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α-Lipoic acid inhibits human lung cancer cell proliferation through Grb2-mediated EGFR downregulation

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ABSTRACT

Background: Alpha lipoic acid (α -LA) is a naturally occurring antioxidant and metabolic enzyme co-factor. Recently, α -LA has been reported to inhibit the growth of various cancer cells, but the precise signaling pathways that mediate the effects of α -LA on non-small cell lung cancer (NSCLC) development remain unclear.

Methods: The CCK-8 assay was used to assess cell proliferation in NSCLC cell lines after α -LA treatment. The expression of growth factor receptor-bound protein 2 (Grb2), cyclin-dependent kinase (CDK)-2, CDK4, CDK6, Cyclin D3, Cyclin E1, Ras, c-Raf, epidermal growth factor receptor (EGFR), ERK1/2 and activated EGFR and ERK1/2 was evaluated by western blotting. Grb2 levels were restored in α-LA-treated cells by transfection of a plasmid carrying Grb2 and were reduced in NSCLC cells via specific siRNA-mediated knockdown.

Results: α -LA dramatically decreased NSCLC cell proliferation by downregulating Grb2; in contrast, Grb2 overexpression significantly prevented α-LA-induced decrease in cell growth *in vitro*. Western blot analysis indicated that α-LA decreased the levels of phospho-EGFR, CDK2/4/6, Cyclins D3 and E1, which are associated with the inhibition of G1/S-phase transition. Additional experiments indicated that Grb2 inhibition partially abolished EGF-induced phospho-EGFR and phospho-ERK1/2 activity. In addition, α-LA exerted greater inhibitory effects than gefitinib on NSCLC cells by preventing EGF-induced EGFR activation.

Conclusion: For the first time, these findings provide the first evidence that α-LA inhibits cell proliferation through Grb2 by suppressing EGFR phosphorylation and that MAPK/ERK is involved in this pathway.

1. Introduction

Non-small cell lung cancer (NSCLC) is the most common lung malignancy and the leading cause of cancer-related mortality worldwide [1]. Most NSCLC patients diagnosed at advanced stages have an average 5-year survival rate of only 16%. The median survival time for patients with metastatic NSCLC is approximately 8–10 months when treated with a combination of chemotherapeutic plans [2].

As a member of the ErbB family of receptor tyrosine kinases (RTKs), the epidermal growth factor receptor (EGFR) mediates downstream signaling networks that contribute to tumor progression via a variety of mechanisms, such as cell proliferation, angiogenesis, invasion and metastasis. Growing evidence has revealed the excessive activation and overexpression of EGFR in NSCLC [3]. Given that EGFR is frequently mutated in NSCLC, small-molecule EGFR tyrosine kinase inhibitors (EGFR-TKIs) were a breakthrough in the treatment of patients with advanced EGFR-mutated NSCLC. However, several patients with EGFR mutations do not show favorable responses to EGFR-TKI treatment; nearly half

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Abbreviations

α-LA	Alpha lipoic acid
EGFR	Epidermal growth factor receptor
Grb2	Growth factor receptor-bound protein 2
NSCLC	Non-small cell lung cancer
TKIs	Tyrosine kinase inhibitors

of drug-resistant patients have the T790M mutation in exon 20 of EGFR, which is considered the main proponent of drug resistance [4].

EGF binding induces receptor dimerization, which triggers intrinsic protein tyrosine kinase activity and leads to EGFR autophosphorylation at multiple tyrosine residues in the cytoplasmic domain of the receptors [5,6]. Growth factor receptor-bound protein 2 (Grb2) was initially discovered as the missing link between EGFR and the MAPK pathway, which is required for signaling by nearly all RTKs [7,8]. Further studies have suggested that Grb2-mediated signaling contributes to a loss of cell cycle control and enhances cell proliferation, motility and invasion [9]. Moreover, elevated Grb2 expression is correlated with poor prognosis and disease progression [10–12]. Considering that Grb2 is ubiquitously expressed and that Grb2 overexpression has been reported in specific cancers, it is important to develop new small molecules targeting Grb2 to overcome chemotherapy resistance in cancers, particularly NSCLC.

Alpha lipoic acid (α-LA) is an endogenous disulfide compound synthesized *de novo* in mitochondria and is a naturally occurring co-factor found in many multi-enzyme complexes that regulate metabolism. α-LA or its reduced form, dihydrolipoic acid (DHLA), scavenges various ROS molecules and has been used as a potent biological antioxidant to treat chronic diseases associated with high levels of oxidative stress [13,14]. Recently, α-LA and its derivatives have been reported to inhibit growth or promote apoptosis in various cancer lines [15–17], but the precise signaling pathways mediating α-LA effects on NSCLC development remain unclear.

