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Fenofibrate down-regulates the expressions of androgen receptor (AR) and AR target genes and induces oxidative stress in the prostate cancer cell line LNCaP

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ABSTRACT

Fenofibrate, a peroxisome proliferator-androgen receptor-alpha agonist, is widely used in treating different forms of hyperlipidemia and hypercholesterolemia. Recent reports have indicated that fenofibrate exerts anti-proliferative and pro-apoptotic properties. This study aims to investigate the effects of fenofibrate on the prostate cancer (PCa) cell line LNCaP. The effects of fenofibrate on LNCaP cells were evaluated by flow cytometry, reverse transcription-polymerase chain reaction, enzyme-linked immunosorbent assays, Western blot analysis, and dual-luciferase reporter assay. Fenofibrate induces cell cycle arrest in G1 phase and apoptosis in LNCaP cells, reduces the expressions of androgen receptor (AR) and AR target genes (prostate-specific antigen and TMPRSS2), and inhibits Akt phosphorylation. Fenofibrate can induce the accumulation of intracellular reactive oxygen species and malondialdehyde, and decrease the activities of total anti-oxidant and superoxide dismutase in LNCaP cells. Fenofibrate exerts an anti-proliferative property by inhibiting the expression of AR and induces apoptosis by causing oxidative stress. Therefore, our data suggest fenofibrate may have beneficial effects in fenofibrate users by preventing prostate cancer growth through inhibition of androgen activation and expression.

1. Introduction

As one of the most frequently diagnosed malignancies, prostate cancer (PCa) is the second leading cause of cancer-related deaths among men in the United States and in other developed countries [1]. Despite an initial response to androgen deprivation therapy, most patients within 2 years progress into hormone refractory or androgen-independent cancer, which is almost incurable [2]. Although the molecular mechanism responsible for the development of hormone independence is poorly understood, androgen receptor (AR) overexpression or mutation is frequent in a large number of castration-resistant PCa [3]. AR, a member of the ligand-activated transcription factor of the steroid receptor superfamily, has a critical function in the development and progression of PCa. androgens, such as testosterone and dihydrotestosterone, are necessary for normal prostate development and maintenance and for stimulating the proliferation and progression of PCa [4,5]. Androgen exerts its biological effects by binding to AR. Upon binding to AR, androgen activates AR transcriptional activity, which leads to its translocation from the cytoplasm to the nucleus. Nuclear AR interacts with androgen response elements in the promoters of target genes, such as the prostate-specific antigen (PSA) gene, and stimulates the transcription of target genes [6]. Therefore, novel effective agents that could potentially block AR signaling need to be developed.

Fenofibrate, a peroxisome proliferator-androgen receptor (PPARa) agonist, is a ligand-inducible transcription factor that belongs to the nuclear-hormone-receptor family and mediates peroxisome proliferation action. Fenofibrate is widely used in treating different forms of hyperlipidemia and hypercholesterolemia [7]. Recent reports have indicated that fenofibrate exerts anti-proliferative and pro-apoptotic properties [8–10]. However, reports on fenofibrate-induced effects on PCa cells are lacking.

This study aims to investigate the effects of fenofibrate on the proliferation and apoptosis of the PCa cell lines LNCaP and PC-3 and to discover the mechanism involved in AR signaling in PCa cells.

2. Materials and methods

2.1. Drugs and reagents

Fenofibrate was purchased from Sigma Chemicals. Malondialdehyde (MDA), total antioxidant capacity (T-AOC), and superoxide

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dismutase (SOD) assay kits were purchased from the Nanjing Jiancheng Bioengineering Institute (Nanjing, China). The production of reactive oxygen species (ROS) was measured using dichlorfluorescein-diacetate (DCFH-DA; Molecular Probes, Sigma). *N*-acetylcysteine (NAC) was purchased from Biosharp. Cell culture medium and supplements were purchased from Gibco. Anti-E2F1 and anti-CCND1 were purchased from Abcam. Anti-P-AKT and anti-AR were purchased from Cell Signaling Technology. Anti-PSA, anti-BCL-2, anti-BAX, anti-GAPDH, and goat anti-rabbit IgG horseradish peroxidase (HRP)-conjugated secondary antibody were purchased from Bioworld. Polyvinylidene difluoride (PVDF) membrane was purchased from Millipore. Chemiluminescence reagents were obtained from Thermo Scientific.

