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# Lysophosphatidic acid induces reactive oxygen species generation by activating protein kinase C in PC-3 human prostate cancer cells

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ABSTRACT

Prostate cancer is one of the most frequently diagnosed cancers in males, and PC-3 is a cell model popularly used for investigating the behavior of late stage prostate cancer. Lysophosphatidic acid (LPA) is a lysophospholipid that mediates multiple behaviors in cancer cells, such as proliferation, migration and adhesion. We have previously demonstrated that LPA enhances vascular endothelial growth factor (VEGF)-C expression in PC-3 cells by activating the generation of reactive oxygen species (ROS), which is known to be an important mediator in cancer progression. Using flow cytometry, we showed that LPA triggers ROS generation within 10 min and that the generated ROS can be suppressed by pretreatment with the NADPH oxidase (Nox) inhibitor diphenylene iodonium. In addition, transfection with $LPA_1$ and $LPA_3$ siRNA efficiently blocked LPA-induced ROS production, suggesting that both receptors are involved in this pathway. Using specific inhibitors and siRNA, phospholipase C (PLC) and protein kinase C (PKC) were also suggested to participate in LPA-induced ROS generation. Overall, we demonstrated that LPA induces ROS generation in PC-3 prostate cancer cells and this is mediated through the PLC/PKC/Nox pathway.



## 1. Introduction

Prostate cancer is one of the most frequently diagnosed cancers in males. The high recurrence rate and metastatic behavior are important challenges in prostate cancer treatment. PC-3 is an aggressive cancer cell line used for investigating the properties of androgen-independent prostate cancer. The reactive oxygen species (ROS) balance in cancer cells has become a new target for cancer therapy, including prostate cancer [1,2]. ROS includes highly reactive molecules such as oxygen anions and free radicals. It is known to be a mediator for many cellular functions, including cell proliferation and adhesion in both normal and cancer cells. ROS scavenger *N*-acetylcysteine suppresses PC-3 cell growth by mediating intracellular ROS production, and NF-κB activity [3]. ROS are mainly produced either endogenously in the mitochondria or through NADPH oxidase (Nox) in the cytoplasm. Several studies have revealed that ROS play an important role in mediating apoptosis in PC-3 cells [4–7], and treatment involving a combination of hydrogen peroxide and X-ray is suggested to induce apoptosis [8]. However, the endogenous molecule that induces ROS accumulation and leads to apoptosis remains unclear.

Lysophosphatidic acid (LPA) is a small lipid growth factor that binds to the endothelial differentiation gene (Edg) family G-protein-coupled receptors, and mediates multiple cellular behaviors such as cell proliferation, adhesion and migration [9]. In prostate cancer, LPA is suggested to act as an autocrine mediator that affects downstream signaling [10]. In PC-3 cells, LPA treatment inhibits autophagy, and promotes cell migration, invasion and survival [11–17]. It was known that LPA activates several downstream signals by inducing ROS production. For instance, LPA-induced interleukin-1β in macrophages is mediated by ROS generation [18]. ROS is also involved in LPA-induced EGFR/MAPK pathway in HeLa cells [19,20], and required for LPA-induced PI3K, PAK1 and ERK activation in breast cancer cells [21]. Our previous study has been shown that LPA enhances vascular endothelial growth factor (VEGF)-C expression by activating ROS production and this pathway is

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$LPA_1$ and $LPA_3$ dependent [22]. Taken together, ROS is an important factor that mediates LPA-induced signaling in both normal, and cancer cells.

Protein kinase Cs (PKCs) are a group of serine/threonine kinases [23]. According to their structure, PKCs can be separated into three groups: classical (c) PKC, novel (n) PKC, and atypical (a) PKC. PKCs trigger ROS production through phosphorylating Nox [24]. Among all the PKC subtypes, atypical PKCζ is shown to induce ROS accumulation in mesangial cells under high-glucose environment [25,26]. In addition, ROS generation in vascular smooth muscle cells is also PKCζ dependent [27]. In human intestinal myofibroblasts, IL-1α-induced COX-2 expression is also dependent on the PKCζ–ROS pathway [28]. These results strongly suggest that PKCζ is responsible for ROS generation in a variety of cell types.