In this study, we report a new potential therapeutic strategy for NSCLC patients. We show that α-LA treatment inhibits cell proliferation by downregulating Grb2 expression in different lung cancer cell lines. In addition, the EGFR-MAPK/ERK signaling pathways have been implicated in α-LA-reduced cell proliferation. Most importantly, α-LA disrupts the interaction of EGFR and Grb2, which prevents the activation of EGFR signaling and the MAPK/ERK pathway. We provide the first evidence showing that α-LA is a chemopreventive agent that can be used as a new clinical strategy for the treatment of NSCLC patients.

2. Materials and methods

2.1. Cell culture and treatment

The human lung carcinoma cell line A549 and human lung adenocarcinoma cell line NCI-H1975 *EGFR* T790M were obtained from ATCC (Manassas, VA, USA). The cells were grown in Dulbecco's modified Eagle's medium/F12 or RPMI-1640 medium supplemented with 10% fetal bovine serum (Gibco), 100 IU/mL penicillin, and 100 μg/mL streptomycin and maintained in an incubator with a humidified atmosphere of 5% CO_2 at 37 °C.

α-LA (purity≥99% by HPLC) and hEGF were provided by Sigma-Aldrich. Gefitinib was purchased from Cell Signaling Technology (Danvers, MA, USA). All compounds were dissolved in sterile dimethyl sulfoxide (DMSO) or acetic acid, and a 10-mM working concentration was prepared and stored at −20 °C. Working solutions were diluted in culture medium just before each experiment.

2.2. CCK-8 assay

For the CCK-8 assay, cells were seeded at a density of 5×10^3 cells per well in 96-well plates for 24 h and subsequently cultured in the presence or absence of different α-LA concentrations for 24–120 h. At the indicated times, the medium was replaced with fresh medium supplemented with CCK-8, and the cells were further incubated for 2 h. The absorbance was determined at 450 nm using a Thermo microplate reader (Waltham, MA, USA). Each experiment assessed at least six samples at a time, and each experiment was performed at least three times.

2.3. Transient transfection

Knockdown of Grb2 expression was achieved using the Grb2-specific siRNA siGrb2-1 purchased from Cell Signaling Technology (Danvers, MA, USA) and siGrb2-2 [18] (5′-CAU GUU UCC CCG CAA UUA UTT-3′) synthesized at GenePharma (Suzhou, China). For overexpression studies, the full-length Grb2 fragment was purchased from Vigene (Rockville, MD, USA) and subcloned into the pENTER expression vector.

Transient transfections were performed using Lipofectamine 2000 (Invitrogen). Briefly, the cells in 6-well plates were transfected with siGrb2 or pENTER-Grb2. Lipofectamine 2000 was incubated with DNA or RNA in serum-free medium for 20 min before being added to cells, with a subsequent incubation for an additional 6 h. After treatment at the indicated time points, the cells were collected for proliferation or western blot analyses.

2.4. Cell cycle

Cells were cultured in either the absence or the presence of 2.0 mM α-LA for 24 h and subjected to cell cycle analysis. Briefly, the cells were harvested and suspended in 70% cold ethanol overnight at 4 °C. After centrifugation, the pellets were washed with cold PBS, suspended in 500 μL of PBS, and incubated with 50 μL of RNase A (20 μg/mL final concentration) for 30 min and then stained with propidium iodide (50 μg/mL) for 30 min in the dark. A total of 10,000 cells per sample were collected and analyzed using a FACSAria BD Biosciences instrument. All experiments were performed in triplicate.

2.5. Western blotting

Whole protein was extracted from cultured cells using extraction buffer (RIPA lysis buffer with proteinase inhibitor cocktail and protein phosphatase inhibitor). The protein concentrations were quantified using a protein assay kit (Bio-Rad). The lysates were separated on 10% SDS-PAGE gels and transferred to PVDF membranes. The membranes were probed overnight at 4 °C using primary antibodies against human Grb2 (Abcam, 1:5000), EGFR/phospho-EGFR (Tyr1068), ERK/phospho-ERK (Thr202/Tyr204), CDK2/4/6, Cyclin D3/E1 and Ras/Raf (Cell Signaling Technology, 1:1000) or β-actin (Cell Signaling Technology, 1:2000) followed by incubation with a horseradish peroxidase (HRP)-linked secondary antibody (Cell Signaling Technology, 1:1000) for 1 h. The signal was detected using ECL (Thermo), and the results were recorded using a GeneGnome XRQ fluorescence chemiluminescence imager (Syngene). Quantification of bands on the membrane was performed with ImageJ software. Protein levels were normalized to the endogenous reference to account for differences in protein loading.

Each sample was analyzed in triplicate, and average values were used for subsequent statistical analyses.

