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Effects of platelet-activating factor and its differential regulation by androgens and steroid hormones in prostate cancers

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Background: Platelet-activating factor (PAF) is an arachidonic acid metabolite that plays an important role in cell proliferation, migration and neangiogenesis, but whether it is involved in the progression of prostate cancer remains undiscovered.

Methods: Clinical prostate specimens were investigated with immunohistochemistry method and *in vitro* cell experiments referred to MTS cell proliferation assay, invasion and migration experiment, quantitative real-time RT-PCR assay, western blotting analysis and ELISA assay.

Results: Platelet-activating factor synthetase, lyso-PAF acetyl transferase (LPCAT1), increased significantly in castration-resistant prostate cancer (CRPC) specimens and CRPC PC-3 cells than that in controls. Intriguingly, PAF induced invasion and migration of PC-3 cells but not LNCaP cells. The PAF receptor antagonist inhibited proliferation of LNCaP and PC-3 cells. Dihydrotestosterone (DHT) treatment caused a decrease in LPCAT1 expression and PAF release in LNCaP cells, which could be blocked by androgen receptor antagonists. Finally, DHT increased LPCAT1 expression and PAF release in PC-3 cells in a Wnt/ β -catenin-dependent manner.

Conclusion: For the first time, our data supported that PAF might play pivotal roles in the progression of prostate cancer, which might throw a new light on the treatment of prostate cancer and the prevention of the emergence of CRPC.

Prostate cancer is the most common malignancy in American men and the second leading cause of male cancer mortality. In 2012, prostate cancer was expected to account for 29% (241 740) of all newly diagnosed cancers and 9% (28 170) of all male cancer deaths (Siegel *et al*, 2012). The development and progression of prostate cancer are initially androgen dependent. Hence, androgen deprivation therapy (ADT) is generally employed to treat advanced or metastatic prostate cancer. However, prolonged androgen deprivation generally results in relapse and androgen-independent tumour growth (Dreicer *et al*, 2011). There is evidence that these castration-resistant tumours continue to depend on AR signalling, which may remain activated despite castrate levels of androgens

and be responsible for tumour progression (Grasso *et al*, 2012). It is not well known, however, whether the responses of target genes to androgen are changed from androgen-dependant prostate cancer (ADPC) to castration-resistant prostate cancer (CRPC). Also, no effective treatment strategy for CRPC is now available in clinical practice.

The arachidonic acid (AA) pathway is a well-studied biological pathway that starts in cells at the plasma membrane of cellular organelles, where it is liberated by the action of PLA₂. Subsequently, AA is converted by COX to produce biologically active eicosanoids, prostaglandins. It is well established that PGE₂, PGF_{2 α} and thromboxane-2, the three main products generated by

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COX-1 and COX-2, may contribute to prostate cancer tumourigenesis via induction of cell proliferation, angiogenesis, invasion and metastasis (Jain *et al*, 2008; Nie *et al*, 2008). However, unwanted side effects of COX inhibitors hampered its widespread use in the prevention of prostate cancer. Targeting other eicosanoids pathways during prostate cancer progression may represent an attractive new strategy for prostate cancer treatment.

Platelet-activating factor (PAF), a remodelling product of AA pathway, is converted by the enzyme acetyl coenzyme A: lyso-PAF acetyl transferase (LPCAT) from lyso-PAF, an immediate precursor catalysed by cPLA₂. Platelet-activating factor then binds to PAF receptors and ultimately leads to the activation of gene expression, stimulating the PLA₂ activities, calcium mobilisation, tyrosine phosphorylation and the generation of cyclic adenosine monophosphate (Jan and Chao, 2004). It has also been reported to be increased in a number of cancers, including human thyroid cancer, meningiomas, breast cancer and oesophageal cancer (Wang *et al*, 2002; Sato *et al*, 2010; Yu *et al*, 2012). It promotes migration, proliferation and neoangiogenesis in breast cancer cells (Bussolati *et al*, 2000). The activity of PAF was demonstrated in tissues of the prostate of rats (Muguruma *et al*, 1993). However, there have been no reports of PAF activity in normal human prostate tissue, human prostate cancer tissue or prostate cancer cell lines to date. In this study, we investigated whether PAF promotes proliferation and migration of prostate cancer cells and the regulation of LPCAT1 expression and PAF production by androgen and other steroid hormones in ADPC and CRPC cells.

MATERIALS AND METHODS

Materials. Dihydrotestosterone (DHT), flutamide, 17 β -oestradiol, progesterone, RU486, MPP, cyclofenil, H89, chelerythrine chloride (CHE), wortmannin, triciribinehydrate, PAF and ABT-491 were obtained from Sigma Chemical Co. (St Louis, MO, USA). Dihydrotestosterone-carboxymethylxime (CMO)-BSA was obtained from Steraloids (Newport, RI, USA). The PAF receptor polyclonal antibody was purchased from Cayman Chemical Co. (Ann Arbor, MI, USA). The LPCAT1 polyclonal antibody was purchased from Proteintech Group, Inc. (Chicago, IL, USA). Matrigel was purchased from BD Biosciences (Bedford, MD, USA). Transwell plates were obtained from Corning Inc. (Corning, NY, USA). The LNCaP and PC-3 cells were obtained from the American Type Culture Collection (The Institution of Biochemistry and Cell Biology, the Chinese Academy of Sciences, Shanghai, China).

