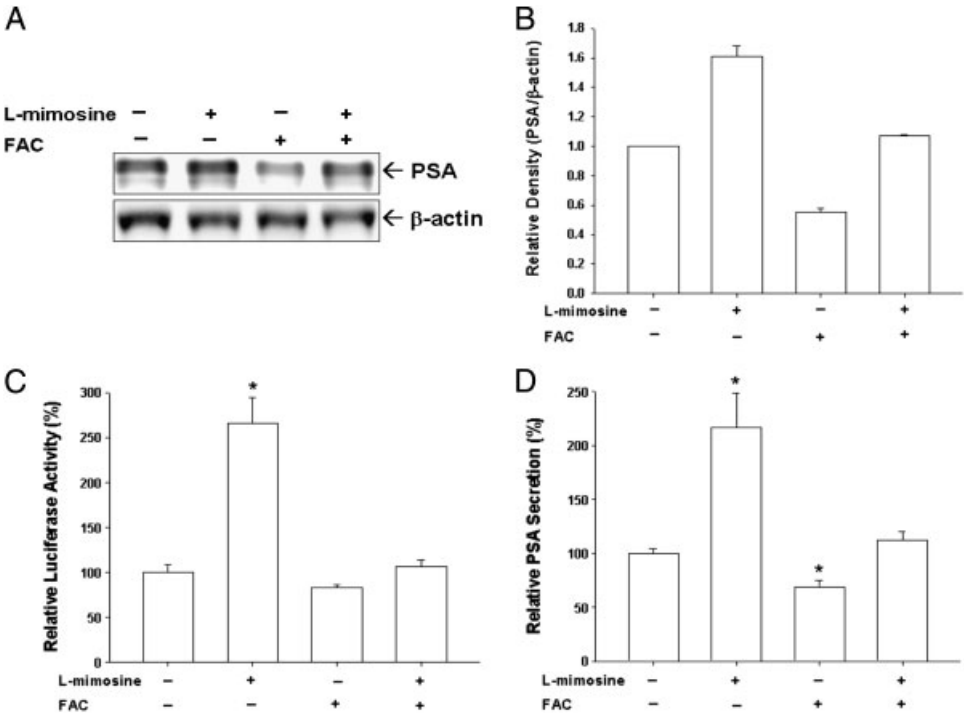

Figure 4. The L-mimosine-enhanced gene expression of PSA is due to iron chelation. (A) Cells were treated with L-mimosine (400 μM) and/or FAC (100 μg/mL) for 24 h. Cells were then lysed and the expressions of PSA and β-actin were determined by immunoblot assay. (B) The quantitative analysis was done by determining the intensity of each band for PSA and β-actin from three independent experiments. Data are presented as the fold-induction (±SE; $n = 3$) of the relative density of the PSA/β-actin (±SE) in relation to the control solvent-treated group. (C) The PSA promoter-reporter vector (pPSABHE)-transfected LNCaP cells were treated with L-mimosine (400 μM) and/or FAC (100 μg/mL) for 24 h. Data are expressed as the mean percent±SE of the stimulations of the PSA reporter activity levels induced by L-mimosine and/or FAC treatments relative to the control solvent-treated group (*$p < 0.01$). (D) Cells were treated with L-mimosine (400 μM) and/or FAC (100 μg/mL) for 24 h. The conditioned media were collected for PSA assay. Data are expressed as the mean percent±SE of four preparations relative to the control solvent-treated group (*$p < 0.01$).

quantitative analysis indicated that cotreatment with curcumin (10 μM) decreased the protein levels of HIF-1α and the HIF-1α downstream gene, VEGF, minimally but not significantly ($p = 0.33$), while hypoxia treatment stabilized the HIF-1α and significantly ($p = 0.01$) increased protein levels of VEGF. Hypoxia treatment did not significantly ($p = 0.5$) affect the protein abundance of the AR but curcumin significantly ($p = 0.001$ and $p = 0.015$, respectively) decreased protein level of AR when the cells were cultured under both normoxia and hypoxia conditions. The AR protein levels were decreased 31 and 26%, respectively, after cotreatment with curcumin for 24 h under the normoxia and hypoxia conditions. The PSA protein level was upregulated 1.8-fold under the hypoxia condition, and the increase was blocked after cotreatment with curcumin for 24 h (Fig. 6A, bottom).