2.6. RNA isolation and real-time PCR

Total RNA was isolated from cell lines using TRIzol reagent (Invitrogen). After treatment with DNase I, the RNA was reverse transcribed into cDNA using the Thermo Scientific Maxima First-Strand cDNA Synthesis Kit for mRNA detection. Real-time quantitative PCR was performed using an Applied Bio-Systems 7500 PCR instrument, and each sample was analyzed in triplicate. The PCR data were normalized to GAPDH mRNA expression mRNA. The primer sequences used are as follows: Grb2 sense 5′-CTG GGT GGT GAA GTT CAA TTC T-3′ and anti-sense 5′-GTT CTA TGT CCC GCA GGA ATA TC-3'; and GAPDH sense 5′-AGC AAG AGC ACA AGA GGA AGA G-3′ and anti-sense 5′-TCT ACA TGG CAA CTG TGA GGA G-3'.

2.7. Statistical analyses

Statistical analyses were performed on data collected from at least three independent experiments. The data are presented as the means ± standard deviation (SD) and were analyzed using GraphPad Prism 5 software. Comparisons among >2 groups were performed with ANOVA using Tukey's test. Comparisons between 2 groups were performed with Student's *t*-test for unpaired data. *P* values less than 0.05 were considered statistically significant.

3. Results

3.1. Grb2 mediates α-LA-reduced cell proliferation

We first examined the effect of α-LA on the proliferation of A549 and NCI-H1975 cells. A CCK-8 assay showed that α-LA dose-dependently suppressed cell proliferation (data not shown). The concentration of α-LA required for 50% inhibition of proliferation was 2.0 mM in both cell lines; thus, 2.0 mM α-LA was used in subsequent experiments (Fig. 1A). Treatment with α-LA has also been reported to cause G1/S-phase arrest in lung cancer cells [17]. To further explore the G1/S-phase arrest caused by α-LA in A549 cells, the cell cycle distribution was analyzed. Compared with that of the control group, the fraction of cells in the G1 population at 24 h was increased in the α-LA-treated group (Fig. 1B). In addition, specific cell cycle regulatory proteins required for the G1/S-phase transition were evaluated by western blot analysis. The