Patients and samples. Study protocols involving human materials were approved by the institutional ethic committee of Changhai Hospital. Fresh prostate specimens were obtained immediately after radical prostatectomy of 12 localised prostate cancer cases. All patients had no prior long-standing ADT or anti-inflammatory treatment. Specimens were thickly sliced immediately. Samples cut from suspected cancerous areas and from apparently tumour-free areas were handled as previous described (Enayetallah *et al*, 2006), frozen in liquid nitrogen and stored at -80°C . Tumour tissues were dissected and separated from the frozen samples after a section of the cancer was pathologically confirmed, and only tumour cell-free samples were included as corresponding non-tumour tissues. Standard histopathological diagnosis, and clinical staging based on the TNM system and Gleason scores, were assessed for each patient. All of them belonged to the stage of PT_{1c-2}N₀M₀ and the Gleason grade ranged from 6 to 9. Nine paired frozen tumour and nontumour samples were homogenised in Trizol reagent (Invitrogen, Carlsbad, CA, USA), and total RNA was extracted and analysed using real-time PCR. Six CRPC specimens derived from TURP (transurethral resection of prostate), and their matched ADT-naive ADPC specimens derived from biopsy,

were obtained from the archives of the Department of Pathology, Changhai Hospital. The 6 CRPC patients received ADT for a median time of 15 months before TURP. Clinicopathologic details of all 18 cases are described in Supplementary Table 1.

Immunohistochemistry. Immunohistochemical stain of LPCAT1 was performed as previously described (Xu *et al*, 2009). The sections were incubated with LPCAT1 antibody (1:100) and were detected with the biotin-streptavidin-peroxidase system (MaiXin Biotechnology, Fuzhou, China) using diaminobenzidine (Sigma-Aldrich, St Louis, MO, USA) as chromogen. Counterstaining was performed with haemalum.

Cell proliferation and invasion. LNCaP and PC-3 cells were cultured in RPMI-1640 medium containing 10% fetal calf serum, 100 units of penicillin per ml and 100 mg of streptomycin per ml at 37°C in a humidified atmosphere of 5% CO₂. Cell proliferation was determined using MTS Cell Proliferation Assay System (Promega, Madison, WI, USA) according to the manufacturer's instruction. Cells in each well received treatment with increasing concentrations of PAF and PAF antagonist ABT-491, or combinations of these agents. Controls received vehicle alone. After 96 h of incubation, 20 μl of CellTiter 96 Aqueous One Solution Reagent (Promega) was added into each well of the 96-well assay plate containing the samples in 100 μl of culture medium. The plate was incubated at 37°C for 4 h and absorbance was recorded at 490 nm using a 96-well plate reader (Bio-Rad, Hercules, CA, USA).

A modified Boyden chamber was used to measure prostate cancer cell invasion. Briefly, Matrigel was thawed overnight at 4°C and diluted 1:50 into RPMI-1640 medium (phenol red-free). The solution (100 μl) was added to the upper surface of Transwell membrane inserts and was allowed to solidify in a sterile environment for 24 h. The PC-3 or LNCaP cells (10^5 cells per ml) were suspended in RPMI-1640 medium and pretreated with the inhibitor ABT-491 (10^{-5}M) for 30 min. Control cultures were treated with equivalent amounts of vehicle. Cells were then plated onto the surface of the inserts in a total volume of 100 μl . The RPMI-1640 medium supplemented with 10^{-9} – 10^{-6}M PAF (400 μl) was added to the lower chamber. Cells were incubated at 37°C for 24 h, followed by a 30-s fixation in 100% methanol, and stained for 3 min by incubating with DAPI. The wells were then counted by using an inverted Olympus IX-55 microscope (Olympus, Tokyo, Japan). A total of 10 high-power ($\times 200$) fields were recorded per insert. Migration assay was conducted with the non-matrigel-coated transwells using the same method. The number of cells migrating was calculated as the mean count per high-power field.

Cell cytotoxicity assay. For assessment of cytotoxicity, lactate dehydrogenase (LDH) release into the extracellular medium was measured using the CytoTox 96 Non-Radioactive Cytotoxicity Assay Kit (Promega), following the manufacturer's instructions.

Quantitative real-time RT-PCR. Following different treatments, total RNA was extracted and quantified by quantitative real-time RT-PCR as previously described (Xu *et al*, 2009). Specific primers for the amplification were 5'-AAAGCTGACCCACCCAATAAC-3' and 5'-CCAATCAGAGGGACTTGTGC-3' for LPCAT1 (accession number NM_017839); and 5'-TGGCACCCAGCACAATGAA-3' and 5'-CTAAGTCATAGTCCGCCTAGAAGCA-3' for β -actin. Real-time PCR was carried out using Rotor-Gene 3000 (Corbett Research, Sydney, Australia) with $\Delta\Delta\text{Ct}$ method, according to the manufacturer's instructions. PCR reaction was set at 94°C (20 s), 60°C (30 s) and 72°C (30 s) in a total of 40 cycles with a final extension step at 72°C for 5 min.

Western blotting analysis. Protein expression levels were determined by western blotting as described before (Xu *et al*, 2009), in

which membranes were incubated with anti-PAF receptor antibody (1:200 dilution in TBST with 5% non-fat milk) or anti-LPCAT1 antibody (1:1000 dilution in TBST with 5% non-fat milk). The intensities of light-emitting bands were detected and quantified using Sygene Bio Image system (Synoptics Ltd, Cambridge, UK). The relative protein expression level was expressed as a ratio over β -actin.

ELISA analysis of PAF. Platelet-activating factor released into the cell culture media was measured by a ELISA Kit (Westang Biotech, Shanghai, China) according to the manufacture's protocol.

