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Research Paper

Silibinin Impairs Constitutively Active TGFα-EGFR Autocrine Loop in Advanced Human Prostate Carcinoma Cells

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Purpose. Epidermal growth factor (EGF) and transformation growth factor-α (TGFα) are potent mitogens that regulate proliferation of prostate cancer cells via autocrine and paracrine loops, and promote tumor metastasis. They exert their action through binding to the cell surface receptor, epidermal growth factor receptor (EGFR), and cause activation of Erk1/2 mediated mitogenic signaling in human prostate cancer (PCA) at both advanced and androgen-independent stages. Thus, we rationalized that inhibiting this mitogenic pathway could be useful in controlling advanced PCA growth.
Methods. LNCaP and DU145 human PCA cells were treated with silibinin (100–200 μM) for different time points, and the levels of TGFα, activated signaling molecules (EGFR, Erk1/2 and Jnk1/2) and Erk1/2 kinase activity were analyzed employing ELISA, immunoprecipitation and/or immunoblotting techniques. The mRNA levels of TGFα were analyzed by RT-PCR.
Results. Treatment of cells (LNCaP and DU145) with silibinin resulted in a decrease in TGFα protein at both secreted and cellular levels together with a decrease in its mRNA level. Silibinin also caused an inhibition of EGFR activation followed by that of Erk1/2 without any change in their protein levels. The kinase activity of Erk1/2 to Elk1 was also inhibited by silibinin at least in DU145 cells. In other study, silibinin caused strong inhibition of Jnk1/2 activation in LNCaP cells while in DU145 cells, a strong induction in Jnk1/2 activation was observed. These results suggest that silibinin impairs TGFα-EGFR-Erk1/2 signaling in both androgen-dependent (LNCaP) and -independent (DU145) advanced human prostate carcinoma cells.
Conclusions. This study, for the first time, identifies the inhibitory effect of silibinin on constitutively active TGFα-EGFR autocrine loop in advanced human PCA cells, which plausible contributes to the strong efficacy of silibinin in PCA prevention and intervention, as reported in recent studies.

KEY WORDS: EGFR; Erk1/2; prostate cancer; silibinin; TGFα.

INTRODUCTION

Prostate cancer (PCA) is the most common non-skin malignancy and the second leading cause of cancer death among men in the United States with an estimated 218,890 new cases this year which will take the lives of an estimated 27,050 people (1). The incidence of PCA shows strong age, race and geographical dependence (2). This disease is uncommon in Asian population; high in Scandinavian countries; and the highest incidence rates are in African American men; the latter being two-fold higher than for American white (2). Other possible risk factors include diet, lifestyle and environmental factors (3). Progression of PCA depends on both genetic and epigenetic factors. Epigenetic mechanisms may be the main driving force for the major changes in gene expression that are responsible for progression of PCA. One of the characterized mechanisms for epigenetic gene regulation is alteration in regulatory feedback loops for growth factors including a shift from paracrine to autocrine control where the receptor and the ligand are produced by the same cells (4).

Epidermal growth factor (EFG) is a mitogenic polypeptide that binds with the EGF receptor (EGFR) and regulates the cellular process (5,6 and references therein). After ligand binding, the EGF receptor is autophoshorylated at tyrosine residues; however, an unrestricted activation may allow the cells to proliferate in the absence of EGF, which indeed could be the case in PCA (5,6 and references therein). A malignant transformation may be associated with the production of growth promoting polypeptide that bind and activate EGFR. Transforming growth factor α (TGFα), a 50 amino acid polypeptide that is structurally similar to EGF (7), has been shown to bind with and activate EGFR, and several studies have identified an autocrine growth factor-receptor interaction among them in both advanced and androgen-independent human prostate carcinoma tumors and derived cell lines (8–10). Together, these studies suggest that targeting epigenetic events such as impairment of tyrosine kinase activity and associated mitogenic signaling pathways could be a practical

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ABBREVIATIONS: EGFR, epidermal growth factor receptor; Erk, extracellular signal-regulated kinase; Jnk, c-Jun N-terminal protein kinase; PCA, prostate cancer; TGFα, transforming growth factor-α.

and translational approach to control the growth and metastatic potential of PCA in humans. Indeed, several naturally occurring agents and those consumed as dietary supplements have received increased attention in recent years to control growth and therapy of PCA by targeting various signaling pathways and the events down-stream of them (10). One such agent is silibinin, which inhibits the growth of various cancer cell lines and tumors in several models including prostate (11–14). Silibinin is a flavonolignan and a major component in silymarin which is a complex mixture of flavonolignans and polyphenols present in milk thistle seeds; and has been extensively used in patients with liver disease for decades (15). In recent studies, silibinin has shown strong anticancer efficacy against both androgen-dependent and -independent advanced human PCA cells (16,17), and accordingly, here we assessed whether the biological activity of silibinin in these cells involves its effect on the production of TGFα and inactivation of constitutively active EGFR and Erk1/2 pathway.