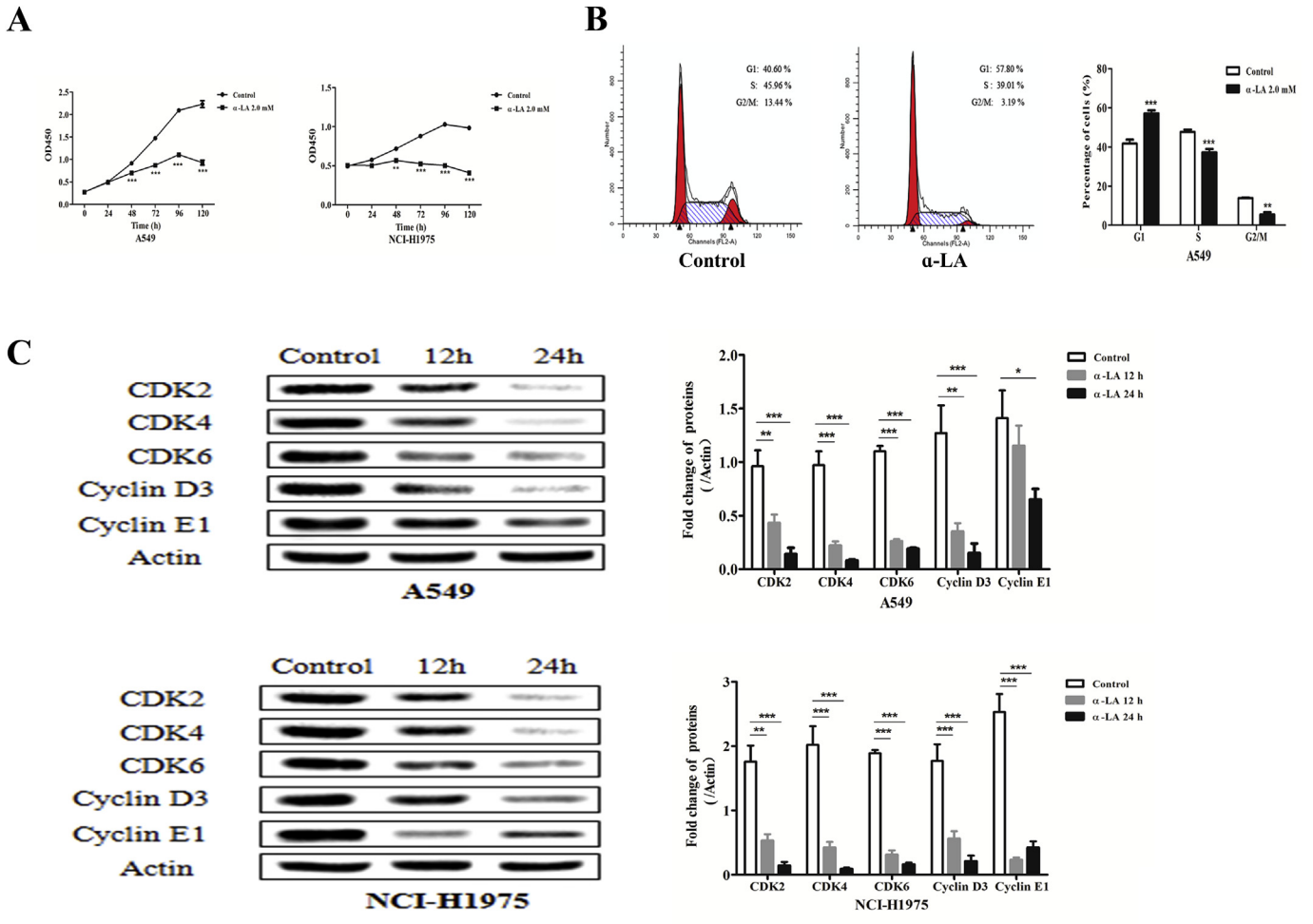

Fig. 1. α-LA reduces cell proliferation through G1/S-phase arrest.
A. Cell proliferation was measured using a CCK-8 assay at the indicated times. Significant differences as indicated by asterisks (**P < 0.01; ***P < 0.001).
B. Changes in cell cycle distribution of A549 cells by 2.0 mM α-LA. Cells were cultured in either the absence or presence of α-LA for 24 h and subjected to cell cycle analysis using a flow cytometer.
C. Expression of cell cycle regulatory proteins was assessed by western blotting in NSCLC cell lines after treatment with 2.0 mM α-LA for 12 and 24 h. Quantification of protein expression normalized to the level of β-actin; means ± SD, n = 3 samples per group, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

results revealed decreased levels of CDK2/4/6 and Cyclin D3/E1 at 12 and 24 h in cells treated with α-LA (Fig. 1C). These analyses suggest that α-LA treatment potently suppresses cell proliferation in association with G1/S-phase cell cycle arrest.

Recent studies have revealed that Grb2 is a key hub in the cell proliferation network [19,20]. To further validate whether Grb2 is involved in the α-LA-induced decrease in cell proliferation, we compared Grb2 levels with or without α-LA treatment. Grb2 was significantly repressed at both the mRNA and protein levels in cell lines after α-LA treatment for 12 and 24 h (Fig. 2A). In addition, knockdown of Grb2 expression using specific siRNAs remarkably attenuated cell proliferation compared with that observed in the scramble RNA control group (Fig. 2B and Supplementary Fig B). In contrast, Grb2 overexpression completely restored the inhibition of cell proliferation induced by α-LA treatment in A549 and NCI-H1975 cells (Fig. 2C). These results strongly suggest that α-LA reduces cell proliferation by downregulating Grb2 levels.

3.2. α-LA suppresses the EGFR-MAPK/ERK pathway in NSCLC cells

Next, we sought to address how α-LA regulates cell proliferation. EGFR activation also regulates cell proliferation characteristics via several pathways, such as MAPK signaling. To verify whether EGFR is involved and which signaling pathway(s) are targeted by α-LA to modulate cell proliferation, we initially examined the effect of α-LA on EGFR phosphorylation and the MAPK pathways. As shown in Fig. 3A, α-LA clearly blocked EGFR phosphorylation at 6, 12 and 24 h, and the levels of Ras and c-Raf were notably decreased at 12 and 24 h. In addition, α-LA treatment also decreased the levels of downstream phospho-ERK1/2 without changing the total ERK protein levels, whereas there was no change in the levels of JNK and p38 pathway components (data not shown). As α-LA treatment attenuated the MAPK-ERK pathway, we further examined whether α-LA exerted this effect via EGFR downregulation. As depicted in Fig. 3B, EGF stimulation robustly induced EGFR phosphorylation, and downstream MAPK signaling was subsequently activated. α-LA also blocked EGF-induced EGFR phosphorylation and downregulated the levels of Ras, c-Raf and phospho-ERK1/2, exhibiting effects on EGFR similar to those of gefitinib (Fig. 3B). Surprisingly, α-LA increased the gefitinib-induced inhibition of EGFR and ERK phosphorylation in EGFR-mutant cells. Therefore, α-LA inhibits NSCLC cell proliferation by targeting the EGFR-MAPK/ERK pathway.