In this study we aimed to investigate the mechanisms of LPA-induced ROS generation in PC-3 cells. Our current results suggest that LPA induces ROS production in PC-3 cells by activating $LPA_1$ and $LPA_3$ receptors, phospholipase C (PLC), PKC, and lead to activation of Nox complex.

## 2. Materials and methods

### 2.1. Cell culture

PC-3 human prostate cancer cells obtained from ATCC were cultured in RPMI 1640 (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum, penicillin (100 U/mL), and streptomycin (100 U/mL) in a humid atmosphere containing 5% $CO_2$ at 37 °C. All experiments were performed after 24 h of cell culture followed by 16 h of starvation.

### 2.2. LPA stimulation and drug treatment

LPA was dissolved in chloroform and methanol in a 1:9 ratio and stored at −20 °C. For each experiment, 100,000 cells were cultured in 3.5 cm diameter plates. After 24 h of culture and 16 h of starvation, LPA was added to a serum-free medium to trigger ROS generation, coupled with 0.005% fatty acid-free bovine serum albumin (BSA) as a carrier. Before LPA stimulation, different inhibitors at the following concentrations were pretreated for 1 h: 100 μM NS398 (COX-2 inhibitor), 1 μM AG1478 (EGFR inhibitor), 100 μM diphenylene iodonium (DPI) (Nox inhibitor), 500 nM calphostin C (PKC inhibitor), 10 μM PKCζ pseudosubstrate, and 5 μM U73122 (PLC inhibitor).

### 2.3. Small interfering (si)RNA transfection

PC-3 cells were transfected with control (sc-37007), $LPA_1$ (sc-43746), $LPA_3$ (sc-37088), and PKCζ (sc-29451) siRNAs (Santa Cruz Biotechnology, Santa Cruz, CA, USA) using Lipofectamine 2000 (Invitrogen). Cells were transfected for 24 h before conducting the experiments. The mRNA knockdown efficiency was confirmed by real-time PCR as described in the following sections.

### 2.4. Reverse transcription (RT) and real-time PCR

TRIzol reagent (Invitrogen) was used to extract total RNA from the PC-3 cells. Complementary DNA was synthesized with 1 μg total RNA using the Toyobo RT-PCR kit (Toyobo, Osaka, Japan). The iCycler iQ Realtime detection system (Bio-Rad, Hercules, CA, USA) with SYBR-Green I (Thermo, Rockford, IL, USA) as a fluorescent dye was used to perform real-time PCR. Gene-specific primers were used and specificity for the primers was checked by melting curve analysis following real-time PCR. Cycling conditions were 95 °C for 3 min, followed by 40 cycles of 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s. To quantify the target gene expression, each gene was normalized using GAPDH as the internal standard. Primers for real-time PCR were $LPA_1$ (F-5′-TTC AAC TCT GCC ATG AAC CCC-3′ and R-5′-CTA AAC CAC AGA GTG GTC ATT-3′); $LPA_3$ (F-5′-TCA GCA GGA GTG ACA CAG GCA G-3′ and R-5′-GGA AGT GCT TTT ATT GCA GAC T-3′); PKCζ (F-5′-GCG TAC TGC GGC CAG TGC-3′ and R-5′-CTT GGC ATA GCT TCC ACG-3′); and GAPDH (F-5′-AAG GTG AAG GTC GGA GTC-3′ and R-5′-TGT AGT TGA GGT CAA TGA AGG-3′).