MATERIALS AND METHODS

Cell Lines and Reagents

Human PCA cell lines LNCaP and DU145 were purchased from American Type Culture Collection (Manassas, VA). Silibinin used in the present study was from Sigma-Aldrich chemical company (St Louis, MO). Primary antibodies to phospho- and total Erk1/2 and Jnk1/2, and secondary anti-rabbit antibody were from Cell Signaling, Inc. (Beverly, MA). Antibody for phospho-EGFR was from BD Biosciences (San Diego, CA) and for anti-EGFR was from Millipore (Billerica, MA). Antibody for β-actin was from Sigma Aldrich. Secondary anti-mouse antibody and ECL detection system were from Amersham (Arlington Heights, IL).

Cell Culture and Treatments

LNCaP and DU145 cells were cultured in RPMI-1640 medium containing 10% fetal bovine serum and 1% penicillin–streptomycin under standard culture conditions (37°C, 95% humidified air and 5% CO_2). At 60% confluency, cultures were treated with desired doses of silibinin (50–200 μM, final concentrations in medium) dissolved in DMSO or DMSO alone for different time points (12–72 h), and cell lysates were prepared in non-denaturing lysis buffer (10 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1% TritonX-100, 1 mM EDTA, 1 mM EGTA, 0.3 mM phenyl methyl sulfonyl fluoride, 0.2 mM sodium orthovanadate, 0.5% NP-40, 5 U/ml aprotinin), as reported earlier (16,17). Protein concentration in lysate was determined using Bio-Rad DC protein assay kit (Bio-Rad laboratories, Hercules, CA) by the Lowry method.

Immunoblot Analysis

Total cell lysates were denatured with 2×-sample buffer, and samples were subjected to SDS-PAGE on 8 or 12% Tris–glycine gels, and separated proteins were transferred onto membrane by Western blotting. Membranes were blocked with blocking buffer for 1 h at room temperature, and probed with primary antibody against desired molecule over night at 4°C followed by peroxidase-conjugated appropriate secondary antibody for 1 h at room temperature and ECL detection.

Erk1/2 Kinase Activity Assay

To assess Erk1/2 kinase activity to Elk1, a nonradioactive assay was used employing a commercial kit and the protocol provided by the vendor (Cell Signaling, Inc., Beverly, MA). Briefly, to immunoprecipitate active MAPK Erk1/2, 200–250 μg protein/cell lysates was incubated with monoclonal phospho-p44/42 MAP kinase antibody, and the resulting immunoprecipitates were then incubated with an Elk1 fusion protein substrate in the presence of ATP and kinase buffer for 30 min at 37°C. The reaction was stopped by adding sample buffer and boiling the samples, and the phosphorylation of Elk1 was then measured by Western blotting using a phospho-Elk1 antibody.

Reverse Transcription Polymerase Chain Reaction (RT-PCR) Assay

Total RNA was extracted with Tri-Zol (Invitrogen, Carlsbad, CA), and the reverse-transcription (RT) reaction was done by using the kit and protocol from Clontech (Mountain View, CA). In brief, the reaction was performed in total 20 μl volume using 1 μl oligo dT primer, 4 μl 5X reaction buffer, 1 μl dNTP mix (10 mM), 0.5 μl recombinant RNase inhibitor and 1 μl MMLV reverse-transcriptase. Five microliters of cDNA obtained in RT was subjected to PCR using the kit and the protocol from Clontech. In brief, the reaction was performed to amplify TGFα and G3PDH products in total 50 μl volume using 1 μl dNTP mix (10 mM), 1 μl 5′ primer (0.4 μM), 1 μl 3′ primer (0.4 μM; oligonucleotide primers specific for human TGFα or human G3PDH) and 5 μl Advantage Taq polymerase in PCR system 9700 PE Applied Biosystem. The primer sequences for TGFα were as: upstream (5′) primer 5′ATGGTCCCCTCGGCT GGACAG 3′ and downstream (3′) primer 5′ GGCCTGCTT CTTCTGGCTGGCA 3′, yielding a product of 297 bp. The G3PDH primers were as: upstream (5′) primer 5′ TGAAGGTCGGAGTCAACGGATTTGGT 3′ and downstream (3′) primer 5′ CATGTGGGCCATGAGGTCCA CCAC 3′, yielding the product of 983 bp. Thirty cycles of PCR were utilized at 94°C for 30 s, 68°C for 3 min, 72°C for 2 min, and finally soaking at 4°C for 20 min. The PCR products were resolved on 1.5% agarose/ethidium bromide gels with 100 bp markers and visualized by UV.

ELISA Assay for TGFα

LNCaP and DU145 cells were grown to 60% confluency in 60 mm dishes under standard culture conditions, and then treated with DMSO alone or varying concentration of silibinin (50, 100, 150 and 200 μM) for varying time points (6–72 h). After these treatments, medium was collected from each dish and stored at −80°C till further assay. At the same time, cells were also collected, washed in ice-cold PBS, and cellular extract was prepared following step by step protocol provided by the manufacture for TGFα ELISA assay kit (Oncogene Research Products, Cambridge, MA). TGFα levels in medium and cellular lysate samples were determined

using the ELISA kit following the protocol provided by the vendor.

Statistical Analysis

Statistical significance of difference between control and treated samples was calculated by one way ANOVA followed by Bonferroni *t*-test for pairwise multiple comparisons. *P* values of less than 0.05 were considered significant. The immunoblot, RT-PCR and ELISA data shown are representative of 2–3 independent experiments with reproducible results.