RNA interferences. For knockdown of β -catenin gene, sequence-specific siRNA targeting human β -catenin (sense 5'-UUUCAAACAGUUGUAUGGUA-3' and antisense 5'-CCAUACAACUGUUUUGAAAAU-3') was designed and synthesised by Gene Pharma (Shanghai, China). The following siRNA sense 5'-UUCUCCGAACGUGUCACGUTT-3'; and antisense 5'-ACGU GACACGUUCGGAGAATT-3' were used as a control. The cultured PC-3 cells were transfected with siRNA- β -catenin and siRNA-negative control using Lipofectamine 2000 (Invitrogen). The cells were then treated with DHT. Production of PAF was measured by ELISA as mentioned above.

Statistics. For illustrative purposes, the results are presented as the mean percent control \pm s.e.m. One-way ANOVA followed by the Student–Newman–Keuls test was used to assess significant differences between each two groups. Significance was set at $P < 0.05$.

RESULTS

Identification of LPCAT1 in clinical specimens. Nine localised prostate cancer tissue specimens from patients undergoing radical prostatectomy were immunostained with LPCAT1 antibody. Positive staining was located mainly in cytoplasm of cancerous cells, and was stronger than normal prostate cells (Figure 1A and B).

The mRNA expression of LPCAT1 was also significantly higher in tumour samples than in the paired nontumour samples (Supplementary Figure 1). To investigate the potential biological and clinical role of LPCAT1 in prostate cancer from ADPC to CRPC stage, we investigated the expression level of LPCAT1 in six CRPC specimens derived from TURP, and their matched ADPC biopsy specimens. The protein expression of LPCAT1 significantly increased in CRPC specimens (5 in 6 samples) compared with their matched ADT-naive specimens (Figure 1C and D).

Effects of PAF on growth of prostate cancer cells. To further understand the potential role of PAF in the progression of prostate cancer, we determined the concentration of PAF in cultured LNCaP and PC-3 cells and found that PAF in PC-3 cells was significantly higher than that in LNCaP cells (Supplementary Figure 2). The expression of PAF receptor (PAF-R) in LNCaP cells, however, was comparable to the PC-3 cells (Figure 2A). When LNCaP and PC-3 cells were grown in the presence of increasing concentrations of PAF antagonist ABT-491 (10^{-8} – 10^{-5} M), a significant inhibition of proliferation was observed in both cells at concentrations between 10^{-7} and 10^{-5} M (Figure 2B). Interestingly, treatment with PAF (10^{-9} – 10^{-6} M) had no effect on proliferation of either LNCaP cells or PC-3 cells (Figure 2C).

Effects of PAF on mobility of prostate cancer cells. To determine whether PAF induces invasion of LNCaP and PC-3 cells through a matrix-coated membrane, cells were exposed to increasing concentrations of PAF (10^{-9} – 10^{-6} M) and PAF antagonist ABT-491 (10^{-8} – 10^{-5} M), or combinations of these agents. Invasion of PC-3 cells was increased by PAF in a concentration-dependent manner. At concentrations of 10^{-8} M, 10^{-7} M and 10^{-6} M, PAF induced a $51.4 \pm 4.37\%$, $77.6 \pm 5.92\%$ and $118.0 \pm 10.49\%$ increase in PC-3 invasion, respectively (Figure 3A). Treatment with ABT-491 inhibited tonic invasion of PC-3 cells (Figure 3B). Similarly, PC-3 cells treated by PAF also demonstrated a significant and concentration-dependent increase in migration. At concentrations of 10^{-8} M,

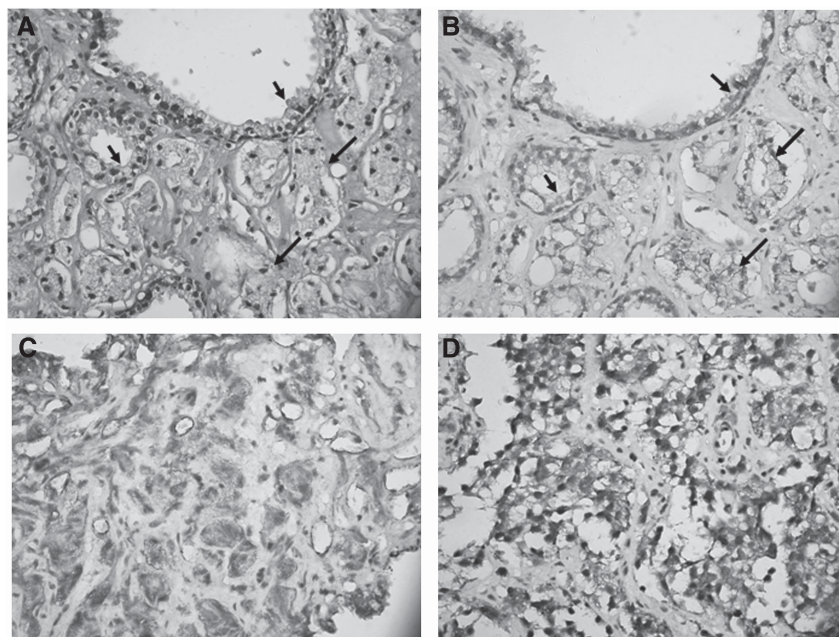


Figure 1. Immunolocalisation of LPCAT1 in human prostate cancer specimens. (A) Representative H&E staining of a clinical prostate cancer sample (Gleason score 4 + 3 = 7). (B) Protein expression of LPCAT1 in the same prostate cancer sample (Gleason score 4 + 3 = 7) by immunohistochemistry: LPCAT1 protein expression of prostate adenocarcinoma cells was strong positive (long arrow), whereas that of normal prostate gland epithelial cells was negative or weak positive (short arrow). In a paired prostate cancer sample, LPCAT1 protein expression of tumour cells significantly increased from (C) ADT-naive biopsy specimen (Gleason score 3 + 4 = 7) to (D) their matched CRPC specimen (Gleason score 4 + 4 = 8). Original magnifications $\times 400$ (A–D).