### 2.5. Flow cytometry

Intracellular ROS was detected by flow cytometry using 2′,7′-dichlorofluorescin diacetate (DCFDA) (Sigma), a cell-permeable dye that emits green fluorescence after redox reaction with ROS. The green fluorescence was then detected in FL1 channel using a Cyflow flow cytometry (Partec, Muester, Germany). After starvation, 2.5 μM DCFDA was added for 10 min, followed by LPA stimulation for 10 min. Treated PC-3 cells were detached from the plate by trypsinization and centrifuged and resuspended with PBS. Intracellular ROS levels were then measured in 10,000 cells per group by flow cytometry. To prevent the effect of light on DCFDA, all procedures were performed in the dark.

### 2.6. Statistical analysis

Data were analyzed by one-way analysis of variance (ANOVA) and Fisher's protected least significant difference (LSD) test (StatView, Abacus Concept, Berkeley, CA, USA). Each result was obtained after three to six independent experiments and a $P$ value of $<0.05$ was considered statistically significant.

## 3. Results

### 3.1. LPA induces ROS generation in PC-3 cells

Previous studies revealed that ROS is involved in LPA-related cell signaling pathways in several cancer cell lines [21,22,29]. To investigate the effect of LPA on ROS generation in PC-3 cells, flow cytometry with DCFDA, a ROS indicator, was used to detect ROS levels. LPA-induced ROS are indicated by gray diagonal (1 μM) and black (5 μM) lines (Fig. 1A). Quantification of the geometric mean indicates that LPA increased the relative level of ROS between 1.5- and 4-fold. We selected the geometric mean of these data for subsequent quantification. LPA induced ROS production within 10 min (Fig. 1B). Other than mitochondria, Nox is suggested as a major generator of ROS. Therefore, we pretreated PC-3 cells with the Nox inhibitor DPI and found that LPA-induced ROS production was abolished by the treatment, suggesting a role of Nox in the pathway (Fig. 1C). DPI pretreatment also suppressed LPA-enhanced VEGF-C mRNA expression (Fig. 1D), same as the effect of NAC pretreatment (Fig. S1A). Here, we demonstrated that LPA induces ROS generation through Nox within 10 min and Nox is essential for LPA-enhanced VEGF-C pathway.

### 3.2. LPA-induced ROS generation is $LPA_1$- and $LPA_3$-dependent in PC-3 cells

LPA activates downstream signaling through binding with Edg family G-protein-coupled receptors [9]. In our previous study, the $LPA_1$ and $LPA_3$ antagonist Ki16425 blocked LPA-induced ROS production in PC-3 cells. To further confirm the roles of either LPA receptor, $LPA_1$ and $LPA_3$ siRNAs were used to block the expression of these receptors. Real-time PCR showed that the knockdown efficiency for $LPA_1$ and $LPA_3$ siRNA treatment were approximately 60%