RESULTS

Silibinin Down-regulates Secreted and Cellular TGFα Levels in Both Androgen-dependent and -independent Human Prostate Carcinoma Cells

Growth factors that act via transmembrane tyrosine kinases are believed to contribute to PCA cell growth and proliferation (18). One of the important growth factors in this process is TGFα, and indeed human prostate tumor-derived cell lines synthesize TGFα in culture (18). Accordingly, in order to assess whether silibinin impairs TGFα-growth factor receptor (e.g. EGFR) autocrine loop in human PCA cells, first we assessed the effect of silibinin on the protein level of TGFα in two different human PCA cell lines namely LNCaP and DU145 cells. In case of LNCaP cells, silibinin treatment at the concentrations of 50, 100, 150 and 200 μM showed a decrease in the levels of TGFα secretion in the range of 2–10% (6 h), 7–11% (12 h), 10–37% ($p<0.01$; 24 h), 33–68% ($p<0.001$; 48 h), and 8–62% ($p<0.001$; 72 h) in culture media (Fig. 1a). Furthermore, under the same silibinin treatment conditions, TGFα cellular levels also decreased by 8%, 16–47% ($p<0.05$), 26–59% ($p<0.05$), 47–72% ($p<0.001$) and 55–80% ($p<0.001$) in cell lysates after 6, 12, 24, 48 and 72 h treatment, respectively (Fig. 1b). Similarly in DU145 cells, the same concentrations of silibinin (50–200 μM) showed a strong decrease in secreted TGFα levels in culture media by 5–28% ($p<0.05$; 6 h), 5–22% ($p<0.05$; 12 h), 27–54% ($p<0.01$; 24 h),