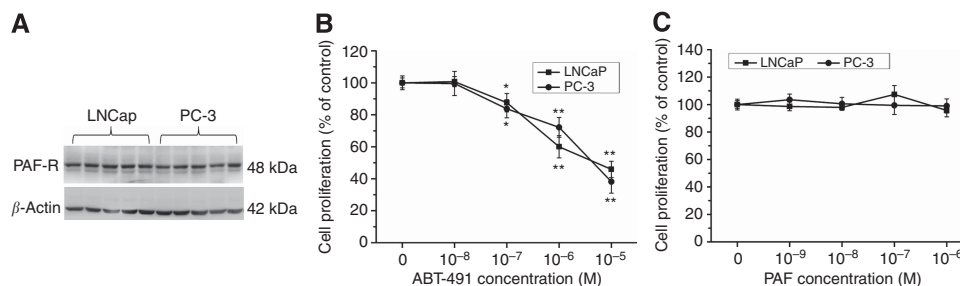


Figure 2. Effects of PAF and a specific PAF receptor antagonist ABT-491 on the proliferation of prostate cancer cells. **(A)** Expression of PAF receptor (PAF-R) in LNCaP cells and PC-3 cells was determined by western blot. **(B)** Prostate cancer cells were exposed to increasing concentrations of ABT-491. **(C)** Prostate cancer cells were treated with increasing concentrations of PAF for 24 h. Values are presented as mean percent control \pm s.e.m. of six different experiments. * $P < 0.05$ and ** $P < 0.01$ compared with control.

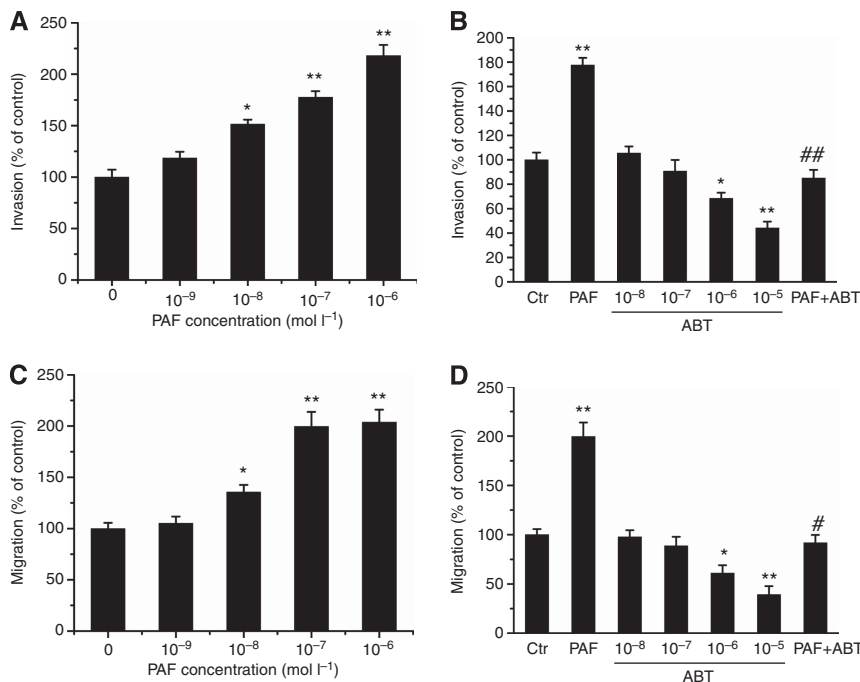


Figure 3. Effects of PAF and ABT-491 on invasion and migration of PC-3 cells. **(A, C)** Effects of increasing concentrations of PAF on invasion and migration of PC-3 cells. **(B, D)** Effects of increasing concentrations of ABT-491 on invasion and migration, and pretreatment with ABT-491 (10^{-5} M) on PAF-induced invasion/migration of PC-3 cells. Data are presented as mean percent control \pm s.e.m. for five cultures ($n = 5$) performed in triplicate. * $P < 0.05$ and ** $P < 0.01$ compared with control; # $P < 0.05$ and ## $P < 0.01$ compared with PAF treatment alone (10^{-7} M).

10^{-7} M and 10^{-6} M, PAF treatment caused an increase in migration to $135.68 \pm 6.91\%$, $199.67 \pm 14.1\%$ and $203.88 \pm 12.13\%$ of control, respectively (Figure 3C). Migration of PC-3 cells induced by PAF was likewise inhibited by the inclusion of ABT-491 (10^{-5} M; Figure 3D). In contrast, PAF did not affect invasion and migration of the androgen-dependent LNCaP cells (Supplementary Figure 3).

To confirm the significant attenuation of invasion and migration observed in PC-3 cells treated with PAF receptor antagonist ABT-491 did not result from a nonspecific loss of cellular viability, PC-3 cells were subjected to a cellular viability assay. As seen in Supplementary Figure 4, treatment of PC-3 with 10^{-5} M ABT-491 resulted in a nonsignificant loss of viability during 24 h of incubation. The decrease in viability of $< 10\%$ at each time point is unlikely to account for the 56.8% attenuation of invasion and the 60.82% attenuation of migration.

