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Inhibition of calcium-independent phospholipase A_2 activates p38 MAPK signaling pathways during cytostasis in prostate cancer cells

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ABSTRACT

The p38 mitogen-activated protein kinase (MAPK) signaling pathways activated during cytostasis induced by Ca^{2+}-independent phospholipase A_2 ($iPLA_2$) inhibition in prostate cancer cells were investigated. $iPLA_2$ inhibition using siRNA, or the selective inhibitor bromoenol lactone (BEL) and it's enantiomers, decreased growth in LNCaP (p53 positive) and PC-3 (p53 negative) human prostate cancer cells. Decreased cell growth correlated to time- and concentration-dependent activation of the mitogen-activated protein kinase p38 in both cell lines. Inhibition of cytosolic $iPLA_2\beta$ using S-BEL, induced significantly higher levels of P-p53, p53, p21 and P-p38 expression than inhibition of microsomal $iPLA_2\gamma$ using R-BEL. Inhibition of p38 using SB202190 or SB203580 inhibited BEL-induced increases in P-p53 (ser15), p53 and p21, and altered the number of cells in G1 in LNCaP cells, and S-phase in PC-3 cells. BEL treatment also induced reactive species in PC-3 and LNCaP cells, which was partially reversed by pretreatment with N-acetyl-cysteine (NAC). NAC subsequently inhibited BEL-induced activation of p38 and p53 in LNCaP cells. In addition, treatment of cells with NAC partially reversed the effect of BEL on cell growth and preserved cell morphology. Collectively, these data demonstrate the novel findings that $iPLA_2$ inhibition activates p38 by inducing reactive species, and further suggest that this signaling kinase is involved in p53 activation, cell cycle arrest and cytostasis.

1. Introduction

Phospholipase A_2 (PLA_2) are esterases that cleave glycerophospholipids at the *sn-2* ester bond to release a fatty acid and a lysophospholipid [1]. Calcium-independent PLA_2 ($iPLA_2$) represent a relatively newer class of PLA_2, compared to the previously characterized cytosolic PLA_2 ($cPLA_2$), and the much older class of secretory PLA_2 ($sPLA_2$) [2]. $iPLA_2$ mediates cell growth in various cell types including kidney cells [3], insulinomas [4] and ovarian cancer cells [5].

We recently demonstrated that pharmacological inhibition of $iPLA_2$ induced cytostasis in prostate cancer cells [6]. In doing so, we used R- and S-enantiomers of bromoenol lactone (R- and S-BEL) to demonstrate roles for both cytosolic $iPLA_2$ ($iPLA_2\beta$, inhibited by S-BEL) and microsomal $iPLA_2$ ($iPLA_2\gamma$, inhibited by R-BEL) in cytostasis. We also used racemic BEL, which inhibits both $iPLA_2\beta$ and γ, to demonstrate that inhibition of $iPLA_2$ in general induces p53-dependent G1 arrest and p53-independent G2/M arrest, and alters the expression of cellular signaling proteins such as the p53 antagonist Mdm2; however, the individual roles of $iPLA_2\beta$ or γ in these cell signaling events were not determined.

Mitogen-activated protein kinases (MAPK) are serine-threonine kinases activated by a cascade of protein–protein interactions. They are crucial mediators of proliferation, adhesion, differentiation, gene expression and apoptosis in several different types of cancer cells [7]. They have also been suggested to mediate the activation of p53 in several studies [8,9]. The most extensively studied MAPK are ERK1/2 (p42/44MAPK), p38 MAPK and c-jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK). MAPK are activated by kinases called MAPK/extracellular signal-regulated kinase or MEK [10].

p38 MAPK and JNK/SAPK are two MAPKs activated in response to cellular stress, inflammation and apoptosis [11,12]. In contrast to ERK, p38 and JNK are commonly known as stress-activated protein kinase (SAPK) as they are activated by diverse cell stresses including irradiation, heat shock, reactive oxygen species, LPS, TNF and IL-1 [13]. The mechanism of activation differs from ERK1/2 as

Abbreviations: ASA, ascorbic acid; ATR, ataxia telangiectasia and rad-3-related kinase; BEL, bromoenol lactone; CM-H2DCFDA, 5-(and-6)-chloromethyl-2,7-dichlorodihydrofluorescein diacetate acetyl ester; ER, endoplasmic reticulum; $iPLA_2$, Ca^{2+}-independent phospholipase A_2; MAPK, mitogen-activated protein kinases; mTOR, mammalian target of rapamycin; NAC, N-acetyl-cysteine; PBS, phosphate-buffered saline; PI, propidium iodide; RS, reactive species.

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p38 and JNK are activated by a different small G-protein called Rac, which activates distinct MEKK and MEK from those involved in ERK1/2 activation [12].

Recent evidence suggests that p38 and JNK regulate anticancer pathways in prostate cancer cells, including cytostasis, cell cycle arrest and apoptosis [8,14–16]. The mechanisms involved are linked to the activation of the tumor suppressor protein p53, which activates proteins involved in cell cycle arrest [8,17], apoptosis [18] and proteins linked to DNA damage such as GADD153 [19,20].

To our knowledge, no study has reported links between $iPLA_2$ and MAPK activation in cancer cells. Recent studies show that thrombin-stimulates both MAPK and $iPLA_2$ activity in ventricular myocytes and vascular smooth muscle cells [21,22]. One of these studies showed that $iPLA_2$ inhibition decreased agonist stimulated MAPK activation [22]. Another study showed that MAPK mediate $iPLA_2$ activity during hypoxia in mouse neural cells [23]. It should be noted that several studies demonstrate links between MAPK and $cPLA_2$ [24–26]; however, $iPLA_2$ and $cPLA_2$ are different proteins, with different substrate preferences, locations within the cell and Ca^{2+} requirements.

While the above studies support the hypothesis that $iPLA_2$ and MAPK mediate cell signaling, they were not performed in cancer cells, nor were the differential roles of $iPLA_2\beta$ or γ investigated. Further, few of these studies investigated the effect of $iPLA_2$ on MAPK activation in the absence of agonists. Such studies are needed to fully understand the role of $iPLA_2$ in cell signaling pathways and its ability to induce p53 and cytostasis. With this hypothesis in mind, we tested the ability of both molecular and pharmacological inhibitors of $iPLA_2$ to alter MAPK in p53-positive prostate cancer LNCaP and p53-negative PC-3 cell lines. Our results suggest the novel finding that $iPLA_2$ inhibition alone activates MAPK and that this activation mediates p53 expression.