The results of the immunoblot assays revealed that the curcumin treatment also blocked the increasing of L-mimosine or DMOG on the PSA protein level in the LNCaP cells (Fig. 6B). The curcumin treatment significantly ($p < 0.01$) blocked the AR protein abundance of the LNCaP cells when combined with L-mimosine or DMOG treatments. Although the L-mimosine or DMOG treatment stabilized the HIF-1α protein and induced the protein levels of VEGF, cotreatment with curcumin did not significantly block the activation effect on the protein levels of HIF-1α ($p = 0.48$ and $p = 0.43$, respectively) and VEGF ($p = 0.94$ and $p = 0.61$, respectively) in the LNCaP cells. The quantitative analysis indicated that the PSA protein level was upregulated 2.4- and 2.1-fold after the L-mimosine or DMOG treatments, respectively, and decreased 53 and 52% after cotreatment with curcumin for 24 h, respectively. Interestingly, the AR protein level increased minimally but not significantly (1.3-fold ($p = 0.09$) and 1.2-fold ($p = 0.1$), respectively) after the L-mimosine or DMOG treatments, but decreased (61 and 67%, respectively) after cotreatment with curcumin for 24 h (Fig. 6B, bottom). The transient gene expression (Fig. 6C) and ELISA assays (Fig. 6D) also showed similar results indicating that curcumin blocked the activation of L-mimosine or DMOG on the PSA expression.