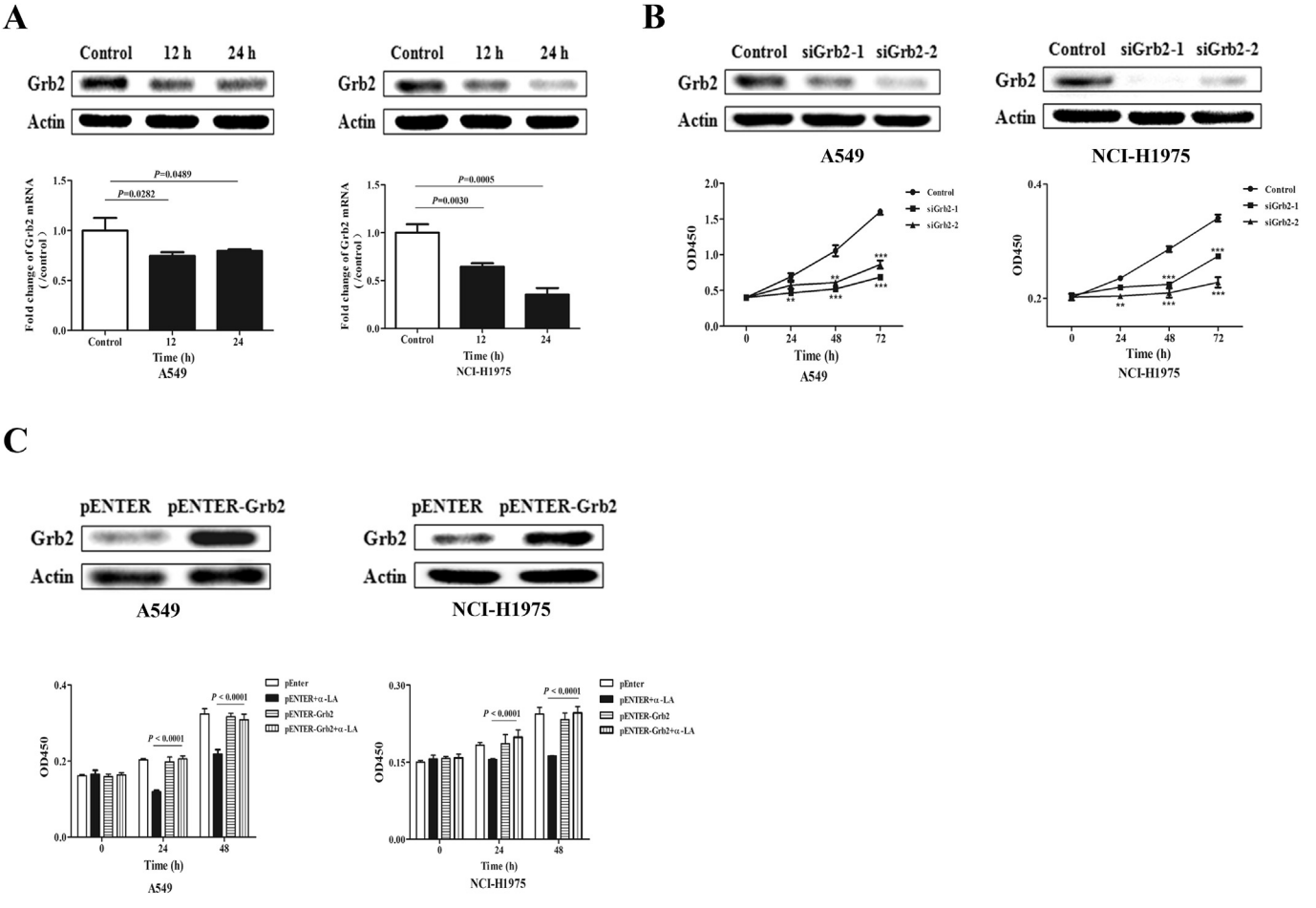

Fig. 2. Grb2 mediates reductions in cell proliferation caused by α-LA.

A. Grb2 levels were measured by real-time PCR and western blotting in NSCLC cell lines after treatment with 2.0 mM α-LA for 12 and 24 h.

B. A549 and NCI-H1975 cells were transfected with siRNA against Grb2; 48 h after transfection, the cells were collected to measure Grb2 protein expression (upper panel). For cell proliferation assays, cells were transfected for 24 h and then seeded in 96-well plates for CCK-8 assays at the indicated times. Significant differences are indicated by asterisks ($^{**}P < 0.01$; $^{***}P < 0.001$).

C. Effect of Grb2 overexpression on cell proliferation was measured using the CCK-8 assay. After transfection with a Grb2-overexpression plasmid, cell proliferation was measured at the indicated times using a CCK-8 assay.

All experiments were independently repeated at least 3 times.