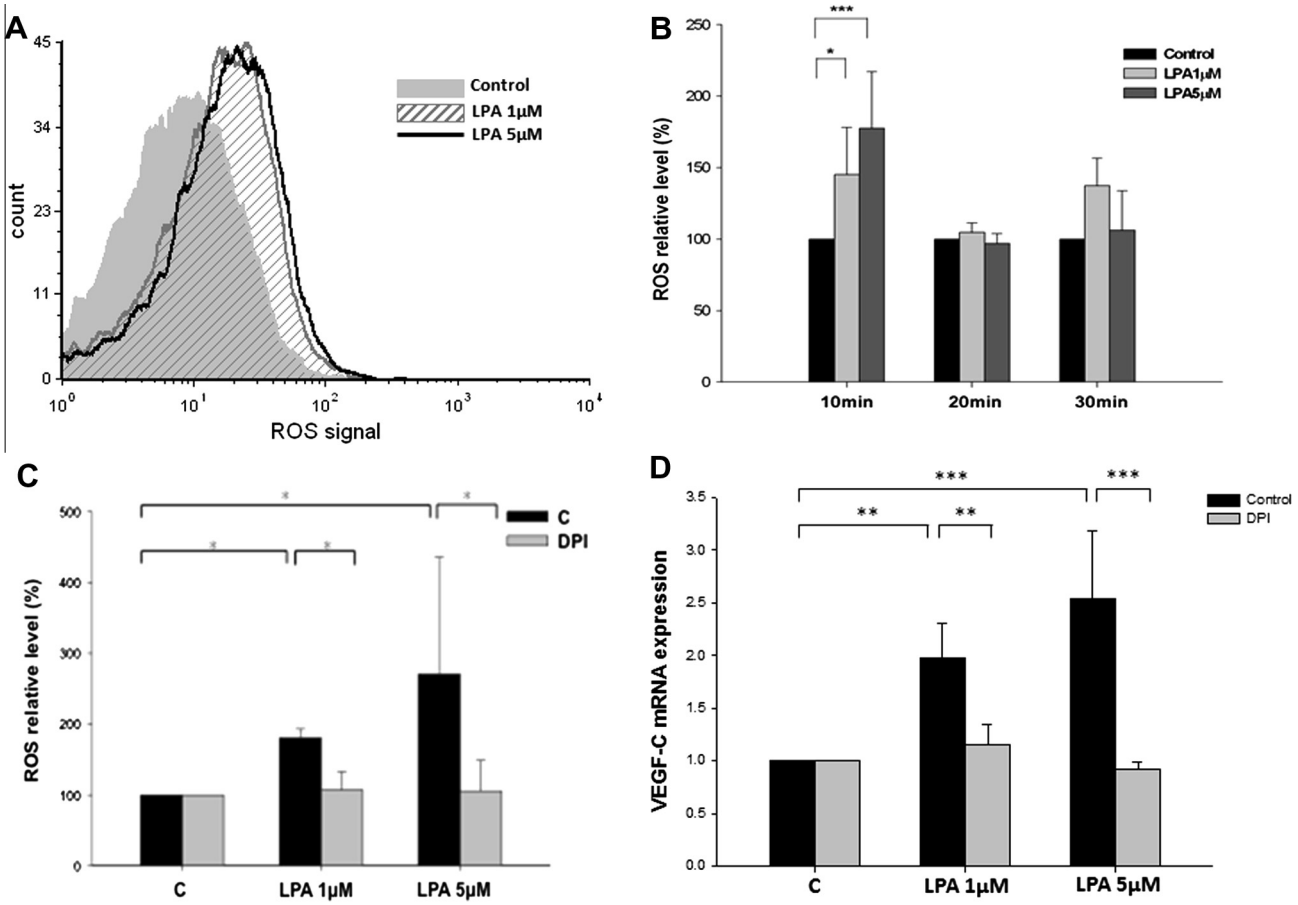


**Fig. 1.** In PC-3 cells, LPA induces ROS generation through Nox within 10 min. (A) PC-3 cells were cultured for 1 day followed by 16 h of starvation before LPA stimulation. Gray-filled area, gray diagonal and black lines represent the control, 1 μM LPA and 5 μM LPA groups, respectively. The geometric mean of the flow cytometry data was used to generate the following bar graph. (B) LPA treatment for three different time intervals: 10, 20 and 30 min. The 10 min treatment was selected for the following experiments. (C) Pretreatment with 100 μM DPI for 1 h, followed by LPA treatment and flow cytometry. (D) Pretreatment with 100 μM DPI for 30 min, followed by LPA treatment. VEGF-C mRNA relative level was analyzed by real-time PCR using GAPDH gene as internal control.

(Fig. 2A). LPA-induced ROS was totally diminished by $LPA_1$ siRNA, suggesting the dominance of $LPA_1$ in the pathway (Fig. 2B), whereas the $LPA_3$ siRNA group showed partially decreased ROS levels (Fig. 2C). We speculate that upon LPA stimulation, $LPA_1$ and $LPA_3$ share common downstream signaling on ROS production whereas $LPA_1$ is a dominant mediator. In conclusion, both $LPA_1$ and $LPA_3$ participate in the signaling pathway by which LPA induces ROS production in PC-3 cells.