2. Materials and methods

2.1. Reagents and antibodies

PC-3 cells (p53 negative human prostate cancer), LNCaP cells (p53 positive human prostate cancer), Ham's F-12K medium, RPMI-1640 and fetal bovine serum were purchased from American Type Culture Collection (Manassas, VA). Penicillin, streptomycin, lipofectamine2000 and Opti-MEM I® media were obtained from Invitrogen (Carlsbad, CA). Mouse anti-p53, p21, Mdm2 and GAPDH monoclonal antibodies, rabbit anti-JNK/SAPK antibodies and goat anti-$iPLA_2\beta$ were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse anti-phospho-p38 (Thr180/Tyr182) and phospho-JNK/SAPK (Thr183/Tyr185) rabbit anti-p38, phospho-ERK1/2 (Thr202/Tyr204) and ERK1/2 antibodies were purchased from Cell Signaling Technology (Danvers, MA). Horseradish peroxidase (HRP)-linked anti-mouse, anti-rabbit and anti-goat IgG were obtained from Promega (Madison, WI). Racemic-bromoenol lactone (BEL), S-BEL ($iPLA_2\beta$ inhibitor) and R-BEL ($iPLA_2\gamma$ inhibitor) were obtained from Cayman Chemical Co. (Ann Arbor, MI). SB202190 and SB203580 were purchased from Calbiochem (San Diego, CA). CM-H2DCFDA was obtained from Invitrogen (Carlsbad, CA), propidium iodide, trypan blue, the bicinchoninic acid assay kit, and all other chemicals not mentioned were obtained from Sigma–Aldrich (St. Louis, MO).

2.2. Cell culture

PC-3 and LNCaP cells were grown under the conditions recommended by ATCC for each cell line. PC-3 cells were cultured in F-12K media supplemented with 10% (v/v) heat inactivated fetal bovine serum (FBS), 100 mg/L streptomycin and 100 U/mL of penicillin G at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. LNCaP cells were grown under the same conditions except that RPMI 1640 media was used. The passages of both cells lines were no more than 30 during all experiments. The doubling time was approximately 35 h for both cell lines. Cells were grown in 12-well plates for immunoblot analysis and cell growth studies. All cells were seeded at 64,000 or 12,800 cells/ml for 24 h prior to pharmacological treatment. Cells were pretreated with respective inhibitors for 30 min prior to treatment, and inhibitors were present throughout the treatment process.

2.3. Assessment of cell morphology

Cell morphology was determined using phase-contrast microscopy as previously described [27] using a Nikon AZ100 fluorescence microscope (Nikon, Melville, NY).

2.4. RNA interference

Cells (at 40% confluence) were transfected with 40 nM of $iPLA_2$-VIA siRNA ($iPLA_2\beta$, Sense: 5′-GGAUCUCAUGCACAUCUCATT-3′, antisense: 5′-UGAGAUGUGCAUGAGAUCCTG-3′ siRNA; 139141; Ambion, Austin, TX) or 40 nM of non-targeting control siRNA (mock-transfected; 12935-300; Invitrogen, Carlsbad, CA). siRNA were diluted in serum-free Opti-MEM I Medium and cells were incubated for 6 h using the Lipofectamine2000 transfection reagent (Invitrogen). After 6 h the siRNA-containing media was replaced with fresh growth media. $iPLA_2$ mRNA and protein levels were determined after 72 and 96 h.

2.5. Real-time PCR

Real-time PCR for assessment of $iPLA_2\beta$ expression was performed as previously described [3]. Briefly, total RNA was isolated using the TRIzol® reagent as directed by the manufacturer (Invitrogen, Carlsbad, CA). cDNA was prepared using the Omniscript RT Kit (Qiagen, Valencia, CA) for 60 min at 37 °C, diluted in the range of $0.1–1.0 \times 10^{-6}$ μg/μl in sterile H_2O, and 25 μl of SuperMix SYBR (Bio-Rad, Hercules, CA) was added along with 1 μl of forward and reverse primers for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or $iPLA_2\beta$ and 5 μl of each cDNA reaction along with sterile H_2O. Negative controls included the absence of cDNA. Each sample was run in duplicate (20 μl per reaction) using a Bio-Rad ICycler sequencer in 96-well PCR plates under the conditions of 95 °C for 3 min, followed by 40 cycles at 95 °C for 10 s and 55 °C for 1 min. For all of the cells, a separate GAPDH reaction was performed for normalization. A standard curve was generated for each set of primers and each set of cells. PCR efficiency was determined by analysis of serial dilutions of cDNA for each primer set. Quantification of RNA was based on comparison of the number of cycles required to reach reference and target threshold values (C_t) as normalized against GAPDH. The primers used for real-time PCR are as follows: GAPDH sense: 5′-AAGGTCGGAGTCAACGGAT-3′, GAPDH antisense: 5′-TGGAAGATGGTGATGGGATT-3′; $iPLA_2\beta$ sense: 5′-TCCTGAAGCGGGAGTTTG-3′, $iPLA_2\beta$ antisense: 5′-GACAGTTTCTG-GAGCATCGTA-3′.

2.6. Immunoblot analysis

After treatment, cells were washed three times using PBS and removed using a rubber policeman in immunoblot buffer (0.25 M Tris–HCl (pH 6.8), 4% SDS, 10% glycerol, 1 mg/ml bromophenol blue and 0.5% (v/v) 2-mercaptoethanol). Protein levels were determined in duplicate wells using lysis buffer that contained 1% (v/v) Triton X-100. Following isolation, cell lysates containing 20 μg total protein was heated to 70 °C for 10 min, separated under reducing conditions on a 12% SDS-polyacrylamide gel, and transferred to a

nitrocellulose membrane. Non-specific binding was blocked by incubating the membrane with 3% (w/v) bovine serum albumin in TBS buffer (10 mM Tris–HCl, pH 7.5, 150 mM NaCl) overnight at room temperature. Membranes were then incubated with the indicated primary antibody for 2 h followed by 2 h incubation in the appropriate secondary antibody. Bands were detected by enhanced chemiluminescence (GE Healthcare, Chalfont St. Giles, UK).

2.7. Cell growth and proliferation analysis

Cell growth and proliferation were determined using cell counting. Cells were seeded at 64,000 cells/ml in 12-well plates (1 ml per well). After the appropriate treatment, cells were trypsinized and constituted in 1 ml cell suspensions. Cells suspensions were stained with trypan blue and living cells (trypan blue unstained) were counted under a microscope using a hemocytometer.

2.8. Cell cycle analysis

Cell cycle was assessed using methods previously described [6,27]. In brief, cells were washed twice with sample buffer [PBS plus glucose (1 g/l)], dislodged using Cellstripper (Mediatech, Herndon, VA), centrifuged at 400 × *g* for 10 min, and suspended in sample buffer. Cells were fixed in ice-cold ethanol (70%, v/v) and stained with propidium iodide (PI) (50 μg/ml) in sample buffer containing RNase A (100 U/ml) for 30 min at room temperature with gentle shaking. Samples were analyzed within 24 h by flow cytometry with a FACSCalibur flow cytometer located in the Center for Tropical and Emerging Diseases at the University of Georgia.