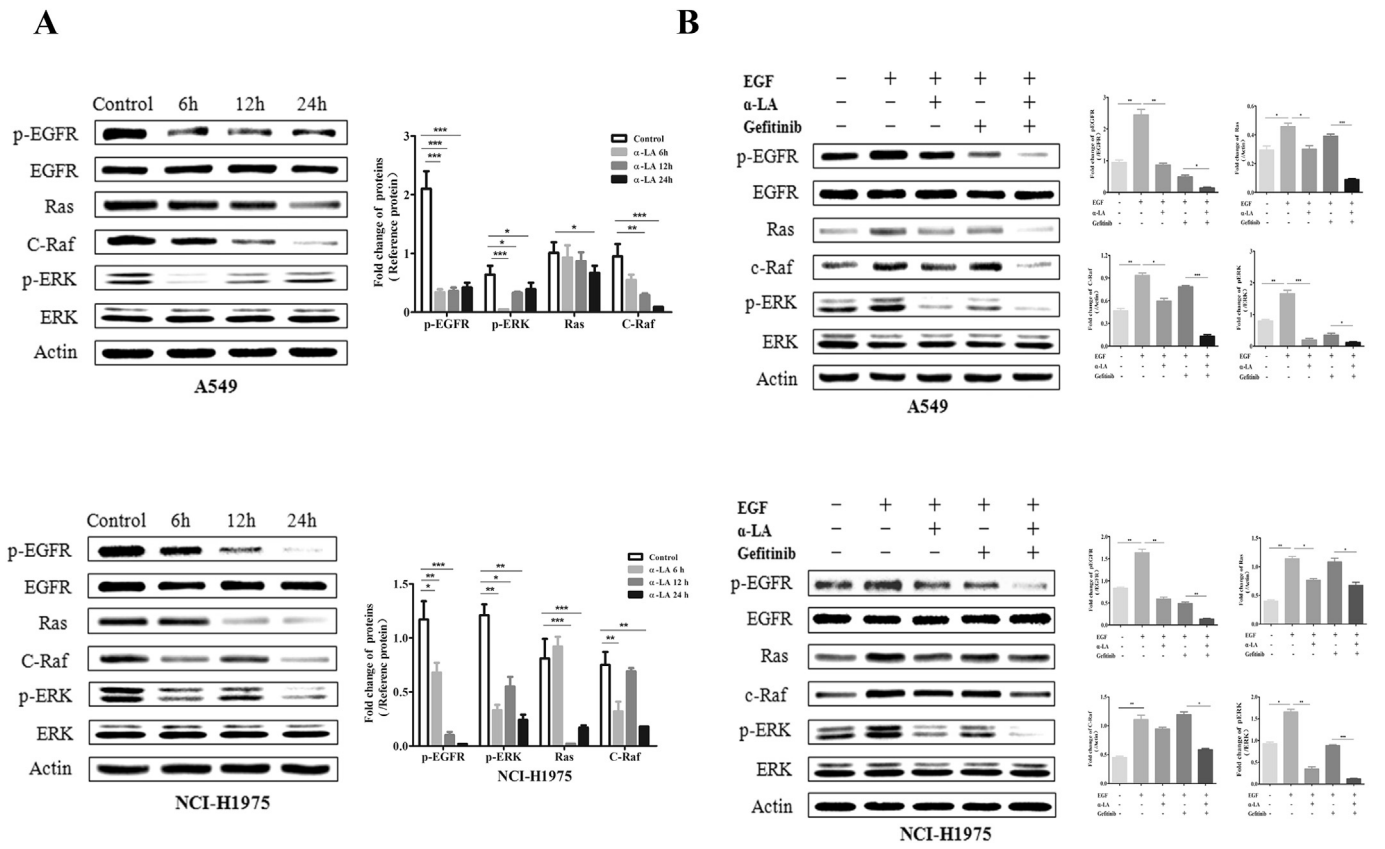

Fig. 3. α-LA treatment reduces EGFR/MAPK-ERK signaling pathway stimulation in A549 and NCI-H1975 cells.
A. Levels of phospho-EGFR, ERK1/2 and Ras, Raf in cells after treatment with 2.0 mM α-LA for 6, 12 and 24 h were assessed by western blotting.
B. Cells were pretreated with 50 ng/mL EGF or 10 μM gefitinib for 2 h and then treated with 2.0 mM α-LA for 10 h. Levels of phospho-EGFR, ERK1/2 and Ras, Raf were analyzed by western blotting.
Quantification of protein expression normalized to the levels of a specific reference protein. Results are expressed as the means ± SD from at least three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

3.3. Grb2 is required for the α-LA-mediated suppression of EGFR-ERK signaling

Because phospho-EGFR and Grb2 were markedly repressed in cells treated with α-LA (Figs. 2A and 3A), we validated whether the EGFR-Grb2 interaction is involved in the α-LA-induced reduction of cell proliferation. To confirm the role of EGFR in the α-LA-mediated reduction of Grb2 expression, a specific EGFR activator or inhibitor was used to directly examine whether EGFR expression is required for α-LA to decrease Grb2 levels in lung cancer cell lines. We observed that Grb2 expression was not altered in EGF- or gefitinib-treated cells (Fig. 4A). Next, to further examine whether Grb2 mediates the α-LA effect on EGFR-ERK signaling, specific siRNAs against Grb2 were used to reduce Grb2 expression. As shown Fig. 4B, the levels of phospho-EGFR and phospho-ERK1/2 were significantly reduced in Grb2-knockdown cells. In addition, Grb2 knockdown also blocked EGF-induced activation of EGFR and ERK1/2. Most importantly, specific RNAs against Grb2 enhanced the inhibitory effects of α-LA or gefitinib on NSCLC cells. In contrast, Grb2 overexpression did not affect the basal levels of phospho-EGFR and phospho-ERK1/2 in cell lines, though it did completely restore the repression of EGFR and ERK1/2 phosphorylation in response to gefitinib or α-LA treatment (Fig. 4C). These findings confirmed that α-LA regulates cell proliferation via the Grb2-mediated repression of EGFR-ERK signaling *in vitro*.