### 3.3. LPA induces ROS generation through PLC in PC-3 cells

LPA activates signal transduction pathways through multiple downstream signaling factors. Our previous study suggests that LPA enhances VEGF-C through cyclooxygenase-2 (COX-2) and EGFR transactivation in HUVEC [30]. In PC-3 cells LPA also enhances VEGF-C expression in a ROS-dependent manner [22]. Therefore, we hypothesize that in PC-3 cells COX-2 and EGFR may participate in the LPA-induced ROS pathway. PC-3 cells were treated with inhibitors of COX-2 and EGFR (NS398 and AG1478, respectively) before LPA stimulation. However, there was no significant difference between the control and treated groups (Fig. 3A), suggesting that LPA-induced ROS generation in PC-3 cells is not mediated through COX-2 or EGFR. As a known downstream signaling molecule of LPA, the role of PLC was determined. Pretreatment with U73122, a PLC inhibitor, suppressed LPA-induced ROS, suggesting the involvement of PLC in the pathway (Fig. 3B). Since PLC is a direct downstream molecule activated by LPA receptors, this finding strengthens our hypothesis that LPA induces ROS in a short interval in PC-3 cells.

### 3.4. LPA induces ROS generation by activating PKC in PC-3 cells

Previous studies have revealed that PKC is an important mediator of ROS production by phosphorylating Nox. Furthermore, PKC is a downstream signaling molecule of PLC. PC-3 cells were treated with a general PKC inhibitor, calphostin C, and LPA-induced ROS production was abolished as expected (Fig. 4A). PKCζ is an atypical PKC implicated in ROS generation. PKCζ pseudosubstrate treatment also inhibits LPA-induced ROS generation; this supports the idea that it is involved in LPA-induced ROS generation in PC-3 cells (Fig. 4B). Furthermore, PKCζ-specific siRNA was used for further confirmation for its involvement. Real-time PCR results showed that PKCζ was knocked down about 80% at mRNA level (Fig. 4C) and LPA-induced ROS production was significantly reduced after PKCζ siRNA treatment (Fig. 4D). Therefore, we concluded that LPA induces ROS generation is mediated through PKCζ in PC-3 cells.

## 4. Discussion

LPA activates multiple cellular signals, including increasing ROS levels [9]. Previous work in our laboratory has shown that LPA enhances VEGF-C expression in a ROS-dependent manner [22]. However, the mechanism by which LPA induces ROS accumulation in