2.2. Cell culture

The LNCaP cell line was obtained from Shanghai Cell Bank, Chinese Academy of Sciences and PC-3 were obtained from KeyGene Biotech (Nanjing, China). LNCaP and PC-3 cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 lg/mL streptomycin at 37 °C in humidified 5% CO_2.

2.3. Western blot analysis

The cells were lysed using radioimmunoprecipitation assay buffer (Keygene, Nanjing, China) supplemented with protease inhibitors on ice for 30 min. Proteins were resolved by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis gels, transferred to PVDF membrane, and then blocked in 5% non-fat milk at room temperature (RT). The blot was incubated first with primary antibodies at 4 °C overnight and then with the secondary HRP-conjugated anti-goat antibody. After washing thrice with Tris-buffered saline with Tween, the bands were detected by chemiluminescence. Protein levels were determined by normalizing to GAPDH.

2.4. RNA extraction and quantitative real-time PCR

Total RNA was extracted using TRIzol reagent (Invitrogen). The concentration and purity of the RNA samples were determined spectroscopically. RNA was reverse-transcripted into cDNAs using a PrimeScript RT reagent Kit (TaKaRa, Dalian, China) according to the manufacturer's instructions. Quantitative RT-PCR assays were conducted using SYBR Premix Ex Taq (TaKaRa) on an Applied Biosystems 7300 real-time PCR system. The primers used for AR were 5′-ATGTCCTGGAAGCCATTGAGCCA-3′ and 5′-CAGAAAGGATCTTGG GCACTTGC-3′. The primers used for PSA were 5′-TACCCA CTGCATCAGGAACA-3′ and 5′-CCTTGAAGCACACCATTACA-3′. The primers used for TMPRSS2 were 5′-GTCCCCACTGTCTACGAGGT-3′ and 5′-CAGACGACGGGGTTGGAAG-3′. The primers used for GAPDH were 5′-GAAGGTGAAGGTCGGAGTC-3′ and 5′-GAAGATGGTGATGG-GATTTC-3′ (synthesized by Invitrogen). The reactions were incubated at 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s and 60 °C for 30 s. For relative quantitation, the $2^{-\Delta\Delta CT}$ method was used. GAPDH was used as an internal control.

2.5. Detection of PSA

LNCaP cells were incubated in a medium with different concentrations of fenofibrate. The medium from each well was collected at different periods and then immediately stored at −80 °C being analyzed. The PSA levels in the cell media were measured by taking PSA enzyme-linked immunosorbent assays (ELISA), as described previously [11].

2.6. MDA, T-AOC, and SOD determination

The cells were seeded at 70% confluence in six-well plates. After 24 h, the cells were treated with different concentrations of fenofibrate for 48 h. Subsequently, the cells were detached by trypsinization, collected by centrifugation, and then resuspended in PBS. The suspensions were used immediately for the assays of MDA, T-AOC, and SOD according to the manufacturer's protocol.

2.7. Cell cycle and apoptosis assay

The cells were seeded at 70% confluence in six-well plates for 24 h and then treated with different concentrations of fenofibrate for 48 h. Subsequently, the cells were collected by trypsinization and then washed twice with cold PBS. For cell cycle analysis, the cells were fixed with 70% ethanol at −20 °C overnight. Subsequently, the cells were incubated in 50 lg/mL of propidium iodide (PI) and 1 mg/mL of RNase for 30 min at RT. The treated cells were analyzed by flow cytometry. For apoptosis analysis, the cells were resuspended in 1× binding buffer at a concentration of 1×10^6 - cells/mL. Approximately 5 μL of annexin V-FITC reagent and 10 μL of PI were added to the cell suspension and then incubated for 15 min under RT in the dark. Stained cells were analyzed by flow cytometry (Becton Dickinson).

2.8. Measurement of ROS generation

For measurement of intracellular ROS, the DCFH-DA method was used. The cells were harvested after the fenofibrate treatments and then washed once with ice-cold PBS. The cells were treated with DCFH-DA (at a final concentration of 10 μmol/L in serum-free medium). After incubation for 20 min at 37 °C in the dark, the cells were washed twice with PBS. Intracellular ROS accumulation was measured by flow cytometry.