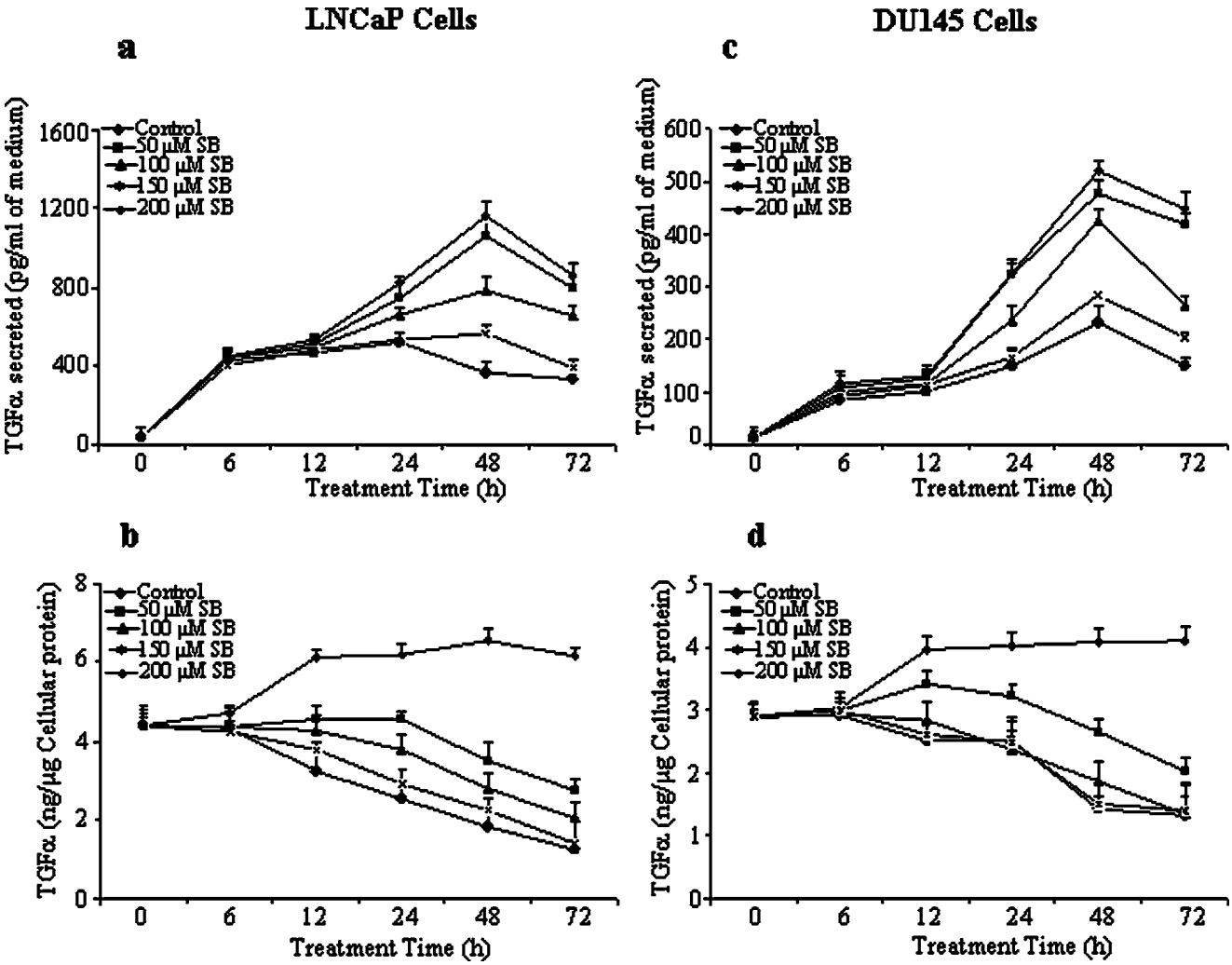

Fig. 1. Silibinin down-regulates secreted and cellular TGFα levels in both androgen-dependent and -independent human prostate carcinoma cells. *LNCaP* (**a** and **b**) and *DU145* (**c** and **d**) cells were treated with either DMSO alone (*control*) or varying concentrations of silibinin for 6, 12, 24, 48 and 72 h as detailed in "MATERIALS AND METHODS". At the end of the treatments, media and cells were collected and TGFα was determined by ELISA using the commercial kit. The data shown are the mean±SE of two independent experiments each done in triplicate. *SB* silibinin

8–55% ($p<0.01$; 48 h), and 7–66% ($p<0.001$; 72 h; Fig. 1c). In addition, as shown in Fig. 1d, cellular protein level of TGFα went down after silibinin (50–200 μM) treatment in the range of 13–25% ($p<0.05$), 20–38% ($p<0.05$), 34–62% ($p<0.05$) and 50–65% ($p<0.001$) for 12, 24, 48 and 72 h, respectively, without an affect at 6 h.

Silibinin also Down-regulates TGFα mRNA Levels in Both Androgen-dependent and -independent Human Prostate Carcinoma Cells

Since we found that silibinin treatment decreases both secreted and cellular TGFα protein levels in LNCaP and DU145 cells, we also evaluated whether this decrease was mediated at the level of transcription by a decrease in TGFα mRNA levels. As shown in Fig. 2a, similar silibinin treatments for 24 and 48 h resulted in a strong and a dose-dependent decrease in TGFα mRNA (24–80%, $p<0.001$) as compared to DMSO-treated control. Importantly, 200 μM dose of silibinin treatment for 48 h caused almost complete decrease in TGFα mRNA levels (98%, $p<0.001$, Fig. 2a) as compared to DMSO-treated control. As expected, silibinin also showed a strong decrease in the TGFα mRNA expression in DU145 cells, though not as profound as in LNCaP cells. As shown in Fig. 2b, in DU145 cells, the same concentration of silibinin (100–200 μM) caused a decrease in TGFα mRNA levels by 10–45% ($p<0.05$; 12 h), 10–58% ($p<0.01$; 24 h), and 41–80% ($p<0.001$; 48 h) as compared to DMSO control (Fig. 2b). Together, these results suggest that silibinin decreases both mRNA and protein (secreted and cellular) levels of TGFα in human PCA cells, which might have an important role in overall effect of silibinin on impairing growth factor-receptor autocrine loop that is constitutively active in these malignant cancer cell lines. Studies were done next to address this anticipation.

Silibinin Inhibits EGFR Activation in Both Androgen-dependent and -independent Human Prostate Carcinoma Cells

Using LNCaP and DU145 cells, next we assessed the effect of silibinin on EGFR activation. As shown in Fig. 3, when these cells are grown under serum conditions, the EGFR is constitutively active in these cells as evidenced by its overall tyrosine-phosphorylated levels, and indeed, treatment of these cells with silibinin caused a strong inhibition in EGFR activation which was not due to a decrease in total EGFR protein levels in both cell lines (Fig. 3a and b). The densitometric quantification of the bands in LNCaP cells (Fig. 3a) showed that compared to DMSO-treated control, silibinin treatment at 100, 150 and 200 μM doses for 24, 48 and 72 h completely inhibited EGFR (Fig. 3a, $p<0.001$) activation; however, similar silibinin treatments for 12 h showed no effect on EGFR activation (Fig. 3a). In case of DU145 cells, none of the silibinin treatments showed any effect on EGFR activation at 12 and 24 h, and lower dose of silibinin (100 μM dose) was ineffective even at 48 and 72 h treatments; however, 150 and 200 μM doses of this agent strongly inhibited EGFR activation (Fig. 3b, $p<0.001$) as compared to the DMSO-treated controls at 48 and 72 h (Fig. 3b). Taken together both TGFα and EGFR activation results, silibinin showed more profound effect on androgen-