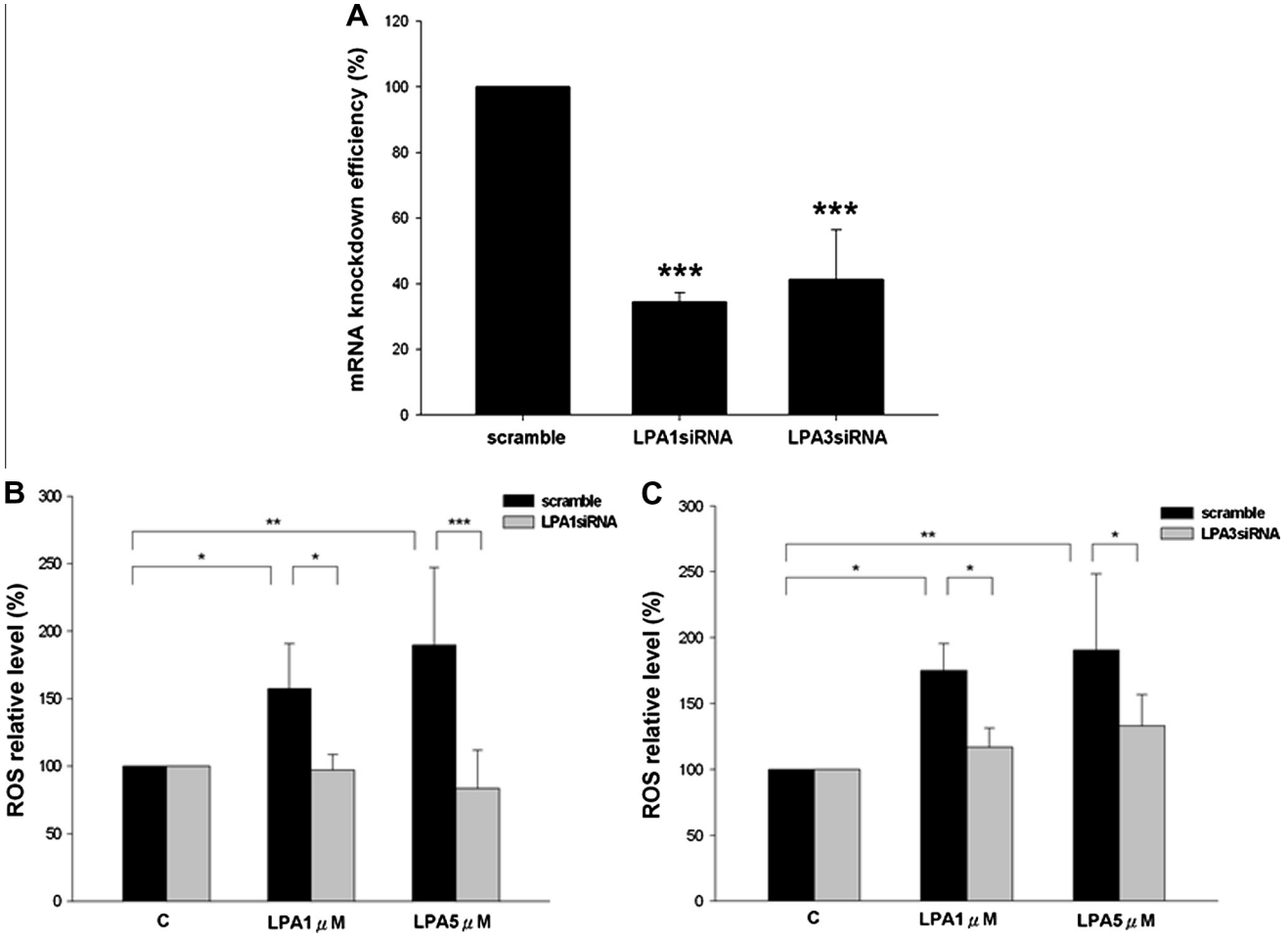


**Fig. 2.** LPA triggers ROS production through both $LPA_1$ and $LPA_3$ receptors. (A) PC-3 cells were transfected with control, $LPA_1$ and $LPA_3$ siRNA for 1 day. siRNA transfection efficiency was confirmed by real-time PCR. (B) and (C) $LPA_1$ siRNA and $LPA_3$ siRNA were transfected for 1 day before LPA treatment and flow cytometry.