4. Discussion

EGFR is one of the most extensively studied tyrosine kinase receptors due to its role in the development of several solid tumors. Even in tumors in which EGFR is not overexpressed, this receptor might be an essential intermediate in the signaling required for cell proliferation and survival. Excess EGFR activation can lead to the sustained signaling of anti-apoptosis, cell proliferation or survival, angiogenesis and metastasis pathways. In recent years, several drugs have been identified that modulate EGFR activation, including molecules directed towards the extracellular domain of EGFR, such as monoclonal antibodies that interfere with intracellular receptor signaling by inhibiting the catalytic kinase domain (i.e., small-molecule TKIs). Although recent meta-analyses have shown that EGFR-TKIs provide significant clinical benefits for patients with EGFR mutations, these studies revealed the presence of a subgroup of patients not carrying EGFR-activating mutations that also benefitted from EGFR-targeted treatment. However, patients with EGFR activation benefit from treatment with EGFR-TKIs for no more than 1 year, after which drug resistance develops [21]. The mechanisms of drug resistance have been associated with the EGFRvIII variant, which eliminates the epitope recognized by some monoclonal antibodies, or with missense mutations in the EGFR kinase domain. In view of the frequent occurrence of intrinsic EGFR resistance mutations, ongoing efforts to identify additional drugs that independently block EGFR are critical.