Effects of androgen and other steroid hormones on PAF synthesis and production. Both androgen-dependent LNCaP cells and CRPC-3 cells have been shown to express LPCAT1 and produce PAF endogenously. To determine whether the expression of LPCAT1 and production of PAF is regulated by androgen and

other steroid hormones, LNCaP and PC-3 cells were treated with increasing concentrations of DHT, oestrogen and progesterone, alone or with their receptor antagonists. Intriguingly, DHT resulted in a dose-dependent decrease in the mRNA and protein expression of LPCAT1 in LNCaP cells (Figure 4A), whereas it had a dose-dependent increase in PC-3 cells (Figure 4B). Maximal effects were both observed at 10^{-6} M of DHT, which decreased LPCAT1 mRNA level to $46.21 \pm 6.48\%$ and protein level to $34.37 \pm 6.14\%$ of control in LNCaP cells, and increased LPCAT1 mRNA level to $345.49 \pm 43.98\%$ and protein level to $233.71 \pm 26.41\%$ of control in PC-3 cells, respectively. The concentration of PAF released into cell culture appeared to correlate well with the decrease or increase in the expression of enzyme protein (Figure 4C and D).

Downregulation of LPCAT1 expression and PAF release by DHT in LNCaP cells was reversed by steroid receptor antagonist RU486 and specific androgen receptor antagonist flutamide (Figure 4A and C). Intriguingly, RU486 and flutamide did not antagonise the effect of DHT in PC-3 cells (Figure 4B and D), indicating that androgen may not act through the traditional steroid receptors in PC-3 cells.

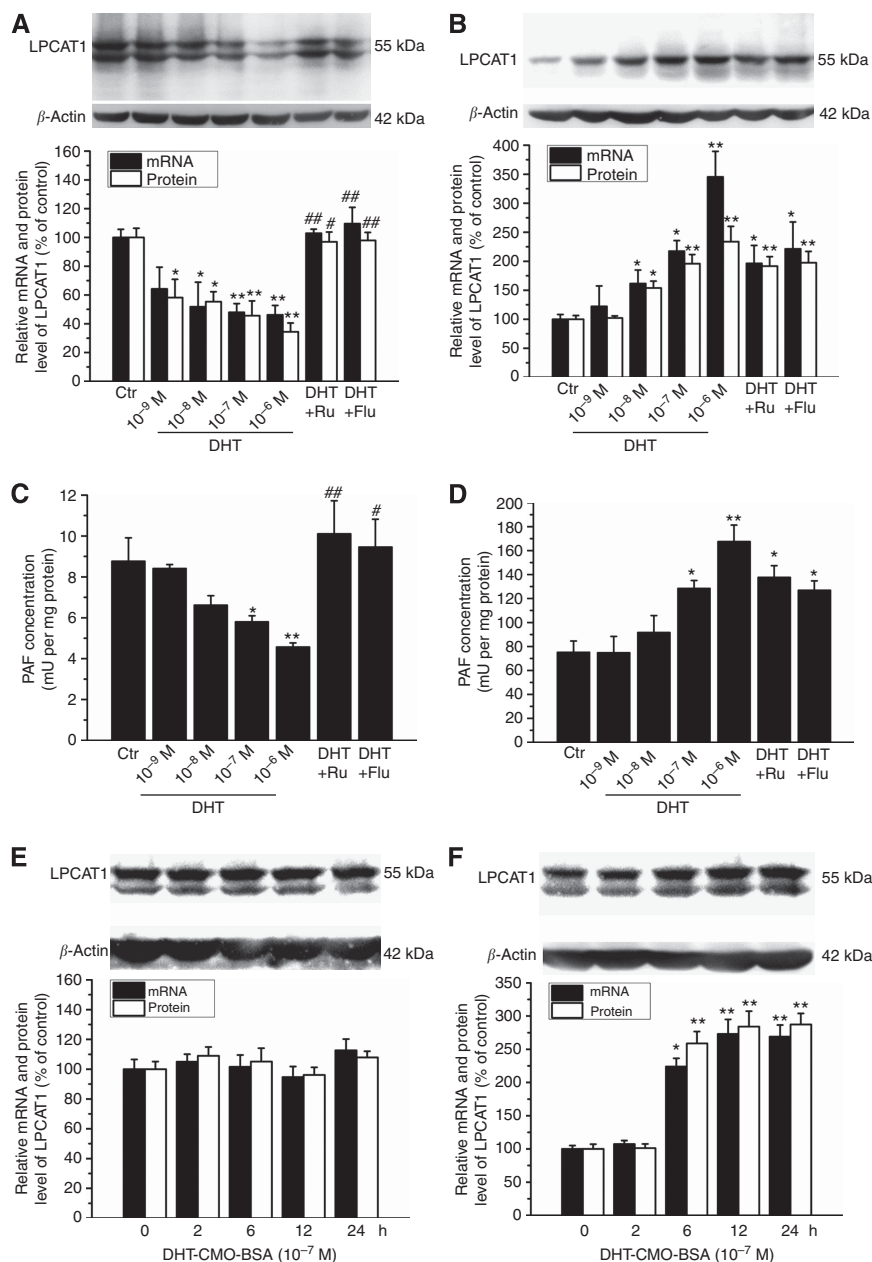


Figure 4. Effects of DHT on LPCAT1 mRNA and protein expression and PAF release in prostate cancer cells. Cells were treated with increasing concentration of DHT, and in combination of DHT (10^{-7} M) and RU486 (10^{-6} M) or flutamide (10^{-6} M) for 24 h. The mRNA and protein levels of LPCAT1 in LNCaP cells (A) and PC-3 cells (B) were measured by real-time RT-PCR and western blot analysis, respectively. Representative protein bands were presented on the top of corresponding histogram. Concentrations of PAF in LNCaP (C) and PC-3 (D) cell culture were determined by ELISA. Time course expression of LPCAT1 in LNCaP cells (E) and PC-3 cells (F) treated by DHT-CMO-BSA are also shown. Flu, flutamide; Ru, RU486. Values are presented as mean percent control \pm s.e.m. (A, B, E, F) or mean \pm s.e.m. (C, D) for five cultures performed in triplicate. * $P < 0.05$ and ** $P < 0.01$ compared with control; # $P < 0.05$ and ## $P < 0.01$ compared with DHT (10^{-7} mol/l $^{-1}$).