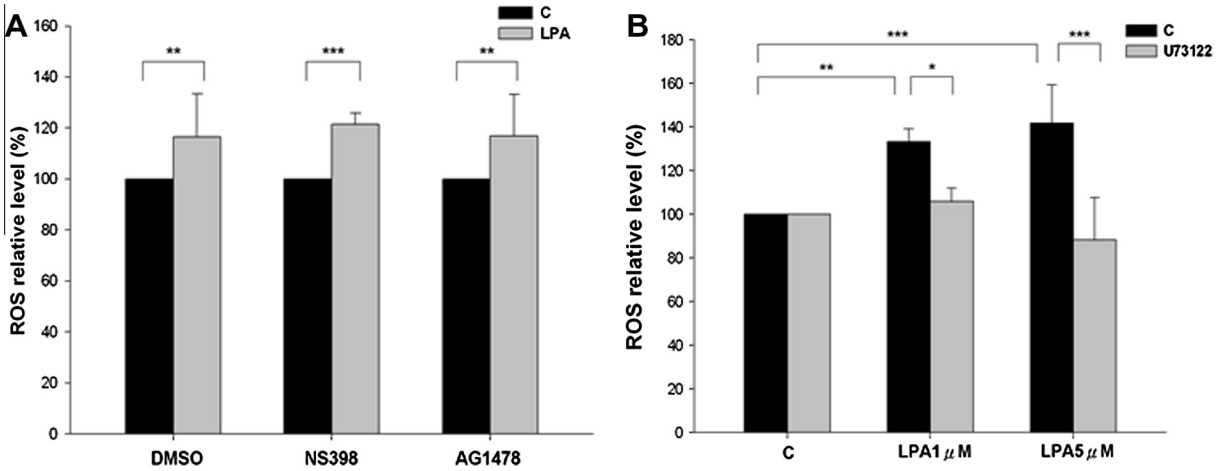


**Fig. 3.** LPA activates PLC to trigger ROS production. (A) 100 μM NS398 and 1 μM AG1478 were pretreated for 1 h to block the COX-2 and EGFR-dependent pathway, followed by LPA treatment and flow cytometry. (B) Pretreatment with 5 μM PLC inhibitor U73122 for 1 h, followed by LPA treatment and flow cytometry.

cancer cells remained unclear. Here, we first demonstrate that LPA induces ROS generation in PC-3 prostate cancer cells by activating the PLC/PKC/Nox pathway shortly after LPA stimulation. Together with our previously published results, we provide a detailed molecular mechanism by which LPA promotes VEGF-C expression and secretion through a ROS-dependent pathway in PC-3 cells. This may contribute to further understanding of pathological lymphangiogenesis and subsequent metastasis of late stage prostate cancer.

Moderate ROS levels may trigger cell growth whereas high levels of ROS may cause apoptosis. As a result, governing ROS accumulation and redox status has recently been recognized as a feasible method for cancer treatment. In this study, we demonstrated that LPA induces ROS generation through two G-protein-