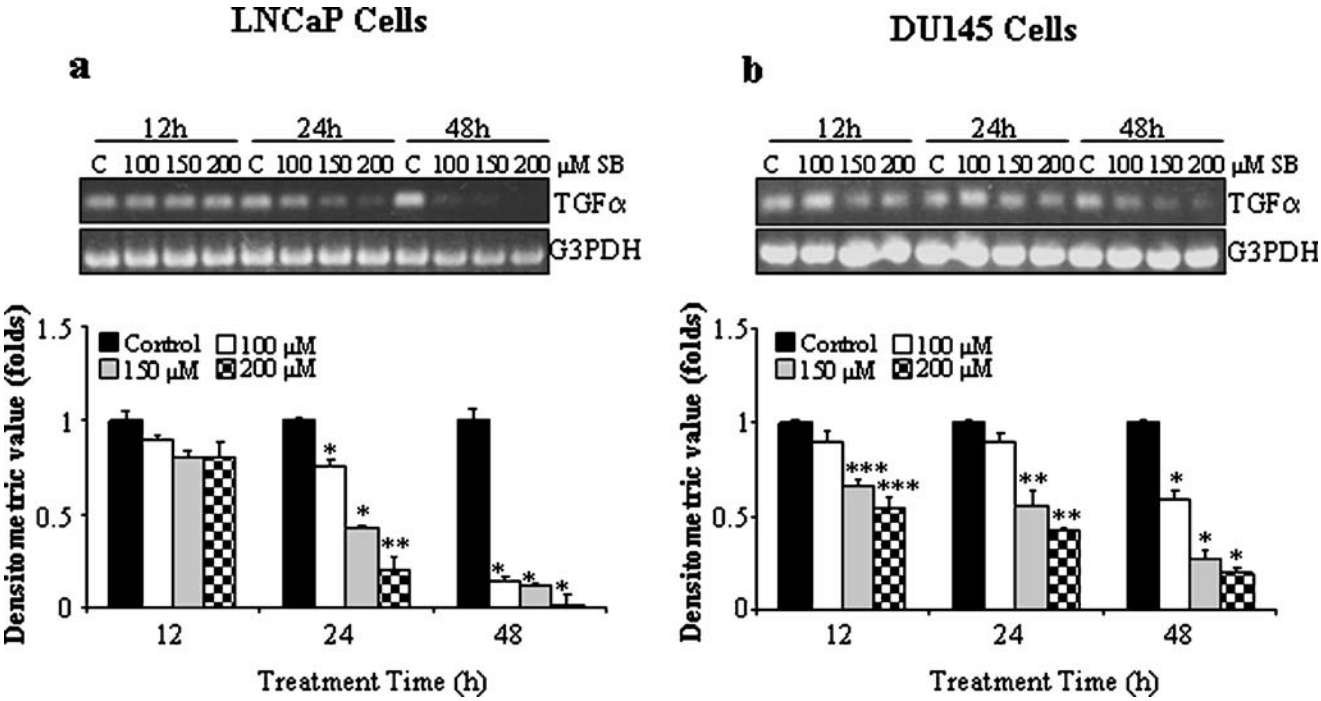

Fig. 2. Silibinin down-regulates TGFα mRNA levels in both androgen-dependent and -independent human prostate carcinoma cells. *LNCaP* (**a**) and *DU145* (**b**) cells were treated with either DMSO alone (*control*) or varying concentrations of silibinin for 12, 24 and 48 h. Different treatments are as labeled in the figure; lanes labeled as "*C*" denote DMSO treatment alone. At the end of the treatment, total RNA was extracted and subjected to RT-PCR and the PCR products were resolved on 1.5% agarose/ethidium bromide gels, as detailed in "MATERIALS AND METHODS". The densitometry data shown as bars below the gels are the mean±SE of two independent experiments. ***, $p<0.05$; **, $p<0.01$; and *, $p<0.001$ indicate statistical significance in silibinin treated groups as compared with the control group. *SB* silibinin