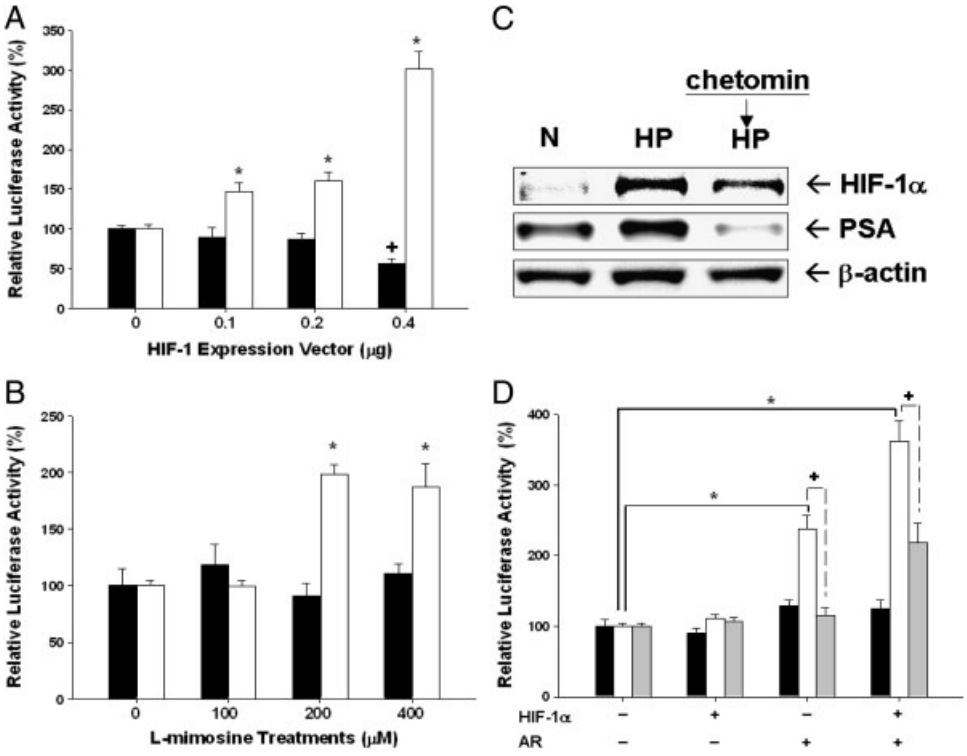

Figure 5. The synergy of HIF-1α and AR by hypoxia is dependent on the enhancer region of the 5′-flanking of the PSA gene. (A) The PSA reporter vectors, pPSABH (−598 to −41; black bars), or pPSABHE (−4801 to −3933 and −41 to −589; white bars) were cotransfected with different concentrations of the HIF-1α expression vector, as indicated, into the LNCaP cells for 72 h. The experimental data are presented as the mean percent ± SE of six preparations of the luciferase activity in relation to the groups cotransfected with 0 μg of HIF-1α (+, $^*p<0.01$). (B) The pPSABH-transfected LNCaP cells (black bars) or the pPSABHE-transfeced LNCaP cells (white bars) were treated with 0–400 μM of L-mimosine for 24 h. Data are expressed as the mean percent ± SE of the stimulations of the PSA reporter activity levels induced by L-mimosine treatments relative to the control solvent-treated group ($^*p<0.01$). (C) Cells were treated with or without 50 nM of chetomin and then cultured under normoxia (N) or hypoxia (HP) for 24 h. Cells were then lysed and the expression of HIF-1α, PSA and β-actin were determined by immunoblot assay. (D) The pPSABH reporter vector was cotransfected with 0.2 μg of the HIF-1α expression vector and/or the AR expression vector into PCJ cells (black bars). The pPSABHE reporter vectors were cotransfected with 0.2 μg of the HIF-1α expression vector and/or the AR expression vector into PCJ cells, and the cells were then treated with (gray bars) or without (white bars) 10 μM of curcumin. (+, $^*p<0.01$).

4 Discussion

Prostate cancer is the most prevalent cancer and the second leading cause of cancer death in American men [25]. PSA is now the most widely used serum marker for detecting and monitoring prostate cancer [8]. Although there is no direct report indicating a significant correlation between hypoxia and PSA levels in vivo, the present study showed that hypoxia induced PSA gene expression and secretion in vitro. The results of the enhancement of PSA gene expression after hypoxic treatment are in agreement with the results of other previous in vitro studies [14, 15]. Our study also showed that treatment with either L-mimosine or DMOG stabilized the HIF1-α protein, which upregulated the PSA protein levels in the LNCaP cells.

Prolyl hydroxylation is important in the degradation of HIF-1α under the normoxic condition. Both L-mimosine and DMOG may exert effects by acting as hyoxia mimetics, inhibiting activity of prolyl 4-hydroxylase [26]. Results from the present study indicated that L-mimosine inhibited the cell proliferation of the LNCaP cells, which is in agreement with earlier reports indicating that the number of G0/G1 cells was elevated after incubation with L-mimosine in the human prostate carcinoma DU145 cells [17]. Our results showed that DMOG stabilized HIF-1α in the LNCaP cells and are in agreement with a previous study, which indicated that DMOG induced the accumulation of HIF-1α protein in the DU145 cells [27]. The present study indicated that 1 mM of DMOG significantly blocked 25% of the ^{3}H-thymidine incorporation in LNCaP cells after treatment for 24 h. Similar results were also found in another study, which indicated that prolonged treatment with 1 mM of DMOG for 44 h induced cell death in prostate carcinoma PC3AR$^+$ cells [28].

Additionally, our results indicated that chetomin treatment suppressed hypoxia-induced PSA expression. Chetomin is a small molecule that disrupts the CH1 domain of p300, precluding its interaction with HIF-1α and thus the subsequent transcriptional activity [29]. The results of various