2.9. Reactive species measurement

The generation of reactive species, including those containing oxygen, was measured using CM-H2DCFDA, which is a non-fluorescent chemical whose fluorescence increases as reactive species production increases. The term reactive species is used, as opposed to reactive oxygen species, because CM-H2DCFDA detects several reactive species, including hydrogen peroxide, hydroxyl radical, peroxyl radical and peroxynitrite anion; however it cannot detect all species, most notably superoxide, and may even detect some non-oxygen radicals [28,29]. LNCaP and PC-3 cells were seeded in 48-well plates at a concentration of 64,000–128,000 cells/ml (400 μL per well) and allowed to grow for 24 h. Cultures in 48-well plates were preloaded with 10 μM CM-H2DCFDA for 30 min at 37 °C. After incubation, the media was replaced with fresh serum-free media for 10 min. Cells were pretreated with either NAC or ascorbic acid for 30 min and then exposed to 10 μM BEL or 5 μM S-BEL or 5 μM R-BEL for 2 h. After treatment, media was aspirated and cells detached using 0.01 M Tris–HCl with 0.5% Triton X-100 (pH 7.4). A 100 μl aliquot was used to measure fluorescence at 485 nm excitation and 520 nm emission with a FLUOstar OPTIMA plate reader.

2.10. Protein determination

Protein concentrations were determined using the bicinchoninic acid assay kit provided by Sigma–Aldrich (Sigma procedure TRPO-562) with bovine serum albumin as a standard using methods previously described [30].

2.11. Statistical analysis

Cells isolated from a distinct passage represented one experiment ($n = 1$). Data are represented as the mean ± SD of at least three separate experiments ($n = 3$). The appropriate analysis of variance was performed for each data set using SAS software (SAS Institute, Cary, NC). Individual means were compared using Fisher's protected least significant difference test with $P < 0.05$ considered as indicative of a statistically significant difference between mean values.

3. Results

3.1. Molecular knockdown of iPLA$_2$ inhibits LNCaP and PC-3 cell growth

Our previous study assessed the effect of iPLA$_2$ inhibition on p53 and p21 signaling pathways in prostate cancer cells using

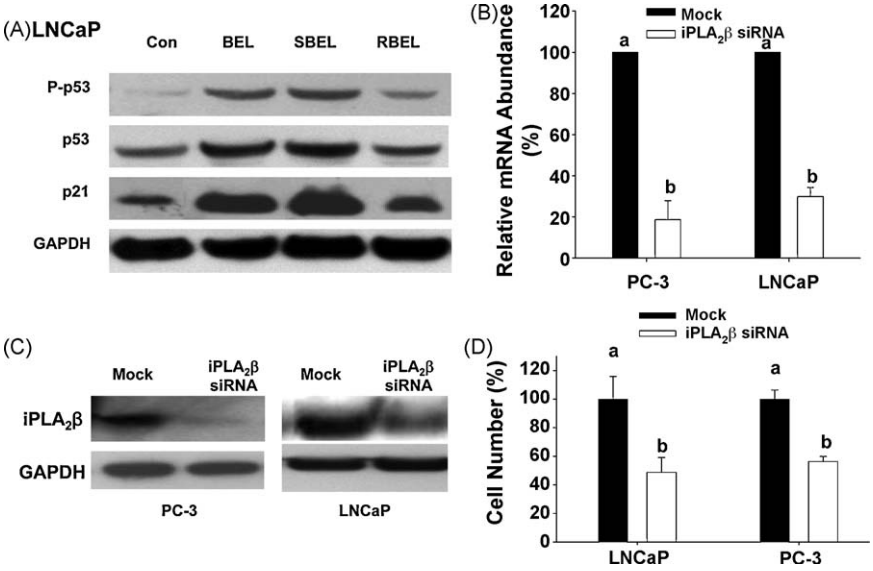

Fig. 1. Inhibition of iPLA$_2$ alters LNCaP and PC-3 cell signaling and growth. (A) LNCaP cells were treated with 10 μM BEL, 5 μM S-BEL or 5 μM R-BEL respectively for 9 h. After treatment, P-p53 (Ser15), p53 and p21 levels were determined using immunoblot analysis, with GAPDH being used as a loading control. iPLA$_2$β siRNA was transfected into PC-3 and LNCaP cells for 72 h followed by real-time PCR analysis (B) or analysis of protein expression after 96 h using immunoblot analysis (C). GAPDH expression is shown as a loading control. All blots are representative of at least three different experiments. (D) iPLA$_2$β siRNA was transfected into PC-3 and LNCaP cells and cells were subcultured into 24-well plates and allowed to grow for 72 h prior to cell counting. Data in B and D are presented as the mean ± SD of at least three separate experiments. Means with different subscripts are significantly different from each other ($P < 0.05$).

racemic BEL, which inhibits both iPLA$_2$β and γ. We also showed that the iPLA$_2$β inhibitor S-BEL, and the iPLA$_2$γ inhibitor R-BEL-induced cytostasis; however, we did not test the ability of R- or S-BEL to alter cell signaling pathways [6]. In separate studies we demonstrated that iPLA$_2$ siRNA decreases activity in multiple cell lines [3,31]; however, these studies did not test the effect of molecular inhibition on cell signaling pathways.

In this study we utilized racemic BEL, R-BEL and S-BEL to study the effect of iPLA$_2$ inhibition on p53 and p21 activation in prostate cancer cells. The concentrations of racemic BEL, R-BEL and S-BEL were based on our previous studies in these same cells [6]. LNCaP and PC-3 cells were used as they represent p53 positive (LNCaP) and negative (PC-3) cells, and are commonly used human prostate cancer cell models. Both racemic BEL and S-BEL increased the expression of phosphorylated-p53 (P-p53), total p53 and p21 after 9 h (Fig. 1A and Supplemental Fig. S1). Treatment of cells with R-BEL only slightly increased the expression of these proteins compared to controls. The levels of induction seen with S-BEL were comparable to racemic BEL. As previously published [6], all of these compounds induced cytostasis and did not induce cell death (data not shown).

Based on the above data, as well as previous studies [3,6,32], we chose to focus our molecular studies on iPLA$_2$β. Transfection of siRNA against iPLA$_2$β into PC-3 and LNCaP cells resulted in 70–80% reductions in the relative mRNA abundance compared to cells transfected with scrambled siRNA (Fig. 1B). The basal overall level of expression of iPLA$_2$β mRNA in control cells did not differ significantly between LNCaP and PC-3 cells (data not shown). Treatment of both cell types with iPLA$_2$β siRNA decreased iPLA$_2$β protein expression as detected using immunoblot analysis (Fig. 1C). Decreases in iPLA$_2$β expression correlated to 50% decreases in cell growth as assessed by cell number after 72 h (Fig. 1D). As previously determined [3,6], iPLA$_2$ inhibition, using siRNA did not induce cell death as assessed by cell morphology and annexin and PI staining (data not shown).