2.9. Dual-luciferase assay

AR promoter was cloned into the downstream of the luciferase gene in the pGL3-REPORT luciferase vector (Invitrogen). For luciferase assay, cells were seeded into 24-well plates and cultured for 24 h. Then, cells were co-transfected with pGL3-REPORT or control reporter plasmid. 24 h after transfection, the cells were exposed to specified concentrations of Fenofibrate for 48 h. Two days later, cells were harvested and lysed in passive lysis buffer and reporter activity was measured using a dual-luciferase reporter assay system (Promega). Firefly luciferase activity was normalized to renilla luciferase activity for each transfected well.

2.10. Statistical analysis

Data were expressed as mean ± SEM. Differences between groups were analyzed using Student's *t* test. Statistical calculations were performed with SPSS 11.0. We considered $P < 0.05$ as statistically significant.

3. Results

3.1. Effect of fenofibrate on cell cycle and apoptosis of LNCaP cells

We first determined if fenofibrate affects the growth and apoptosis of LNCaP PCa cells. After the cells were treated with different concentrations of fenofibrate for 48 h, the percentage of G1 phase and apoptosis increased in a dose-dependent manner (Fig. 1A and B). After the cells were treated with 50 μmol/L fenofibrate, the protein levels of BCL-2, CCND1, and E2F1 decreased and the

protein level of BAX increased (Fig. 1C). In contrast, fenofibrate affect the apoptosis of PC-3 cells after the cells were treated with high concentrations of fenofibrate (Fig. 1D). However, after PC-3 cells were treated with high concentrations of fenofibrate for 48 h, the percentage of G1 phase increased (Fig. 1E).

3.2. Fenofibrate transcriptionally down-regulates AR expression together with impaired Akt activation

Fenofibrate has an important function in inducing G1 cell cycle arrest and apoptosis in LNCaP cells. Therefore, we investigated the effect of fenofibrate on AR expression. As shown in Fig. 2A and B, fenofibrate significantly decreased AR mRNA and protein expressions in a dose-dependent manner. Next, we investigated the potential mechanism of fenofibrate-mediated AR transcription. As shown in Fig. 2D, fenofibrate markedly decreased AR promoter activity in a dose-dependent manner. Akt was previously shown to affect AR stability and function [12]. Thus, we further speculated that reduced AR expression was an indirect effect resulting from the fenofibrate-induced inhibition of Akt signaling. Phosphorylated Akt (pAkt) was also reduced in a dose-dependent manner (Fig. 2B).

3.3. Fenofibrate decreases the expressions of PSA and TMPRSS2 in LNCaP cells

The effects of fenofibrate on AR target genes (PSA and TMPRSS2) in LNCaP cells were determined through Western blot, qRT-PCR, and ELISA. Fenofibrate decreased TMPRSS2 mRNA level and PSA mRNA and protein expressions in a dose-dependent manner (Fig. 2A and B). We also measured PSA in cell culture supernatants. As shown in Fig. 2C, PSA was significantly reduced compared with the mock control in time- and dose-dependent manners.