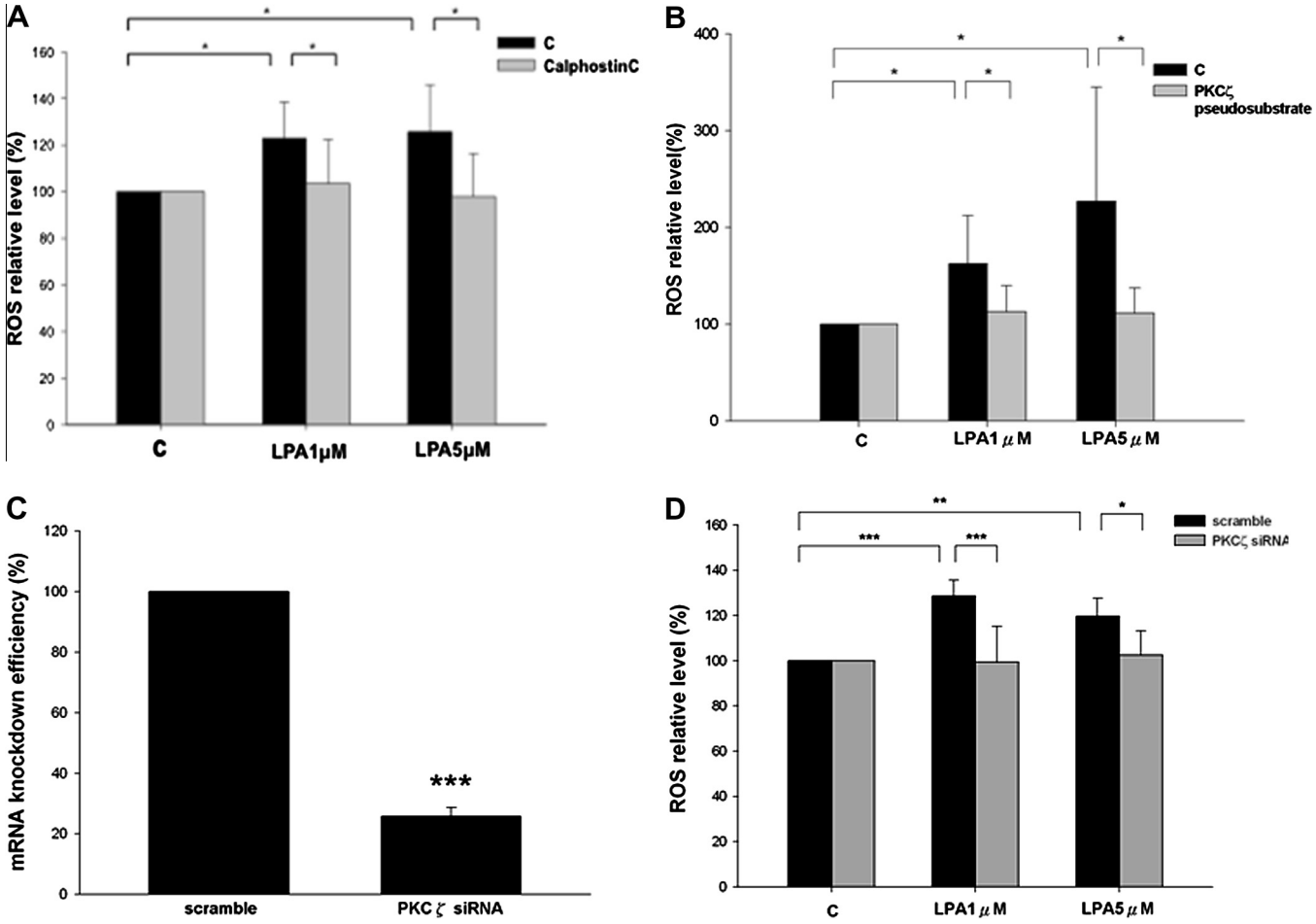


**Fig. 4.** LPA-induced ROS generation pathway is protein kinase C (PKC)-dependent. (A) PC-3 cells were pretreated with 500 nM Calphostin C for 1 h, followed by LPA treatment and flow cytometry. (B) PC-3 cells were pretreated with 10 μM PKCζ pseudosubstrate for 1 h to block PKCζ-specific pathways, followed by LPA treatment and flow cytometry. (C) PKCζ-specific siRNA is transfected into PC-3 cells for one day to knockdown PKCζ mRNA expression. Transfection efficiency was confirmed by real-time PCR. (D) PC-3 cells transfected with PKCζ siRNA were treated with LPA, followed by flow cytometry.

coupled receptors, $LPA_1$ and $LPA_3$. In addition to platelets, many cancer cells produce LPA as an autocrine mediator, including prostate cancer cells [10]. This self-produced LPA may efficiently stimulate downstream signaling. Taken together, our findings suggest $LPA_1$ and $LPA_3$ as potential targets to control ROS level and signaling pathways related to ROS accumulation. Furthermore, ROS blockage may downregulate VEGF-C expression in PC-3 cells, which in turn may decrease lymphangiogenesis and subsequent metastasis.

PKC is known to cause ROS accumulation by activating the Nox complex [23,31]. PKCζ is an atypical PKC and activates Nox activity to increase ROS levels under high-glucose conditions in mesangial cells [25,26]. Here, we provided evidence that PKCζ is involved in LPA-induced ROS production in PC-3 cells. However, the mechanism by which LPA activates PKCζ in PC-3 cells remains unknown. Since the structure and regulation of atypical PKCs is different from that of classical and novel PKCs [23], the effect of LPA on activating PKCζ requires further investigation.

In conclusion, our study revealed the mechanism of LPA-induced ROS production in PC-3 prostate cancer cells. We identify both $LPA_1$ and $LPA_3$ as important receptors in this LPA-related pathway. PLC participates in this rapid process, while COX-2 and EGFR are not involved. Furthermore, PKC and NAPDH oxidase were confirmed to be involved in this signaling process. PKCζ, one of the PKC subtypes, plays an essential role in LPA-induced ROS generation. These findings may contribute to our understanding of the effect of redox status in late stage prostate cancer and reveal the roles of LPA in the ROS regulation processes.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.09.104.

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