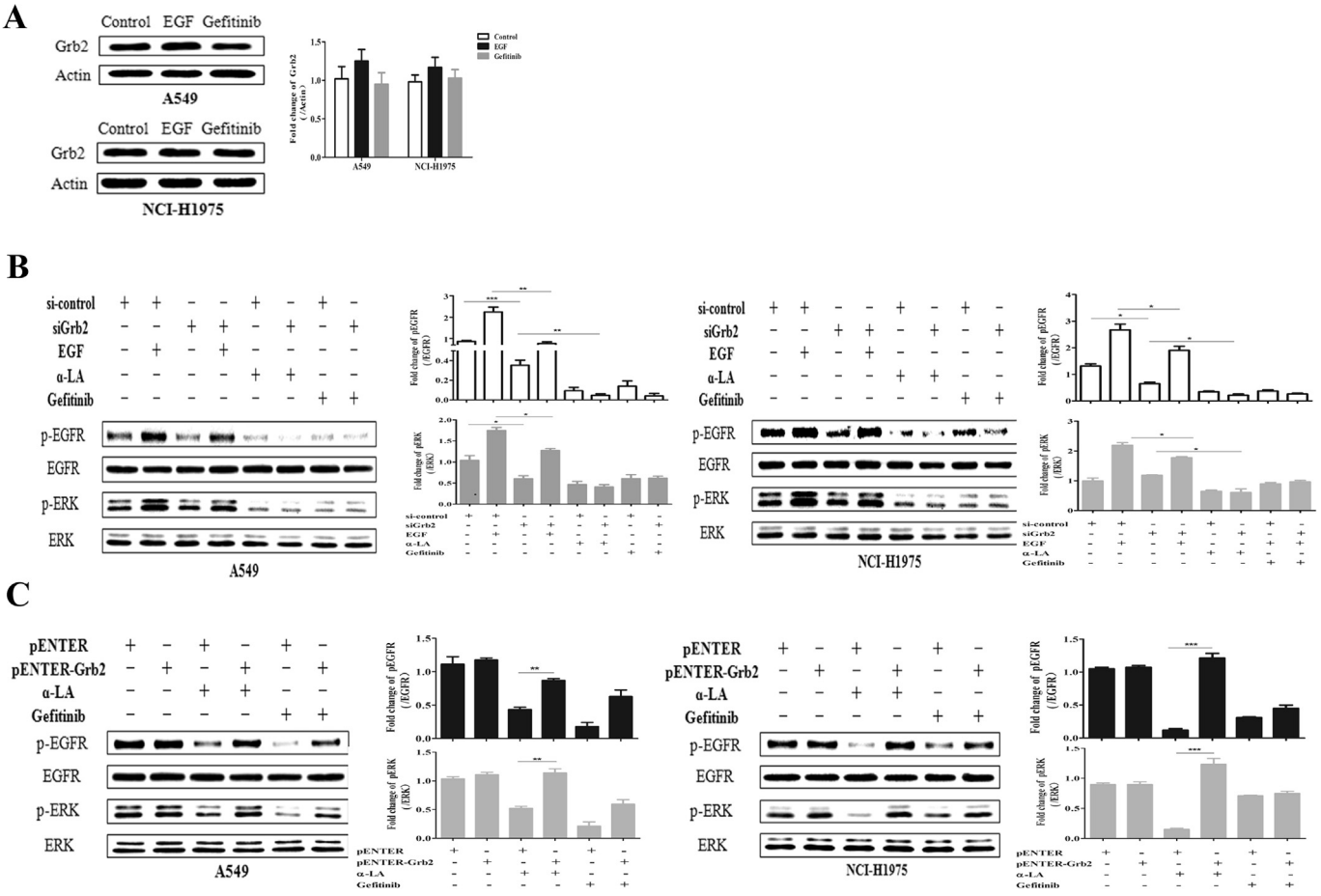

Fig. 4. α-LA treatment reduces EGFR and ERK phosphorylation by downregulating Grb2 levels.
A. Western blotting was used to assess Grb2 protein levels in cells after treatment with EGF or gefitinib for 12 h.
B. Cell were transfected with 50 nM control siRNA (si-control) or Grb2-specific siRNA (siGrb2) for 48 h and then treated with EGF, gefitinib or α-LA for 12 h. Levels of phospho-EGFR and ERK1/2 were analyzed by western blotting. Results are expressed as the means ± SD from at least three independent experiments.
C. Cells were transfected with the control plasmid (pENTER) or the Grb2 plasmid (pENTER-Grb2) for 48 h and then treated with EGF, gefitinib or α-LA for 12 h. Levels of phospho-EGFR and ERK1/2 were analyzed by western blotting. Results are expressed as the means ± SD from at least three independent experiments.
Quantification of protein expression normalized to the level of a specific reference protein. Results are expressed as the means ± SD from at least three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

Activated EGFR primarily acts via the Ras and Raf/MAPK/ERK downstream pathway, in which the adaptor protein Grb2 is a key player through direct or indirect interaction with the autophosphorylated receptor [22]. Grb2-mediated signaling has been implicated in the onset, progression and metastasis of several human cancers. Recent studies have reported that Grb2 inhibition significantly reduced fat accumulation, improved glucose metabolism, ameliorated oxidative stress and activated MAPK pathways. Recently, the analysis of Grb2-associated proteins and the functional consequence of specific binding events as well as extensive structural studies of Grb2-ligand interactions have aroused widespread interest. A wide variety of experimental strategies have been used to block Grb2-mediated signaling. Analyses of Grb2-associated binding events and Grb2-ligand interactions were facilitated by the development of peptide or synthetic small-molecule antagonists with pharmaceutically desirable potency. To date, the SH2 domains of Grb2 antagonists have been rigorously examined as anti-cancer drug candidates. In contrast, the Grb2 SH3 domain has both structural and functional complexities that should be addressed in the development of selective and potent anti-cancer drugs, which is a complex process.

α-LA is a biological antioxidant used to treat clinical diseases such as diabetes and Alzheimer's disease. α-LA has long been known to be a crucial coenzyme that protects mitochondria against respiration-linked oxidative stress, increases functional life span and preserves the genomic and structural integrity of organelles [23]. Recently, α-LA was also reported to suppress the proliferation or apoptosis of multiple type cancer cells by inhibiting related molecular signaling pathways. In NSCLC cells, α-LA not only blocked cell proliferation but also showed anti-cancer activity very similar to that of gefitinib. More importantly, α-LA possesses an eight-carbon disulfide chemical backbone structure, which is substantially different from typical EGFR-TKIs. This molecule provides a new structural scaffold for future modification and the development of potent anti-cancer agents against NSCLC.

In summary, the results of this study demonstrate that α-LA, a novel antioxidant, targets the EGFR-Grb2 interaction and inhibits EGFR-MAPK/ERK signaling in NSCLC cell lines, enhancing the effect of EGFR-TKIs. α-LA decreased Grb2 levels and suppressed the activation of EGFR, limiting cell growth and proliferation. However, several limitations of this study should also be noted. First, we used two NSCLC cell lines in this study, and further studies should include larger numbers and types of cell lines, particularly those of cells involved in multiple types of EGFR-mutant cancers. In

addition, the precise mechanism regulating the α-LA-mediated disruption of the EGFR-Grb2 interaction must be explored *in vivo*. Finally, the clinical stages of NSCLC patients should be thoroughly analyzed in further studies. Nevertheless, these results support the novel idea that α-LA can be used to supplement EGFR-TKIs in NSCLC clinical therapy.

Funding

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Competing interests

The authors declare that they have no conflicts of interest concerning the content of this article.

Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.bbrc.2017.10.030.

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