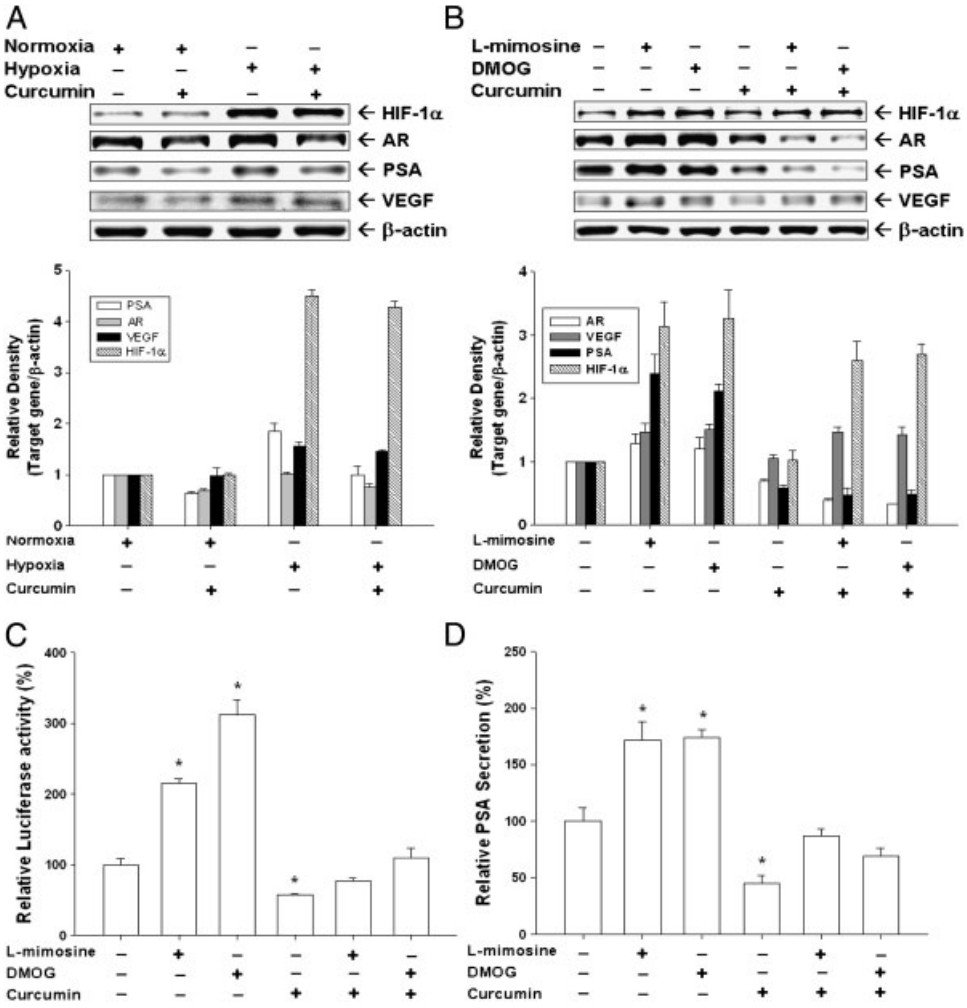

Figure 6. Curcumin blocks the increasing of hypoxia and prolyl 4-hydroxylase inhibitors on PSA expression. Cells were treated with or without 10 μM of curcumin and then cultured under hypoxia (1% O_2) or normoxia (21% O_2) for 24 h. The cells were then lysed and the expressions of HIF-1α, PSA, VEGF and β-actin were determined by immunoblot assay (A, top). The quantitative analysis was done by determining the intensity of each band for target genes and β-actin from three independent experiments. Data are presented as the fold-induction (±SE; $n = 3$) of the relative density of the target genes/β-actin (±SE) of treatments in relation to the normoxia treatment (A, bottom). Cells were treated with L-mimosine, DMOG and curcumin as indicated for 24 h. The cells were then lysed and the expressions of HIF-1α, PSA, VEGF and β-actin were determined by immunoblot assay (B, top). The quantitative analysis was done by determining the intensity of each band for target genes and β-actin from three independent experiments. Data are presented as the fold-induction (±SE; $n = 3$) of the relative density of the target genes/β-actin (±SE) of treatments in relation to the solvent control group (B, bottom). (C) The promoter activity of the pPSABHE-transfected LNCaP cells after treatment with L-mimosine (400 μM), DMOG (500 μM) and/or curcumin as indicated for 24 h. Data are expressed as the mean percent±SE of stimulations of the PSA reporter activity levels induced by different treatments relative to the control solvent-treated group (*$p < 0.01$). (D) Cells were treated with L-mimosine, DMOG and/or curcumin as indicated for 24 h. The conditioned media were collected for PSA assay. Data are expressed as mean percent±SE of four preparations relative to the control solvent-treated group (*$p < 0.01$).

transient gene expression assays demonstrated the effect of L-mimosine or hypoxia on PSA gene expression is dependent on the DNA fragment located between −4801 and −3933 upstream of the translation initiation site of the PSA gene, which in agreement with a previous study suggesting a putative hypoxia response element located in the 5′-flanking region (−3951 to −3947) of the PSA gene [15]. In combination with our results, this suggests that hypoxia-enhanced PSA gene expression is dependent on the putative HIF-1α response element on the enhancer region of the PSA gene. Moreover, we used the PCJ cells, a subculture cell line of prostate carcinoma PC-3 cells selected by the PSA reporter assay [22], to conduct the transient gene expression assay using the PSA reporter vector. Our results revealed that AR synergy with HIF-1α induced PSA expression and indicated that hypoxia-induced PSA gene expression is also