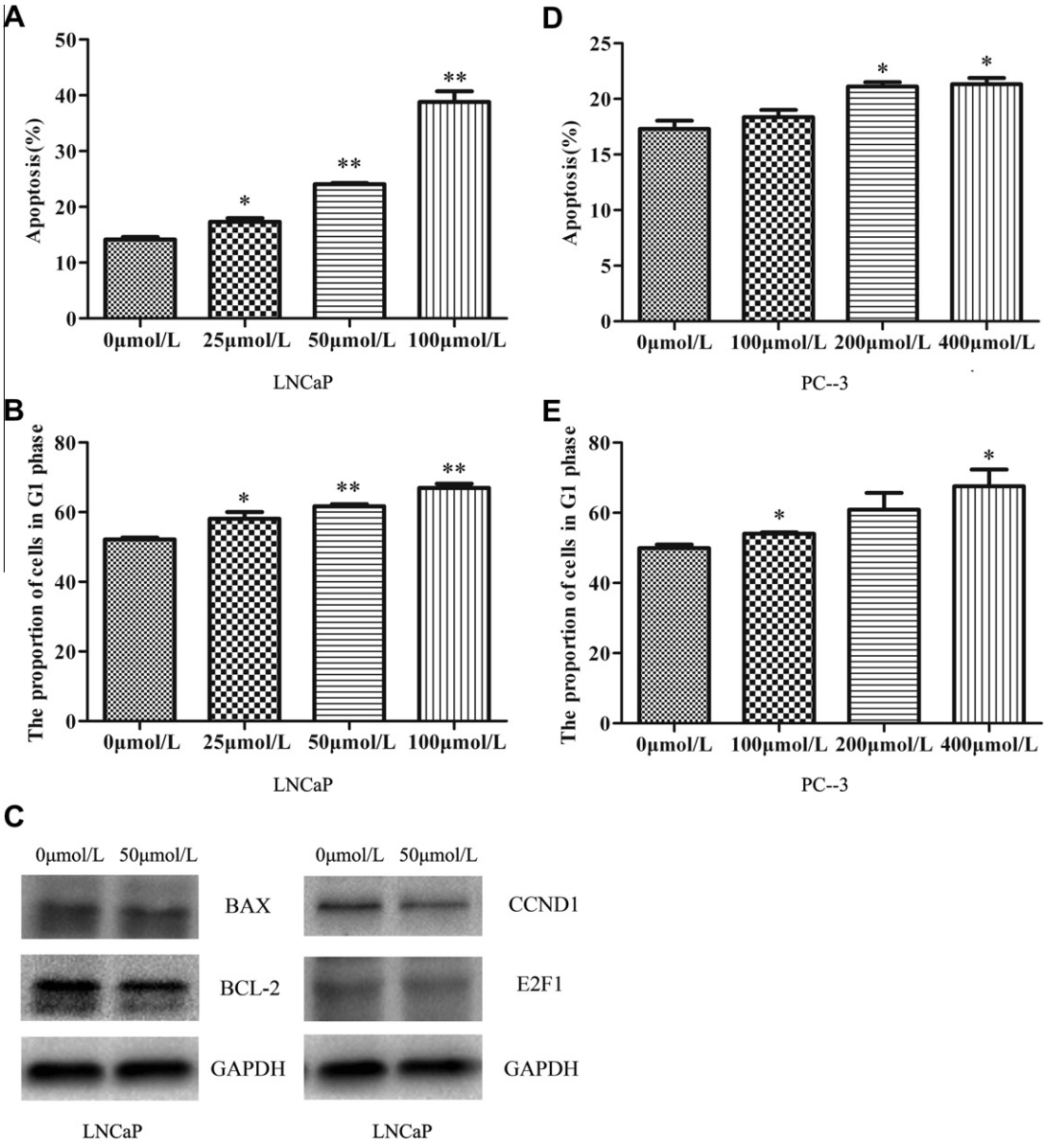

Fig. 1. Fenofibrate induces cell cycle arrest in G1 phase and apoptosis in LNCaP cells. (A) Apoptosis measured by flow cytometry analysis using Annexin-FITC and PI staining following treatment with different concentrations of fenofibrate for 48 h. (B) After treatment with different concentrations of fenofibrate for 48 h, LNCaP cells were collected, and cell cycle analyses with propidium iodide were performed using flow cytometry. (C) LNCaP cells were treated with 50 μmol/L fenofibrate for 48 h and lysed for Western blot analysis for cyclin D1, E2F1, Bcl-2, and Bax. The expression of GAPDH was detected as a loading control. (D) Effect of fenofibrate on apoptosis of PC-3 cells. (E) Effect of fenofibrate on cell cycle of PC-3 cells. Data are expressed as means ± SEM. $^{*}P < 0.05$ as compared with the control group. $^{**}P < 0.01$ as compared with the control group.