3.2. iPLA$_2$ inhibition induces p38 activation in PC-3 and LNCaP cells

We tested the hypothesis that iPLA$_2$ inhibition alters p38 activation by treating LNCaP and PC-3 cells with racemic BEL, R-BEL, S-BEL, or iPLA$_2$β siRNA, and assessing p38 phosphorylation (Fig. 2). Treatment of both PC-3 and LNCaP cells with racemic BEL caused concentration- and time-dependent increases in the phosphorylation of p38 (P-p38) (Fig. 2A–D). P-p38 was induced as early as 5 min after exposure to BEL, and remained elevated after 2 h of exposure. Concentrations of BEL as low as 2.5 μM also increased p38 phosphorylation after 30 min (Fig. 2B and D). These data were confirmed by siRNA studies demonstrating that transfection of cells with iPLA$_2$β siRNA increased p38 phosphorylation compared to cells transfected with scrambled

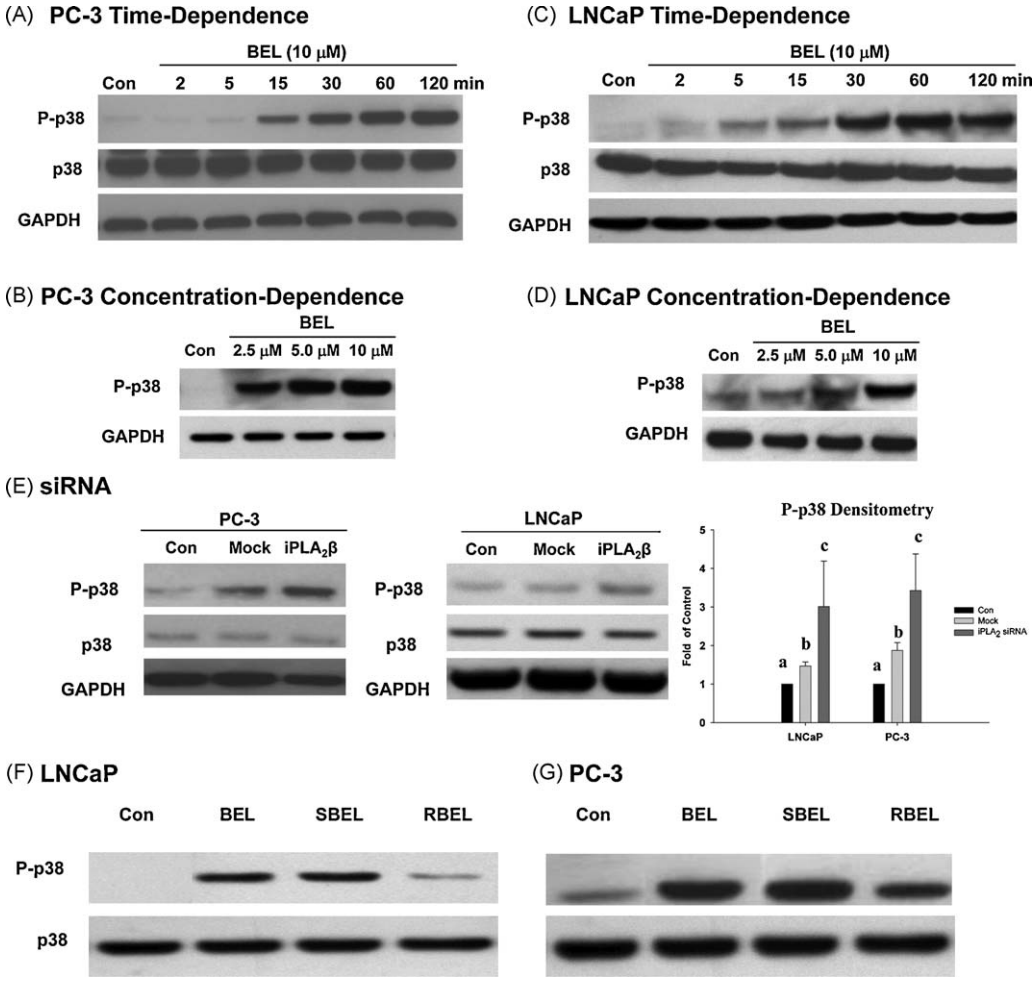

Fig. 2. Effect of iPLA$_2$ inhibition on p38 activation in PC-3 and LNCaP cells. PC-3 cells (A and B) or LNCaP cells (C and D) were exposed to 10 μM BEL for either 0–120 min (A and C) or to 0–10 μM BEL for 30 min (B and D), prior to analysis of p38 phosphorylation (P-p38 at Thr180/Tyr182) using immunoblot analysis. (E) iPLA$_2$β siRNA was transfected into PC-3 and LNCaP cells for 96 h prior to isolation of cell lysate and analysis of P-p38 and p38 expression. GAPDH expression is shown as a loading control. The densitometry of P-p38 is also shown using regular p38 for normalization. LNCaP cells (F) and PC-3 cells (G) were treated with 10 μM BEL, 5 μM S-BEL or 5 μM R-BEL, respectively, for 2 h followed by analysis of p38 and P-p38 using immunoblot analysis. All blots are representative of at least three different experiments.