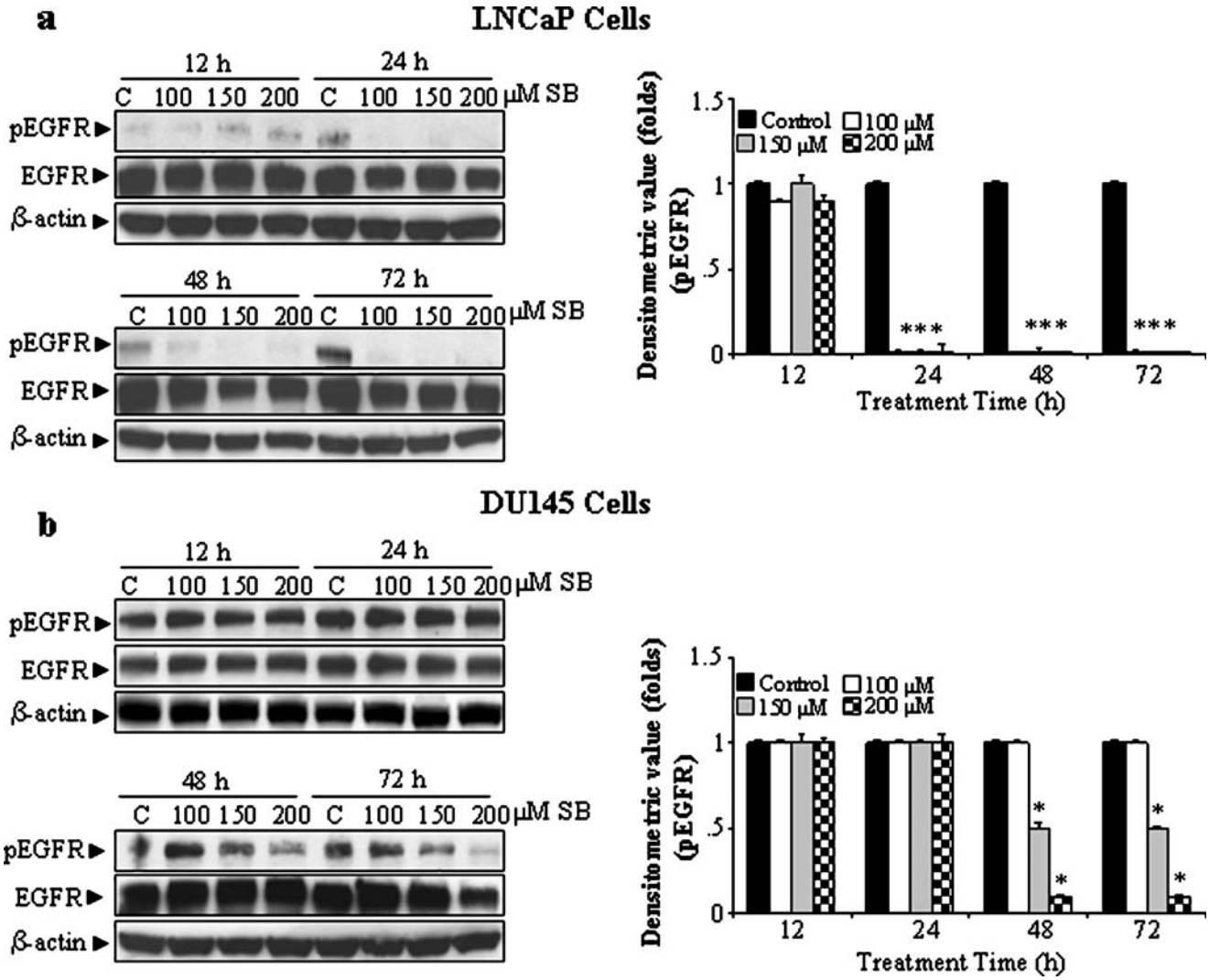

Fig. 3. Silibinin inhibits EGFR activation in both androgen-dependent and -independent human prostate carcinoma cells. *LNCaP* (**a**) and *DU145* (**b**) were cultured as described in "MATERIALS AND METHODS", and treated with either DMSO alone (*control*) or varying concentrations of silibinin. Different treatments are as labeled in the figure; lanes labeled as "*C*" denote DMSO treatment alone. At the end of the treatments, total lysates were prepared and subjected to SDS-PAGE followed by western immunoblotting. Membranes were probed with anti-phospho EGFR, total EGFR and β actin antibodies followed by peroxidase conjugated appropriate secondary antibodies, and visualized by ECL detection system. The densitometry data shown as bars at the right side of the blots are the mean±SE of two independent experiments. ***, $p<0.05$; and *, $p<0.001$ indicate statistical significance in silibinin treated groups as compared with the control group. *SB* silibinin

dependent PCA LNCaP cells as compared to androgen-independent PCA DU145 cells.

Effect of Silibinin on Erk1/2 Activation and Kinase Activity, and Jnk1/2 Activation

Based on the inhibitory effect of silibinin on EGFR activation in both LNCaP and DU145 cells, we also assessed its effect on the ultimate cytoplasmic mitogenic signaling targets; MAPK/Erk1/2 and Jnk1/2 activation. Silibinin (100–200 μM) treatment of LNCaP cells for 24 h caused a moderate decrease in phospho-Erk1/2 levels with no observable response in 12 h treatment; however, similar silibinin treatments for 48 and 72 h showed a moderate to strong inhibitory effect in phospho-Erk1/2 levels in a dose-dependent manner without any noticeable change in total Erk1/2 levels except at the highest dose and treatment time that showed strong decrease in total Erk1/2 as well (Fig. 4a). In terms of Erk1/2 kinase activity to Elk1, interestingly no change was evident in silibinin treated LNCaP cells for 48 h as compared to DMSO-treated control (Fig. 4b). In case of DU145 cells, the activation of Erk1/2 was biphasic after silibinin treatment where 12 h showed no effect on the activation of Erk1/2 and 24 h treatment with silibinin at higher doses (150 and 200 μM) showed a marked increase in phosphorylated levels of Erk1/2 as compared to DMSO-treated control without any change in total Erk1/2 levels (Fig. 4c). However, silibinin treatment at 100–200 μM doses for 48 and 72 h revealed the strong inhibition of Erk1/2 activation as compared to DMSO-treated control (Fig. 4c). Consistent with these results, silibinin treatment for 48 h also showed up to 70% decrease in Erk1/2 kinase activity to Elk1 as compared to the control (Fig. 4d).

Next, we examined the effect of silibinin on Jnk1/2 activation in both PCA cell lines (LNCaP and DU145)