AR-dependent. Another study using the PC-3 cells also showed similar results [15]. In their transient gene expression assay using the PSA specific reporter vector, they showed that overexpression of HIF-1α or AR increased the PSA promoter activity; moreover, AR enhanced the effect of HIF-1α on PSA promoter activity with or without androgen stimulation. In the present study, our research indicated that curcumin has little impact on the HIF-1α protein levels under the hypoxia or treatment of the prolyl hydroxylase inhibitors, but significantly blocks the protein expression of AR in the LNCaP cells. In combination with other studies [5, 6, 14, 30], the results of this study suggest AR is the major factor involved in the induction of PSA expression by hypoxia; and the decrease in AR protein levels by curcumin attenuated the increasing effect of hypoxia on PSA expression, even without affecting the HIF-1α.

Curcumin, (diferuloylmethane; also called turmeric, haldi or haridra in the East and curry powder in the West), the yellow pigment in Indian saffron, is a commonly used spice and coloring agent in food. It has a variety of beneficial biological effects, has been consumed by people for centuries as a dietary component and has been shown to possess potent in vitro and in vivo antitumor promotion in several cancer cells including prostate [31–34]. Several studies have indicated that PSA is a molecular target of curcumin in the prostate. The function of curcumin is to enhance AR degradation that blocks AR activation and downregulated PSA expression in vitro and in vivo [6, 7, 35].

One previous study indicated that treatment of prostate carcinoma PC3 cells with curcumin or EF24, a novel curcumin analog, led to the inhibition of HIF-1α protein levels and, consequently, inhibition of HIF transcriptional activity [36]. Another study using HepG2 cells indicated that curcumin treatment decreased the protein levels of HIF-1α and VEGF [37]. In their studies, a significant decrease in the protein levels of HIF-1α or VEGF was only found in high dosage ($>25\,\mu M$) curcumin treatments. However, our study indicated that curcumin has only a miminal impact on the effects of hypoxia or prolyl hydroxylase inhibitors on the HIF-1α protein levels and the expression of HIF-1α-regulated gene, VEGF, in the LNCaP cells. The different results may be due to different cell types or the lower dosage of curcumin ($10\,\mu M$) used in the present study.

The results of the immunoblot assay using whole cell lysis indicated that hypoxia has only a minimal effect on AR expression. These results are in agreement with a previous report, which indicated that hypoxia increases AR activity but not AR expression [14]. Our results also showed that L-mimosine-induced PSA gene expression was iron-dependent, since increases in its effect were blocked by cotreatment with FAC, an iron donor. A previous study indicated no significant correlation between serum iron levels and prostate cancer [38]. However, another study indicated that high serum ferritin levels were observed more often in normal men than in prostate cancer patients with significantly higher concentrations of PSA, suggesting a negative correlation between body iron status and the risk of prostate cancer [39]. The regulator of intracellular iron on PSA gene expression is still unknown and requires further investigation.

Three important essays derived from the results of the present study are summarized as follows: (i) Hypoxia-induced PSA expression is both HIF-1α- and AR-dependent. Curcumin treatment decreases the protein levels of AR but not HIF-1α, which blocks the activation of hypoxia on PSA expression. (ii) Prolyl 4-hydroxylase inhibitor, L-mimosine and DMOG, mimic the hypoxia that upregulates PSA gene expression in prostate carcinoma cells in vitro. This phenomenon suggests that pharmacists, who want to develop prolyl 4-hydroxylase inhibitors as antitumor prodrugs for prostate cancer, should be aware of the side effect of inducing PSA expression and the supplement of curcumin can prevent this side effect from prolyl 4-hydroxylase inhibitors used as antitumor treatments for prostate cancer. (iii) Low-dosage curcumin treatment does not block the inducing of HIF-1α and VEGF by prolyl 4-hydroxylase inhibitors, but decreases the protein level of AR that blocks PSA gene expression. This phenomenon suggests that a low dosage of curcumin would block PSA expression but would not affect the therapeutic applications of prolyl hydroxylase inhibitors on myocardial ischemia, cerebral ischemia, anemia and kidney disease.

In conclusion, curcumin either from diet or from food supplement is important for preventing prostate disease. Our study indicates that curcumin treatment reduces the expression of PSA, a tumor maker of prostate cancer, under the hypoxia micro-environment.

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