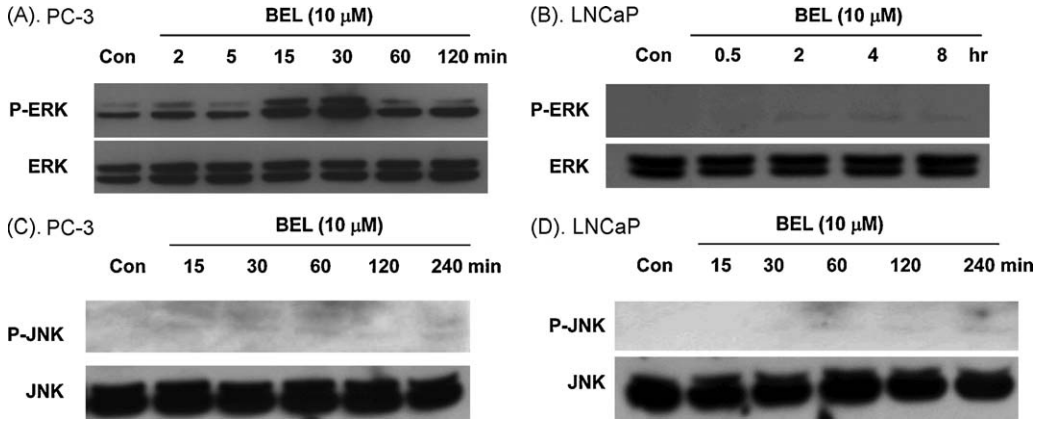

Fig. 3. Effect of iPLA$_2$ inhibition on ERK1/2 and JNK activation in PC-3 and LNCaP cells. PC-3 (A) and LNCaP cells (B) were exposed to 10 μM BEL for 0–120 min (PC-3 cells) or 0–8 h (LNCaP cells only), followed by analysis of P-ERK1/2 (Thr202/Tyr204) expression using immunoblot analysis. Total ERK1/2 expression is shown as a loading control. PC-3 cells (C) and LNCaP (D) were exposed to 10 μM BEL for 0, 15, 30, 60, 120 or 240 min followed by analysis of P-JNK (Thr183/Tyr185) expression using immunoblot analysis. Total JNK expression is shown as a loading control. The two observable bands are JNKI (46 kDa) and JNKII (54 kDa) respectively. All blots are representative of at least three different experiments.

control siRNA (Fig. 2E). To study the differential roles of iPLA$_2$β and γ in these processes, we assessed p38 phosphorylation in cells treated with racemic BEL, S-BEL or R-BEL. The results show that S-BEL-induced higher levels of P-p38 compared to R-BEL (Fig. 2F and G). The level of p38 phosphorylation seen with S-BEL was similar to that seen with racemic BEL. Collectively, these data demonstrate the novel finding that inhibition of iPLA$_2$β alone induced p38 activation in prostate cancer cells. In contrast to p38, BEL only transiently increased ERK1/2 phosphorylation in PC-3 cells (Fig. 3A), and did not induce ERK1/2 in LNCaP cells (Fig. 3B). siRNA against iPLA$_2$β did induce slight increases in P-ERK after 96 h in PC-3 cells (Supplemental Fig. S2). Additionally, BEL did not significantly induce JNK activation in either cell type within 4 h (Fig. 3C and D). Based on these data we focused our studies on p38.

3.3. Inhibition of p38 alters BEL-induced p53 and p21 activation and cell cycle

To investigate the functional role of p38 on p53 and p21 expression we exposed LNCaP cells to the p38 specific inhibitor SB202190 or SB203580 prior to exposure to BEL. LNCaP cells were used, as opposed to PC-3 cells, because PC-3 cells do not express p53. As previously demonstrated, treatment of LNCaP cells with BEL (10 μM) increased p53 expression, compared to control cells (Fig. 4A). BEL also induced p53 activation based on increase in

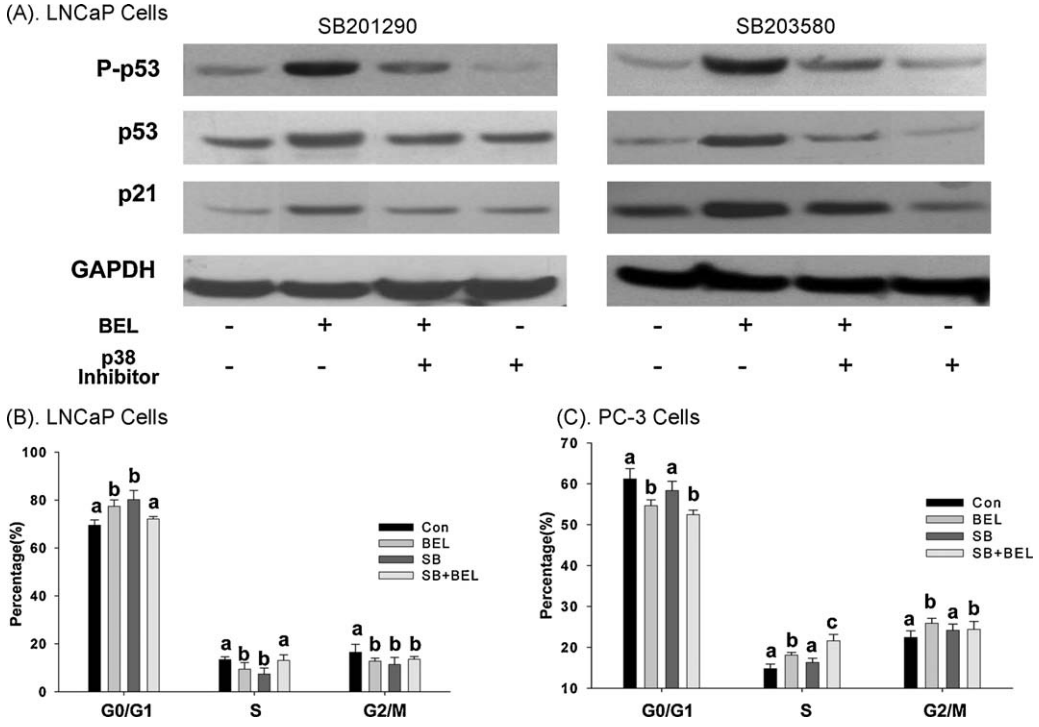

Fig. 4. Effect of p38 inhibitors on BEL-induced p53 and p21 expression and cell cycle in PC-3 and LNCaP cells. (A) LNCaP cells were treated with SB202190 (10 μM) or SB203580 (10 μM) for 30 min prior to exposure to 10 μM BEL for 12 h, followed by analysis of P-p53 (ser15) p53 and p21 expression using immunoblot analysis. GAPDH expression is shown as a loading control. LNCaP (B) and PC-3 cells (C) were treated with SB202190 (10 μM) for 30 min prior to exposure to 10 μM BEL for 24 h, followed by analysis of cell cycle using flow cytometry. All blots are representative of at least three different experiments. Data in B and C are presented as the mean ± SD of at least three separate experiments. Means with different subscripts are significantly different from each other ($P < 0.05$).