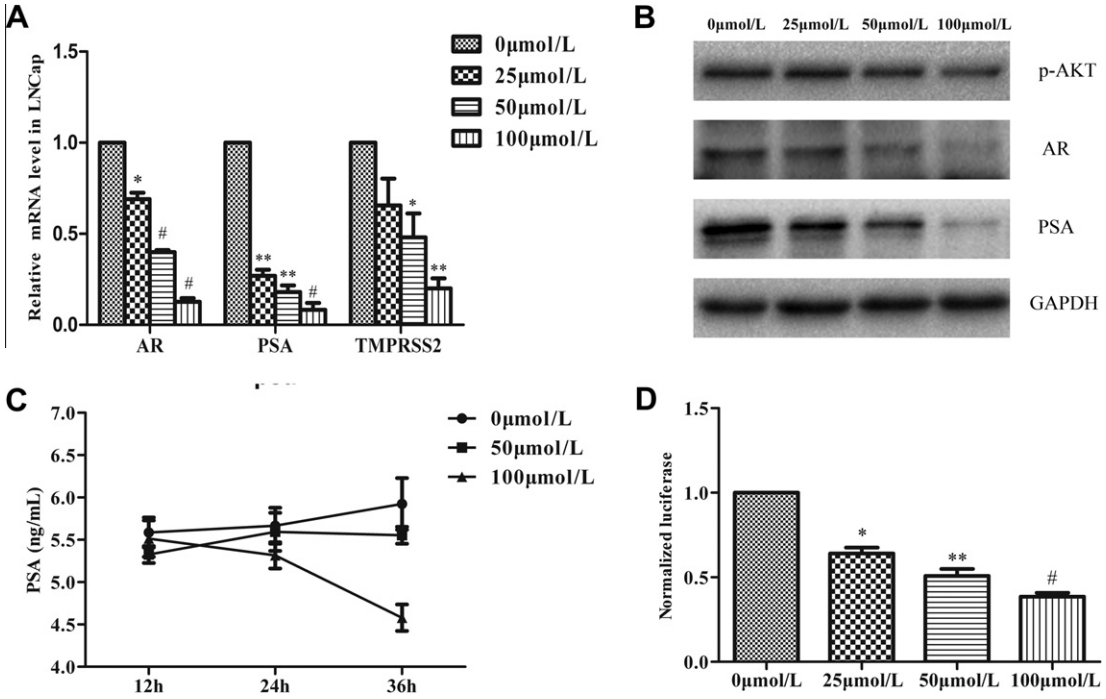

Fig. 2. Fenofibrate reduces the expressions of AR and AR target genes (PSA and TMPRSS2), and inhibits Akt phosphorylation. (A) Fenofibrate significantly decreases the mRNA expressions of AR and AR target genes. (B) Fenofibrate reduces AR and PSA protein expression and inhibits Akt phosphorylation. (C) PSA was measured in cell culture supernatants at different concentrations and different time lengths. (D) AR activity was determined in total cell lysates by measuring luciferase reporter gene activity. Data are expressed as means ± SEM. $^{*}P < 0.05$ as compared with the control group. $^{**}P < 0.01$ as compared with the control group. $^{\#}P < 0.001$ as compared with the control group.

3.4. Increase of intracellular ROS⁻ after fenofibrate treatment

After the cells were treated with different concentrations of fenofibrate for 48 h, DCF mean fluorescence intensity was increased significantly and positively correlated with fenofibrate concentration (Fig. 3). The result showed that fenofibrate had enhanced intracellular ROS levels in the LNCaP cells.

3.5. Effects of NAC on fenofibrate-induced increases of ROS and apoptosis

We examined the effect of NAC on the level of ROS and apoptosis after fenofibrate treatment. *N*-acetylcysteine (NAC) is a general antioxidant and a GSH precursor able to ameliorate and ROS-mediated injury. Pretreatment of LNCaP cells with NAC significantly attenuated the increase in apoptosis and intensity of DCF fluorescence in LNCaP cells caused by fenofibrate (Fig. 4A and B). These data suggest that fenofibrate may exert its function in LNCaP cells by enhancing intracellular ROS.