To further investigate this AR-independent regulation of DHT on LPCAT1 expression, DHT-CMO-BSA, in which DHT is conjugated to BSA to prevent the permeability of DHT into the cell, was used to treat LNCaP and PC-3 cells. It was shown that DHT-CMO-BSA could increase the mRNA and protein expression of LPCAT1 in PC-3 cells as early as 6 h, whereas the expression of LPCAT1 in LNCaP cells was not affected by DHT-CMO-BSA (Figure 4E and F).

Aside from the androgens, 17β -oestradiol (10^{-7} M) was found to decrease the expression of LPCAT1 and PAF release in both LNCaP and PC-3 cells, which was blocked by RU486 and oestrogen receptor (ER)- β antagonist cyclofenil but not by ER- α antagonist MPP in each cell line (Figure 5). Progesterone at a concentration of

10^{-7} M did not significantly affect LPCAT1 expression and PAF release in LNCaP and PC-3 cells (data not shown).

Wnt/ β -catenin signalling pathway is involved in androgen effects on PAF in PC-3 cells.

To investigate the possible signalling pathways mediating nontraditional genomic effects of androgen on PAF production in PC-3 cells, various protein kinase inhibitors were examined to see whether they affected DHT-induced PAF production. None of the inhibitors exhibited antagonistic effect on DHT-induced PAF production in PC-3 cells (Supplementary Table 2). Using transfection of siRNA to inhibit the expression of β -catenin, the critical molecular component of canonical Wnt signalling, significantly reversed the DHT-induced PAF production

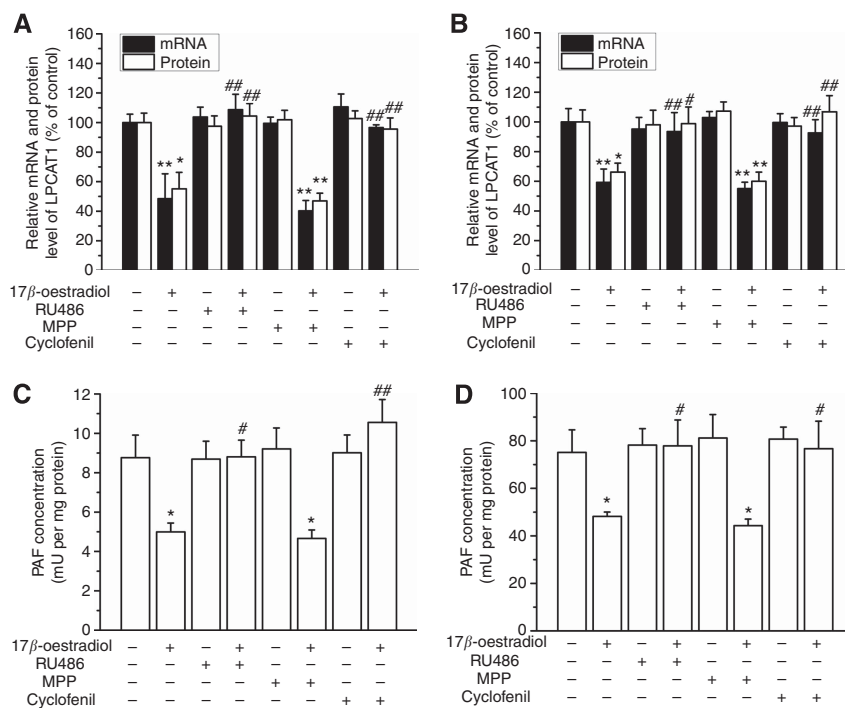


Figure 5. Effects of 17β -oestradiol on LPCAT1 mRNA and protein expression and PAF release in prostate cancer cells. Cells were treated with 17β -oestradiol (10^{-7} M) in the presence or absence of RU486 (10^{-6} M), MPP (10^{-6} M) and cyclofenil (10^{-6} M) for 24 h. (A, C) LNCaP cells and (B, D) PC-3 cells. Values are presented as mean percent control \pm s.e.m. (A, B) or mean \pm s.e.m. (C, D) for five cultures performed in triplicate. * $P < 0.05$ and ** $P < 0.01$ compared with control; # $P < 0.05$ and ## $P < 0.01$ compared with 17β -oestradiol (10^{-7} mol/l $^{-1}$).

in PC-3 cells (Figure 6A and B). Further western blot analysis confirmed that DHT could significantly promote the expression of β -catenin in PC-3 cells but not in LNCaP cells. Not so surprisingly, the elevated β -catenin in PC-3 cells could not be reversed by flutamide, the antagonist of DHT (Figure 6C).