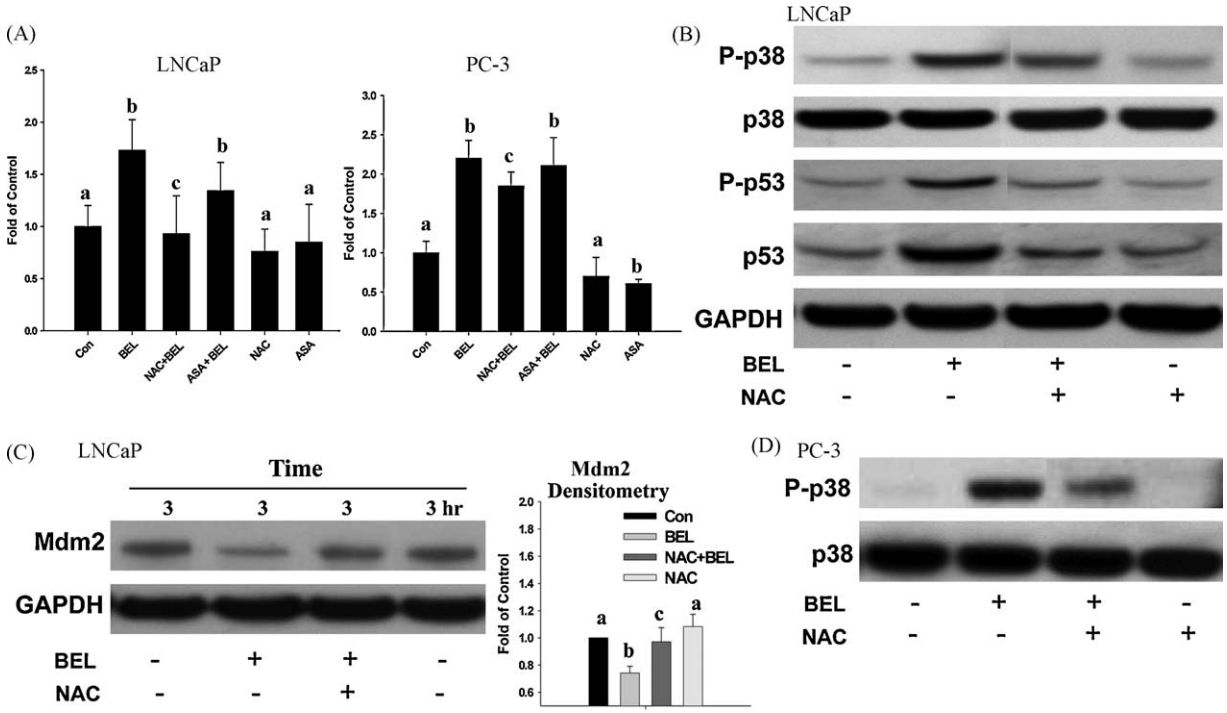

Fig. 5. BEL treatment induces reactive species in prostate cancer cells. (A) LNCaP and PC-3 cells were loaded with CM-H2DCFDA for 30 min, followed by exposure to 2.5 mM NAC or 0.5 mM ascorbic acid (ASA) for 30 min prior to exposure to 10 μM BEL for 2 h and analysis of fluorescence at 485 nm excitation and 520 nm emission. (B) LNCaP cells were treated with NAC for 30 min prior to exposure to 10 μM BEL and analysis of p38, P-p38, P-p53 (ser15), p53 and GAPDH expression after 2 h (p38 and P-p38) or 8 h (P-p53, p53 and GAPDH). (C) LNCaP cells were treated with 2.5 mM NAC for 30 min prior to exposure to BEL for 3 h and analysis of Mdm2 expression. (D) PC-3 cells were treated with 2.5 mM NAC for 30 min prior to treatment with 10 μM BEL and analysis of p38 and P-p38 expression after 2 h. Data in A are presented as the mean ± SD of at least three separate experiments. Means with different subscripts are significantly different from each other ($P < 0.05$). All blots are representative of at least three different experiments.

phospho-p53 (Ser15) (Fig. 4A). In contrast, exposure of cells to SB202190 (10 μM) or SB203580 (10 μM) for 30 min prior to BEL treatment decreased p53 and phosho-p53 (Ser15) expression, compared to cells treated with BEL alone (Fig. 4A). Similar results were seen for p21 expression. Interestingly, SB202190 did not alter the basal level of p53 or p21 expression, while SB203580 slightly decreased these levels.

To test the functional role of p38 in cell cycle alterations induced by $iPLA_2$ inhibition we exposed cells to either solvent controls or SB202190 for 30 min prior to exposure to BEL (10 μM) for 24 h. BEL treatment caused a small, but significant increase in cells in G1 in LNCaP cells (Fig. 4B). Interestingly, there was also an increase in the number of cells in the G1 phase after exposure to SB202190 alone. In contrast, treatment of cells with both SB202190 and BEL decreased the percent cells in G1, compared to cells exposed to either BEL or SB202190 alone.

Even though PC-3 cells do not express p53, we still determined the effect of SB202190 on BEL-induced G2/M arrest (Fig. 4C). PC-3 cells treated with BEL (10 μM) for 24 h exhibited an increase in cells in both the S and G2/M phases of the cell cycle, compared to control (Fig. 4C). Interestingly, treatment of cells with SB202190 prior to exposure to BEL, actually increased the percent of cells in S-phase compared to cells exposed to BEL alone, but had no effect on BEL-induced increase in the G2/M phase. We also tested the hypothesis that $iPLA_2$ inhibition-induced cytostasis was altered by p38 inhibitors; however, we could draw no conclusions from these studies because the p38 inhibitors alone were cytostatic (Supplemental Fig. S3).

3.4. Inhibition of $iPLA_2$ induced reactive species in prostate cancer cells

We investigated the hypothesis that $iPLA_2$ inhibition activates p38 by reactive species-mediated mechanisms because several studies demonstrate that MAPK are activated by reactive species [10,33,34]. To do this, LNCaP and PC-3 cells were exposed to 10 μM BEL for 2 h and intracellular reactive species were assessed using CM-H2DCFDA staining. BEL treatment alone significantly increased reactive species levels in both cell lines (Fig. 5A). Treatment with S-BEL also increased reactive species formation to levels comparable to that seen with racemic BEL alone, while R-BEL only had a marginal affect (Supplemental Fig. S4). Treatment of both cell lines with NAC prior to exposure to BEL significantly decreased BEL-induced reactive species (Fig. 5A). In contrast, ascorbic acid (ASA) did not alter the formation of reactive species in the presence of BEL. Larger decreases were seen in LNCaP cells compared to PC-3 cells; however, the decreases in PC-3, albeit small, were still statistically significant. Additionally, treatment of LNCaP cells with NAC, prior to BEL exposure, decreased BEL-induced increases in phosphorylated-p38, phosphorylated-p53 (ser15) and p53 (Fig. 5B). NAC treatment also reversed BEL mediated decrease in the p53 antagonist Mdm2 in LNCaP cells (Fig. 5C) and inhibited the ability of BEL to induce p38 phosphorylation in PC-3 cells (Fig. 5D).

3.5. Treatment with NAC reverses the effect of BEL on cell growth and morphology

To test the functional consequence of NAC on cell growth we exposed LNCaP and PC-3 cells in log phase growth to 2.5 mM NAC for 30 min prior to exposure to 5 μM BEL for 48 h. Analysis of cell morphology showed that BEL decreased growth in both cell types (Fig. 6). This finding was supported by cell counts (Fig. 6B and D). Treatment with 2.5 mM NAC alone did not significantly alter cell morphology or growth; however, NAC inhibited the ability of BEL to reduce cell number.