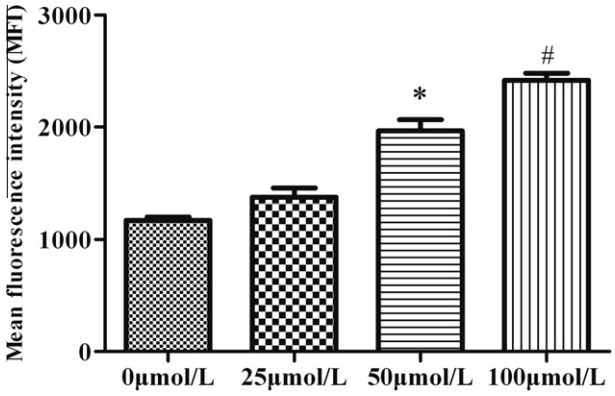

Fig. 3. Effect of fenofibrate on intracellular ROS. Flow cytometry revealed that fenofibrate induced intracellular ROS in a dose-dependent manner. Data are expressed as means ± SEM. $^{*}P < 0.05$ as compared with the control group. $^{\#}P < 0.001$ as compared with the control group.

3.6. T-AOC, SOD, and MDA activity

T-AOC in all fenofibrate groups was significantly reduced compared with that in the control group of LNCaP cells. The activity of the antioxidant enzyme SOD was markedly decreased by fenofibrate treatment in a dose-dependent manner. MDA, the stable metabolite of lipid peroxidation products, was significantly increased in a dose-dependent manner (Table 1).

4. Discussion

The PPARα agonist fenofibrate has been used as a lipid-lowering medicine since 1975. Fenofibrate was identified as a liver tumor promoter in rodents. However, this effect has not yet been observed in humans [13]. Growing evidence demonstrates that the PPARα agonist fenofibrate has anti-tumor effects, which may be attributed to its anti-proliferative and pro-apoptotic activities [8–10]. Fenofibrate likely exerts its function in a PPARα-independent manner [14,15].

The present study is the first to prove that fenofibrate induces cell cycle arrest in G1 phase and apoptosis in LNCaP cells. Cyclins D1 and E2F1 are crucial cell cycle-regulatory genes. A marked reduction in the protein levels of cyclins D1 and E2F1 was noted after exposure to fenofibrate. These results suggest that fenofibrate-induced G1 arrest may be mediated by down-regulating the protein levels of cyclins D1 and E2F1. Bcl-2-related proteins were proven to play important functions in either inhibiting or promoting apoptosis [16]. In the present study, Western blot analysis showed a significant decrease in Bcl-2 protein level without affecting the expression for bax protein level after 48 h of

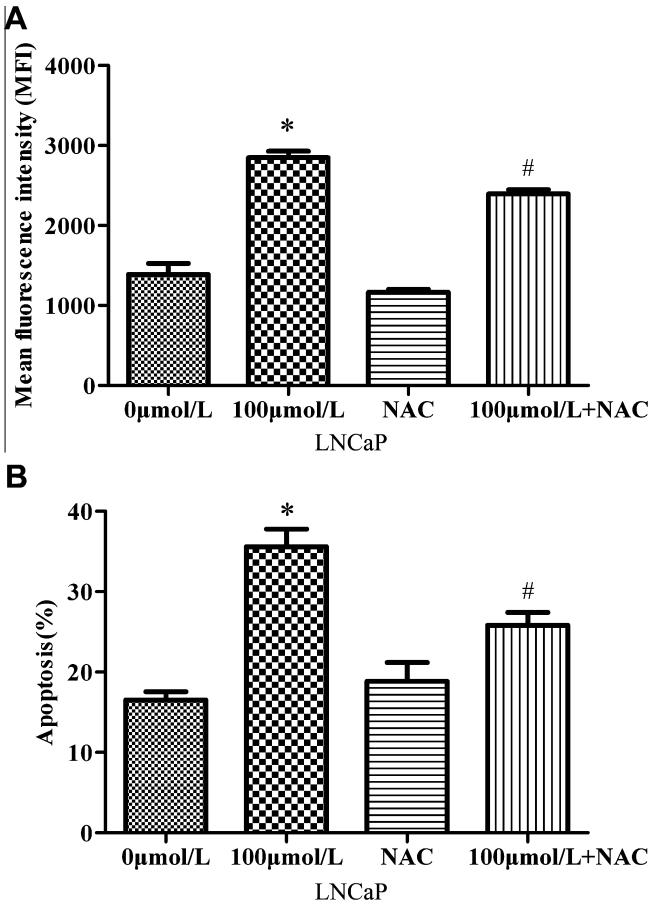

Fig. 4. Effect of *N*-acetylcysteine (NAC) on fenofibrate-induced intracellular ROS and apoptosis. (A) Effect of NAC on fenofibrate-induced intracellular ROS. (B) Effect of NAC on fenofibrate-induced apoptosis. Data are expressed as means ± SEM. $^{*}P < 0.05$ as compared with the control group. $^{\#}P < 0.05$ as compared with the 100 μmol/L fenofibrate group.