DISCUSSION

Platelet-activating factor, a potent phospholipid second messenger found in high abundance in various human cancers, has unique pleiotropic biological properties in addition to platelet activation. This study demonstrated, for the first time, the production of PAF as well as the expression of its receptor PAF-R and synthetase LPCAT1 in human prostate cancer. The expression of LPCAT1 increased in prostate cancer tissue compared with matched tumour-free tissue, and further increased in CRPC specimens after ADT. Similarly, PAF produced by CRPC-3 cells is much higher than that produced by androgen-dependent LNCaP cells. These results suggested that endogenous PAF might exert biological functions on prostate cancer cells in a paracrine or autocrine manner, and play important roles in the transition from ADPC to CRPC.

Platelet-activating factor receptors have been identified in human breast cancer cells, pancreatic cancer cells and bladder cancer cells (Youlyouz *et al.*, 2002). In our studies, we determined first the comparable protein expression of PAF-R in both LNCaP cells and PC-3 cells. The PAF-R antagonist ABT-491 was shown to inhibit LNCaP and PC-3 cell proliferation, which is consistent with a previous discovery that specific PAF-R antagonist, Y-24180, inhibited cell proliferation in a concentration-dependent manner in prostate cancer cells (Jan and Chao, 2004). These results indicate that endogenously produced PAF mediate a tonic growth of LNCaP and PC-3 cells. The fact that exogenous PAF had no effect on proliferation of LNCaP or PC-3 cells might suggest that PAF may be a second messenger rather than a primary message involved in

signalling cascades leading to cellular proliferation. The potential exact mechanism, however, needs further research work in future.

In this study, we demonstrated that PAF signalling is directly involved in PC-3 cell invasion and migration, similar with previous conclusions in human umbilical vein endothelial cells (HUVECs) and breast cancer (Bussolati *et al.*, 2000; Axelrad *et al.*, 2004), indicating that the PAF signalling cascade alone is sufficient to induce invasion and migration. In HUVECs, PAF increases the ratio of active/inactive membrane type 1 matrix metalloproteinase (MT1-MMP) and thus results in increased activation of MMP2, the key enzyme that regulates degradation of extracellular matrix and facilitates invasion. Whether the mechanism underlying PAF-induced invasion in PC-3 cells is also related with activation of MMP2 needs further investigation. Otherwise, PAF was shown to increase COX-2 expression and its downstream substance PGE₂ production in oesophageal squamous cell carcinoma (Wang *et al.*, 2002). In the oncogenesis and angiogenesis of prostate cancer, PGE₂ plays a crucial role via a number of mechanisms, such as resulting in increased proliferation and decreased apoptosis in androgen-sensitive and -insensitive prostate cancer cells (Uotila *et al.*, 2001; Jain *et al.*, 2008; Patel *et al.*, 2008). It is interesting to further investigate if PAF could indirectly regulate prostate cancer cells proliferation and invasion via bypass way of induced COX-2 and PGE₂ production.

Despite the awareness of the important role of AR signalling in prostate cancer, the exact effect of ADT on AR signalling has not been well characterised. In this study, we expand our understanding that PAF may be a new potential signalling message involved in mechanisms underlying androgen and steroids effects on prostate cancer cells. The expression of LPCAT1 and the production of PAF were downregulated by DHT treatment and reversed by steroid receptor antagonist RU486 and specific androgen antagonist ABT-491 in serum and phenol-free (androgen free) LNCaP cultures. This suggested that androgen may exert a tonic inhibitory effect on PAF release in ADPC cells in an AR-dependent manner. Androgen deprivation therapy impairs this tonic inhibition and makes PAF increase dramatically, which offsets

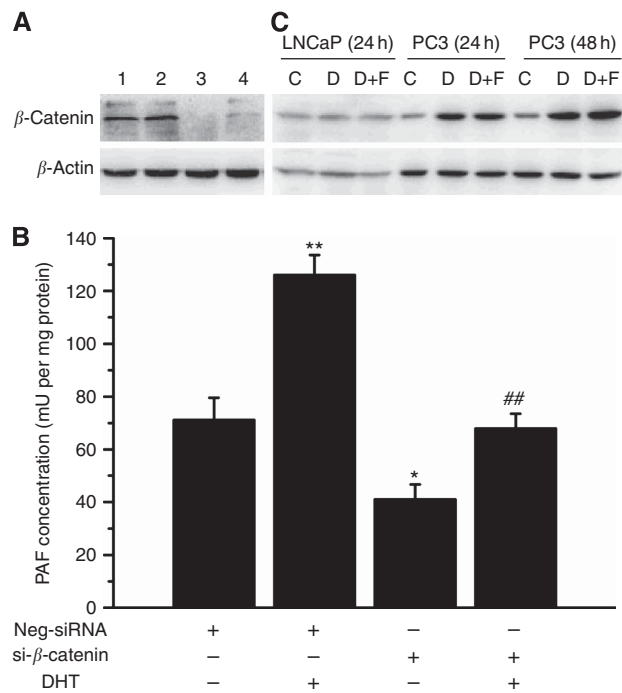


Figure 6. Dihydrotestosterone-induced PAF release in β -catenin knockdown PC-3 cells. **(A)** Representative bands for protein expression of β -catenin in cells transfected with β -catenin siRNA. (1, 2) Cells transfected with Neg-siRNA; (3, 4) cells transfected with β -catenin-specific siRNA. **(B)** Effects of β -catenin knockdown on PAF release in PC-3 cells in the presence or absence of DHT (10^{-7} M). Values are presented as mean \pm s.e.m.; $n=4$. * $P<0.05$ and ** $P<0.01$ compared with negative control siRNA; ## $P<0.01$ compared with DHT (10^{-7} M) treatment in cells transfected with negative control siRNA. **(C)** Representative bands for protein expression of β -catenin in LNCaP cells and PC-3 cells. Cells were treated with DHT (10^{-7} M) in the absence or presence of flutamide (10^{-6} M) for 24 h (LNCaP and PC-3 cells) or 48 h (PC-3 cells). C (control), 1:10 000 ethanol in PBS; D, DHT; F, flutamide.