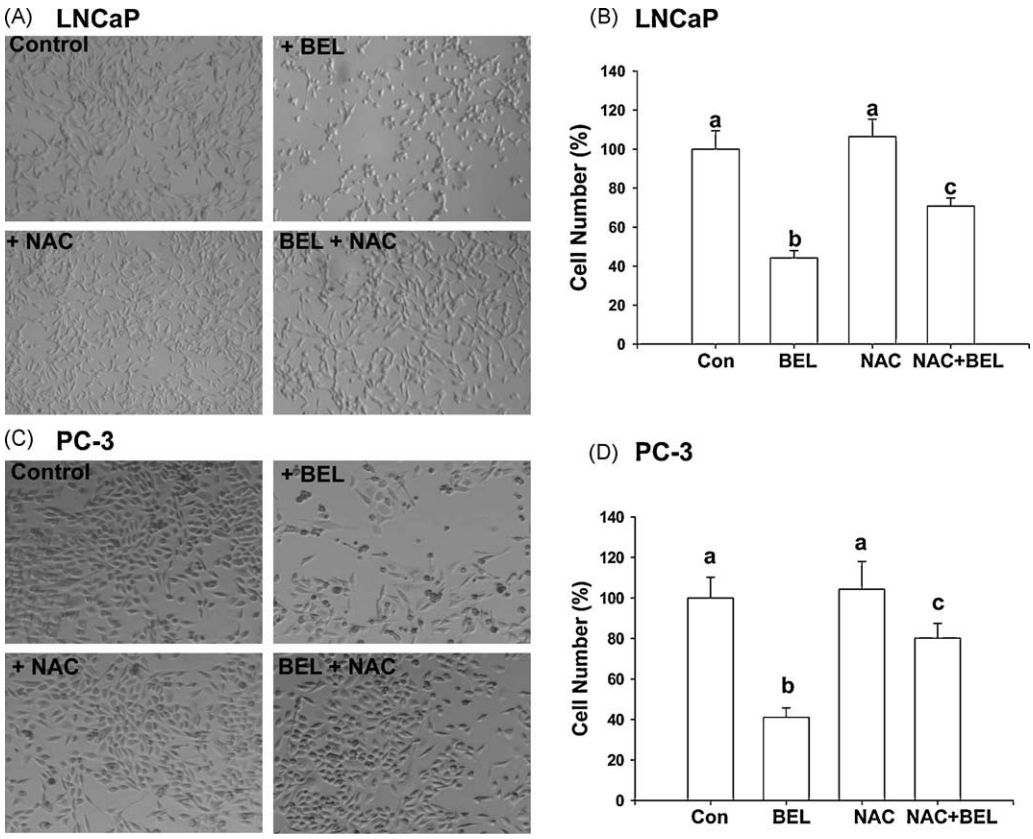

Fig. 6. Treatment with NAC reverses the effect of BEL on cell growth and morphology. LNCaP and PC-3 cells were seeded in 12-well plates for 24 h after which media was replaced with serum-free media. After 2 h, cells were treated with vehicle (DMSO), 5 μM BEL, 2.5 mM NAC, or NAC plus BEL. After 48 h, the cells were visualized using microscopy at 25× magnification, followed by analysis of cell number (C and D). Data in A and B are representative of at least three different experiments. Data in C and D are presented as the mean ± SD of at least three separate experiments. Means with different subscripts are significantly different from each other ($P < 0.05$).

4. Discussion

$iPLA_2$ is essential to phospholipid remodeling in many cells [35]. It also mediates cell growth in many cell types, including pancreatic, ovarian, kidney and prostate cancer cells [5,6,31,32]. The cell signaling events involved are only just beginning to come to light but include alterations in p53, p21 and cell cycle progression. Roles for MAPK or reactive species in such events have not been previously reported.

Our previous work demonstrated that both S-BEL and R-BEL decreased LNCaP and PC-3 cell growth [6], but their differential roles on cell signaling were not addressed. Results from this current study support the conclusions that S-BEL has a more prominent effect on p53, p38 MAPK and p21 expression and reactive species formation than R-BEL. The effect of S-BEL on cell signaling was validated using siRNA against $iPLA_2\beta$. These results support the hypothesis that $iPLA_2\beta$ plays a more prominent role in p53, p21 and p38 signaling than $iPLA_2\gamma$ during $iPLA_2$ inhibition.

It is important to point out that these data do not suggest that $iPLA_2\gamma$ has no roles in cell signaling during cytostasis. That fact R-BEL alone induces some levels of p38, p53 and p21 expression supports this hypothesis. Further, R-BEL does decrease cell growth in several cell types, as does $iPLA_2\gamma$ siRNA; however, typically not as effectively as S-BEL [3,6]. The differential effects of $iPLA_2\gamma$ inhibition, compared to $iPLA_2\beta$, may result from the exclusive membrane location of $iPLA_2\gamma$, which is confined to the endoplasmic reticulum, the mitochondria and peroxisomes [36–38]. Thus, $iPLA_2\beta$ cytosolic location may allow it greater access to cellular membranes, especially the plasma and nuclear membranes. This may account for the greater effect of S-BEL on p38 MAPK. It is also possible that $iPLA_2\gamma$ may activate a differential set of signaling pathways, the identity of which are the subject of future studies.

These results directly demonstrate that p53 is not required for $iPLA_2$ inhibition to induce cytostasis. Such results are promising as a majority of malignant prostate tumors are negative for p53 [39]. These results also demonstrate the novel finding that $iPLA_2$ inhibition induces p38 activation in two different human prostate cancer cell models. Further, the data suggest that $iPLA_2$ may regulate p38 MAPK. These results are pertinent because p38 are often involved in cell death and senescence [13].

The ability of $iPLA_2$ inhibitors to activate p38 in prostate cancer cells leads to questions about its function under these conditions. The ability of either SB202190 or SB203580 to decrease BEL-induced p53 phosphorylation (ser15) and p53 expression in LNCaP cells suggests that p38 is being used to activate this tumor suppressor protein. The decrease in p21 expression induced by p38 inhibitors in the presence of BEL is mostly likely a result of p53 inhibition. Decreases in either p53 or p21 are mostly likely the cause of alterations in cell cycle in cells treated with SB202190 or SB203580 prior to BEL exposure.

Neither SB203580 nor SB202190 completely blocked BEL induction of p53. This suggests that other pathways for p53 activation exist during $iPLA_2$ inhibition. This hypothesis is supported by a recent study reporting that ATR (ATM and Rad3-related kinase) mediates $iPLA_2$ inhibition-induced p53 induction in insulinoma cells [40]. Interestingly, we found that SB202190 plus caffeine, an ATR inhibitor, blocked BEL activation of p53, while neither of those two totally reversed p53 by themselves (Supplemental Fig. S5). These data suggest that both ATR and p38 may mediate p53 activation in prostate cancer cells.