Table 1
Activity of T-AOC, SOD and level of MDA in different fenofibrate concentration group.

Group (μmol/L)	T-AOC (U/mgprot)	SOD (U/mgprot)	MDA (nmol/mgprot)
0	6.02 ± 0.36	34.13 ± 2.09	0.90 ± 0.11
25	3.96 ± 0.36*	28.98 ± 1.43	1.51 ± 0.21
50	3.34 ± 0.05**	25.75 ± 1.05*	2.53 ± 0.26**
100	2.45 ± 0.27**	19.24 ± 0.53**	4.10 ± 0.17**

Data are expressed as means ± SEM.
* $P < 0.05$ as compared with the control group.
** $P < 0.01$ as compared with the control group.

fenofibrate treatment. These results suggest that fenofibrate-induced apoptosis may be mediated by down-regulating Bcl-2 anti-apoptotic protein and increasing the Bax/Bcl-2 ratio in LNCaP cells.

Fenofibrate can induce G1 arrest and appotosis of androgen-dependent prostate cell line LNCaP at low concentration. However, fenofibrate only induce G1 arrest of androgen-independent prostate cell line PC-3 at high concentration. These results suggest that fenofibrate-induced apoptosis and G1 arrest may be mediated by down-regulating AR protein.

A previous study revealed that Akt phosphorylation may be involved in the crossroads of multiple cellular signaling pathways [17]. Fenofibrate inhibits Akt phosphorylation in many cell lines [18,19]. In the present study, Akt phosphorylation was measured by Western blot using anti-pAkt monoclonal antibody. A marked decrease in pAkt protein level was observed after exposure to fenofibrate.

AR could activate the transcription of cell growth-related and survival-related genes via androgen-mediated signal transduction. Therefore, we investigated if AR is a novel molecular target of fenofibrate. Our results showed that fenofibrate decreased AR mRNA and protein expressions in a dose-dependent manner. To the best of our knowledge, PSA, a key AR target gene, is the best biomarker for PCa screening and is one of the most important indicators of treatment efficacy. In the present study, fenofibrate was found to downregulate intracellular and secreted PSA protein levels in LNCaP cells. These findings suggest that fenofibrate exerts its anti-proliferative property by inhibiting the expression of AR.

Previous studies demonstrated that PPARα agonists can induce intracellular ROS accumulation [8,20,21]. Thus, the effect of fenofibrate on intracellular ROS and O^{2-} was determined by the DCFH-DA and dihydroethidium methods, respectively. ROS, such as O^{2-}, H_2O_2, and hydroxyl radical, are usually side products of normal metabolism or environmental stress. ROS at low concentrations can cause mitogenic to proliferative effects; when ROS generation exceeds the cellular antioxidant defenses, cell damage and cell death ensue [22]. In the present study, the intracellular ROS in LNCaP cells induced by fenofibrate were found to be concentration dependent. Lipid peroxides, such as MDA, are easily accessible biomarkers of ROS damage. By contrast, the production of antioxidant enzymes, such as T-AOC and SOD, serves as a defense mechanism against ROS. In the present study, fenofibrate induced oxidative stress by increasing MDA. As a result, the activities of T-AOC and SOD in LNCaP cells significantly decreased. These findings suggest that the pro-apoptotic effects of fenofibrate might be mediated by up-regulating the intracellular concentration of ROS. Pretreatment with NAC not only significantly reduced the increase of ROS but also decreased the percentage of apoptotic LNCaP cells caused by fenofibrate.

In conclusion, fenofibrate exerts an anti-proliferative property by inhibiting the expression of AR and induces apoptosis by causing oxidative stress. Thus, combining fenofibrate with other drugs may increase the therapeutic potential. Further studies are necessary to evaluate the influence and significance of fenofibrate in the progression of PCa.

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