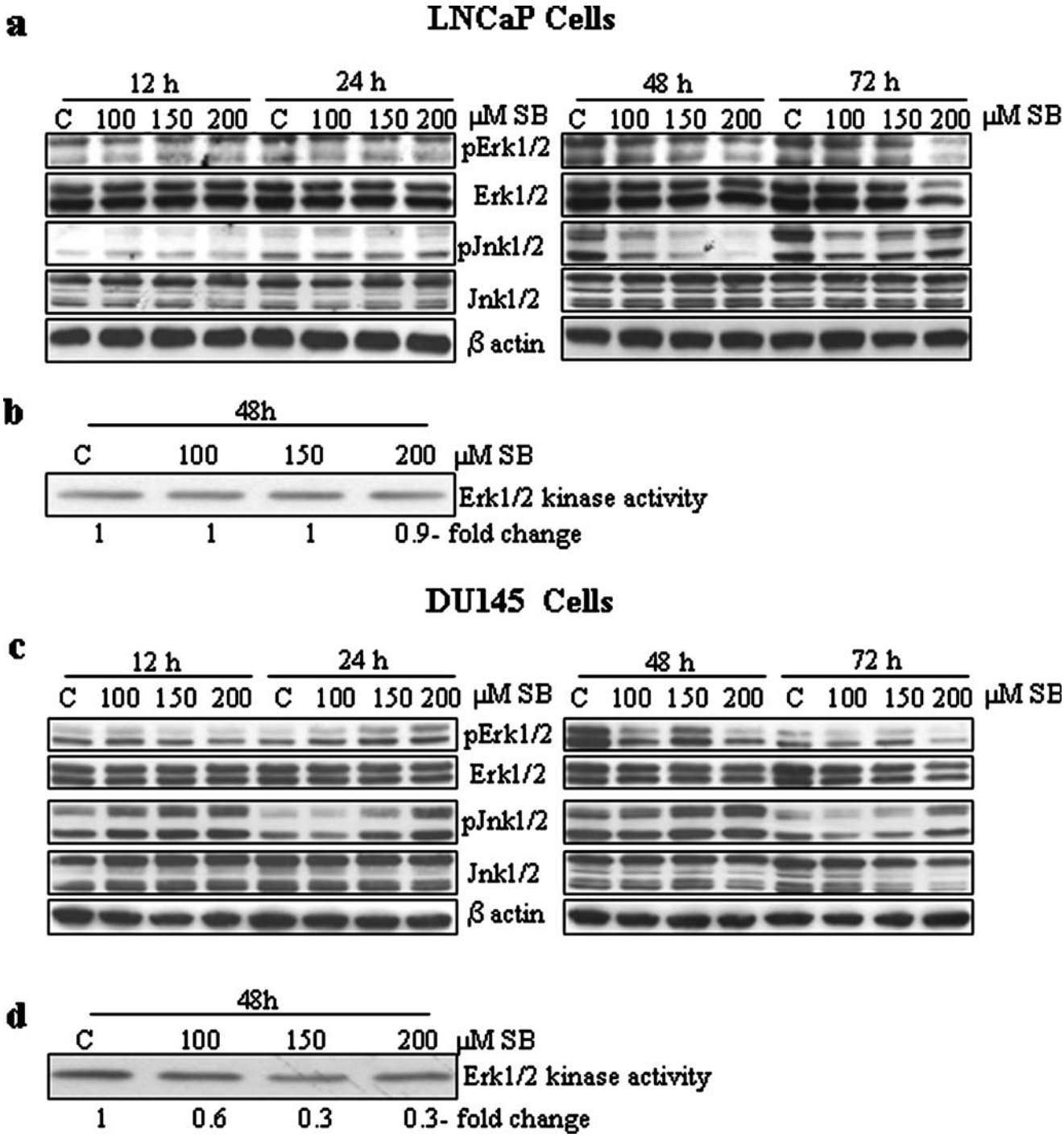

Fig. 4. Effect of silibinin on Erk1/2 activation and kinase activity, and Jnk1/2 activation. *LNCaP* (**a** and **b**) and *DU145* (**c** and **d**) were cultured as described in "MATERIALS AND METHODS", and treated with either DMSO alone (*control*) or varying concentrations of silibinin. Different treatments are as labeled in the figure; lanes labeled as "*C*" denote DMSO treatment alone. At the end of the treatments, total lysates were prepared and subjected to SDS-PAGE followed by western immunoblotting. Membranes were probed with anti-phospho Erk1/2, phospho Jnk1/2, total Erk1/2 and Jnk1/2 antibodies (**a** and **c**) followed by peroxidase conjugated appropriate secondary antibodies, and visualized by ECL detection system. The same membranes were then stripped and re-probed with anti-β actin antibody to confirm equal protein loading. Erk1/2 kinase activity to Elk1 (**b** and **d**) was determined by immunoprecipitating active Erk1/2 with monoclonal phospho-Erk1/2 antibody, followed by incubation with GST-Elk1 protein substrate in the presence of ATP and kinase buffer, and phosphorylation of Elk1 was measured by Western blotting using antiphospho-Elk1 antibody as detailed in "MATERIALS AND METHODS". *SB* silibinin

because this stress kinase pathway is also implicated in modulating cell growth. Compared to DMSO-treated control LNCaP cells, treatment with silibinin (100–200 μM) for 48 and 72 h showed strong inactivation of Jnk1/2 as evidenced by a 50–90% reduction in phospho-Jnk1/2 protein levels (Fig. 4a, lower panel) without any noticeable change in their total protein levels (Fig. 4b, lower panel). In case of DU145 cells, however, silibinin (150–200 μM) treatment for 12–72 h caused

a strong activation (2–7 fold) of Jnk1/2 (Fig. 4c, lower panel) without any change in total Jnk1/2 protein level. Taken these results together, it seems that silibinin treatments for longer time periods cause an inhibition in the activation of Erk1/2 in both LNCaP and DU145 cell lines, which is consistent with its observed effects (Figs. 1, 2, 3) on TGFα levels and EGFR activation; however, more studies are needed to address the observed effects of silibinin on Jnk1/2 activation in these cell lines in future.