Pretreatment of LNCaP cells with SB202190 reversed the increase in G1 cells induced by BEL. Reasons why BEL reverses the effect of SB202190 on cell cycle alterations are not currently known. One hypothesis is that $iPLA_2$ may also act as a downstream effector of p38 via a negative feedback mechanism [22,23]. We were unable to directly study the effect of p38 inhibitors on cytostasis induced by $iPLA_2$ inhibition as these compounds were cytostatic on their own (Supplemental Fig. S3). This limitation will hopefully be addressed in future studies.

Interestingly, BEL transiently induced P-ERK1/2 in PC-3 cells but not in LNCaP cells, as did $iPLA_2\beta$ siRNA (Supplemental Fig. S2). Reasons for the differential induction of P-ERK1/2 may be related to the fact that PC-3 cells express a higher basal level of ERK1/2 (Fig. 3A and B). The role of ERK1/2 activation may be as a survival signal to resist the stress of $iPLA_2$ depletion. This hypothesis is supported by data demonstrating that the ERK1/2 selective inhibitor PD98059 intensified BEL-induced cell cytostasis (Supplemental Fig. S2B).

Reactive species, including those containing oxygen, are major stimulators of p38 [13]. The ability of NAC to inhibit BEL-induced increases reactive species, in correlation with p38 activation, suggests that $iPLA_2$ inhibition induces p53 using reactive species-mediated activation of p38. This hypothesis is supported by the fact that NAC inhibited BEL-induced decreases in Mdm2 in correlation with p53 expression.

How exactly $iPLA_2$ inhibition induces reactive species, and what type of species is induced, is under study. Inhibition of $iPLA_2$ increases lipid peroxidation in several cell models [36,38,41]. Thus, lipid peroxides may be mediating the cascades of signaling events seen during inhibition. Another possibility is BEL is inactivating cys/thiol groups near the active site of $iPLA_2$, which mediates cellular redox regulation [42]. This action may increase thiol oxidation. This hypothesis is supported by the fact that NAC, but not ASA, altered BEL-induced cell signaling and cytostasis; however, it should be pointed out that NAC also decreases lipid peroxidation.

It should be noted that cysteines are not directly targeted by BEL [43]. In fact, BEL itself does not interact with cysteine in $iPLA_2$. Rather, the specific reaction of BEL with cysteine bonds in $iPLA_2$ results from the ring opening of BEL, which results from serine-mediated hydrolysis. This is believed to form a specific reactive α-bromoketone that alkylates cysteines located in $iPLA_2\beta$ [42].

To our knowledge, the finding that NAC almost completely reverses the growth inhibition effect of $iPLA_2$ inhibition is a novel finding. Several studies have attempted to reverse $iPLA_2$ inhibition-induced cytostasis with only moderate success. For example, neither LPA nor lyso-phosphatidylcholine, metabolites of phospholipid metabolism by $iPLA_2$, reversed the effect of BEL on INS-1 cell proliferation [4]. Another study in ovarian cancer cells demonstrated that LPA only had a "weak" reversal effect on BEL-induced decreases in cell proliferation, and did not release cells from BEL-induced G2/M arrest [5]. Unpublished studies from our laboratory determined that p53 inhibition, calcium chelation, ER calcium depletion or epidermal growth factor activation also failed to reserve the effect of BEL on cell growth in prostate cancer cells (data not shown). Similarly, arachidonic acid, LPA and lyso-phosphocholines also gave negative results. MAPK inhibitors, including the p38 inhibitors used in this study, were partially effective at reversing cell cycle arrest.

The above data, in combination with the robust effect of NAC on reactive species, p38, p53 and p21, support the hypothesis that inhibition of $iPLA_2$ decreases cell proliferation via mechanisms that are not directly related to the production of mitogenic lipid signals. Rather, we propose that inhibition of $iPLA_2$ increases reactive species that initiate a broad series of cell signaling events including MAPK, p53 and p21 activation (Fig. 7). The nature of the reactive species is probably thiol based on the ability of NAC, but not ascorbic acid, to alter these signaling events and cell growth. The p53-independent mechanisms used by $iPLA_2$ inhibitors to decrease PC-3 cell growth remain undetermined, but may also involve p38 and reactive species, which also increased in PC-3 cells. As mentioned above, p38 is a stress-induced kinase and has multiple targets other than p53, including many pro-apoptotic proteins located in the mitochondria, mTOR and ER stress proteins [34,44,45]. $iPLA_2$ is expressed in both the ER and mitochondria, so it is possible that $iPLA_2$ inhibition decreases cell growth by ER- or mitochondrial dysfunctions.

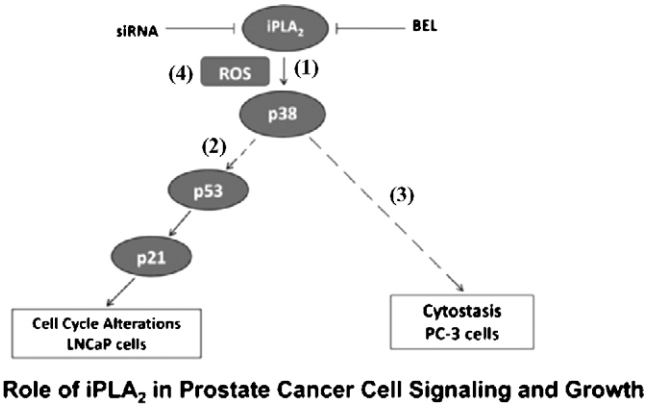

Fig. 7. Proposed pathway for activation of MAPK in prostate cancer cells during $iPLA_2$ inhibition. (1) Inhibition of $iPLA_2$ using either BEL or siRNA results in activation of p38 in both LNCaP and PC-3 cells. (2) Activation of p38 in LNCaP cells increases expression of p53 and p21 and induces cell cycle alterations. (3) In PC-3 cells the mechanisms involved in decreased cell growth are unknown. (4) Reactive species may be involved in BEL-induced p38 activation. The dashed lines represent possible indirect interactions.

In conclusion, this study used both molecular and pharmacological techniques to demonstrate that $iPLA_2$ inhibition-induced cytostasis in prostate cancer cells and demonstrated the novel finding that p38 is activated under these conditions. Inhibition of p38 altered cell cycle arrest induced by $iPLA_2$ inhibition, and decreased activation of p53 and p21 in LNCaP cells. Activation of p38 correlated to reactive species formation, which was inhibited by NAC, but not ascorbic acid. NAC reversed the effect of BEL on cell proliferation. These data support the hypothesis that $iPLA_2$ mediates cell growth in prostate cancer cells, identifies novel signaling pathways mediated by this protein, and suggests that thiol-based reactive species may be involved.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bcp.2010.02.005.

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