therapeutic actions elicited by ADT, partially if not all. Meanwhile, the increasing PAF after androgen deprivation may act as an important signalling message to facilitate the transition to an aggressive phenotype of CRPC. In contrast, treatment of DHT in CRPC-3 cells increased LPCAT1 expression and PAF production in a rapid and AR-independent manner. Nongenomic actions of androgens have been previously described in prostate cancer cells. Tong and Tai (2000) have reported that ERK kinase inhibitor and tyrosine kinase inhibitor attenuated androgen-induced 15-PGDH expression that was also shown to be AR independent. The sequential responses to hormonal manipulation, known as secondary hormonal therapy, also relies on the fact that CRPC is not necessarily androgen independent and is susceptible to further hormonal manipulation (Reis, 2012). Abiraterone, a CYP17 inhibitor that effectively blocks the conversion of androgens from nongonadal precursors, thus reducing testosterone to undetectable levels, has recently been proved to prolonged overall survival among patients with metastatic CRPC even after previous chemotherapy (de Bono *et al*, 2011). Although better evidence is necessary, the present study also supports the rationale of an important role for androgen depletion, as low-level DHT can promote malignant CRPC cells progression through Wnt/ β -catenin pathway.

Androgen signalling is now known to be influenced by several other signal transduction pathways, and these points of crosstalk might be key factors of the abnormal behaviour of the prostate cells in disease states. Wnt is a complex signalling pathway whose end

point involves activation of transcription from LEF-1/TCF transcription factors and it is known to be involved in the development and progression of numerous human epithelial tumours including prostate cancer. β -Catenin protein, a particularly critical molecular component of canonical Wnt signalling, is now known to promote androgen signalling through its ability to directly bind to the AR protein and to enhance the ability of liganded AR to activate transcription of androgen-regulated genes (Truica *et al*, 2000; Pawlowski *et al*, 2002; Song *et al*, 2003; Masiello *et al*, 2004). Several different studies have reported an upregulation in the expression of Wnt ligands and β -catenin in cancer cells compared with normal prostate cells (McKiernan and Benson, 2004; Ricke *et al*, 2008). In our study, knockdown of β -catenin in PC-3 cells significantly decreased production of PAF supported this previous work, and led us to hypothesise that increase of Wnt ligands and β -catenin in prostate cancer cells may promote cell proliferation and invasion by inducing PAF production. Here we further demonstrated that β -catenin was not influenced by DHT in LNCaP cells but significantly elevated in DHT-treated PC-3 cells, which could not be reversed by the antagonist of DHT. Knockdown of β -catenin in PC-3 cells significantly decreased the production of PAF induced by DHT supported the important role of β -catenin in the regulation of DHT on PAF in PC-3 cells. These results suggested that in PC-3 cells, β -catenin interacts with AR signalling pathways on regulation of PAF, even though the effect seems to be AR independent. Androgen regulation on PAF release in a Wnt/ β -catenin-dependent pathway in CRPC stage may partially contribute to its development. However, in this study we did not find an obvious variation in the expression and activation of LEF-1/TCF, one of the validated downstream transcription factors of Wnt/ β -catenin pathway (data not shown). Further studies on the cross-talk between AR and Wnt/ β -catenin signalling pathway are needed so as to identify underlying mechanism of CRPC that may finally lead to new strategies to neutralise aggressive CRPC.

Oestrogen is used as a second-line hormone therapy for prostate cancer, based on its indirect suppression of androgen levels. It is known that oestrogens acting via ER α mediate aberrant epithelial cell proliferation, prostatic inflammation and malignancy (Imamov *et al*, 2004; Ricke *et al*, 2008; Setlur *et al*, 2008). In contrast, effects of oestrogen mediated by ER β are beneficial. Previous studies have reported anti-proliferative and pro-apoptotic activity of ER β agonist in the prostate, independent of systemic androgens, targeting castrate-resistant epithelial cells (Weihua *et al*, 2002; Imamov *et al*, 2004; McPherson *et al*, 2008; Ricke *et al*, 2008; McPherson *et al*, 2010). Likewise, in this study, we presented that 17 β -oestradiol could decrease the expression of LPCAT1 and PAF release in both LNCaP and PC-3 cells via ER β -mediated pathway. It provides a new line that beneficial effects of ER β activation could also be mediated by the downregulation of proliferative and invasive signalling message, namely PAF, other than previously uncovered TNF- α pathway, either in ADPC or CRPC cells. In the latter circumstance, ER β agonist may offer some therapeutic advantage other than castration. Although ER (as well as PR and GR) has not been identified to have a similar interaction with β -catenin as AR (Pawlowski *et al*, 2002), the mechanism underlying ER β effect on PAF is likely to be classical genomic response.

In conclusion, androgen differentially regulates LPCAT1 and PAF expression in androgen-dependent LNCaP cells and CRPC-3 cells via different signalling pathways. The increase of PAF after castration may play an important role in transition from ADPC to CRPC, and further accelerate the progression of aggressive phenotype of CRPC cells. This study indicates the potential clinical application of PAF antagonist, either alone or in combination with existing androgen blockade, may be potentially effective for the prevention of the emergence and development of CRPC.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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