DISCUSSION

The present study identifies the effect of silibinin on critical signaling molecules in human PCA LNCaP and DU145 cells, and based on the data presented here supports the notion that silibinin could be an effective cancer preventive and therapeutic agent against PCA with defined mechanism of action. Herein, we examined the effect of silibinin on the regulation of TGFα, EGFR and mitogenic signaling in androgen-dependent (LNCaP) and -independent (DU145) prostate cancer cell lines. Our results demonstrate that silibinin inhibits TGFα expression and secretion and down-regulates EGFR-Erk1/2 activation in both LNCaP and DU145 cells, which possibly are the key contributors to its growth inhibitory effects in these cell lines as reported recently (17).

The EGFR has been implicated in epithelial cell malignant transformation and is found in all PCA cell lines, though androgen-independent cell lines express more EGFR than the androgen-dependent cell lines (10). The high expression of EGFR and associated growth factors, and their interactions are known to cause autocrine and paracrine loops for both mitogenic and anti-apoptotic signaling leading to uncontrolled growth and metastasis (8–10,19). Growth factors are known to be involved in neoplastic development. In this regard, the evidence exists for an autocrine growth factor pathway in transformed cells (8–10,20) together with their over expression and that of growth factor receptors which also causes activation of post receptor pathway (8–10,21). TGFα, the member of the family of mitogenic and autocrine growth factors, shows homology to EGF and exert its biological effects via the EGFR (10). TGFα regulates proliferation of PCA cells via both autocrine and paracrine loops and can promote tumor metastasis (8–10). TGFα binding to the cell surface receptor EGFR initiates an intracellular phosphorylation cascade resulting in the activation of MAPKs (8–10). Following this mitogenic stimulation, MAPK/Erk1/2 translocates to the nucleus and activates transcription factors that promote cell division and/or differentiation (8–10,22,23). Accordingly, the strong inhibitory effect of silibinin on cellular and secreted TGFα protein levels together with a decrease at mRNA levels in both LNCaP and DU145 cells is an important biological finding. Silibinin also strongly inhibited the activation of EGFR in both LNCaP and DU145 cells, which could be because of the fact that TGFα is not available to bind with EGFR and activate it after silibinin treatment. Our results revealed that silibinin was more effective to inhibit EGFR activation in LNCaP cells as compared to the DU145 cells that could possibly be because EGFR is more constitutively active in DU145 cells. It is known that androgens regulate proliferation of PCA cells by increasing TGFα secretion, and accordingly, based on our current findings and earlier work (17); it is possible that silibinin may be playing a role in initially inhibiting androgen signaling followed by inhibitory effects on TGFα secretion and EGFR activation. However, in case of DU145 cells which are androgen-independent, silibinin inhibitory effects on TGFα secretion may be by direct or some other mechanism.

Downstream of EGFR, MAPK/Erk1/2 activation is the key event to drive proliferation, and similar to constitutively active EGFR, an increase in constitutive activation of MAPK/Erk1/2 signaling has also been reported in human PCA as it progresses to a more advanced and androgen-independent malignancy (24). Furthermore, some studies have also shown that MAPK/Erk1/2 is constitutively active in DU145 cells, and that Erk1/2 kinase activity decides activation of transcription factors for cell proliferation (8–10). Consistent with our other findings in the present study, we observed that silibinin inhibits Erk1/2 phosphorylation in both LNCaP and DU145 cells, and also inhibits Erk1/2 kinase activity to Elk1 at least in DU145 cells. Overall, consistent with the involvement of activated MAPK/Erk1/2 pathway possibly via TGFα/EGFR autocrine loop in human PCA, we observed that impairment of TGFα-EGFR signaling by silibinin results in the inhibition of MAPK/Erk1/2 activation in both androgen-dependent and androgen-independent cell lines. It has been shown that Erk1/2 signaling pathway determines cell growth and inhibition through cell cycle regulation (25). However, in transformed cells, cell cycle progression could be a mitogenic signal dependent or independent process (26). In addition, the cyclin-dependent kinase inhibitors (CDKIs), $p21^{WAF1/CIP1}$ (p21) and $p27^{KIP1}$ (p27), play an important role in mammalian cell cycle regulation and these CDKIs are also regulated by Erk1/2 activation (25). Consistent with our findings in the present study showing the inhibitory effects of silibinin on TGFα-EGFR-Erk1/2 activation pathway, we have also reported that silibinin causes G1 arrest and an induction of both p21 and p27 in LNCaP and DU145 cells (17,27). Together, these observations suggest a strong possibility that down-regulation of Erk1/2 by silibinin causes p21 and p27 up-regulation resulting in cell cycle arrest in these human PCA cell lines. Lastly, we would like to emphasize here that silibinin concentrations used in this study are pharmacologically achievable without any toxicity as reported in our animal studies (28), and also in recently completed phase I clinical trial for silibinin in human PCA patients (29). In conclusion, the findings in the present study together with *in-vivo* studies published earlier from our group (12,30) suggest that silibinin is a promising chemopreventive agent for the intervention of human prostate cancer, and accordingly, a phase II pilot clinical trail is currently ongoing at our institution with this natural agent.

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