Gefitinib circumvents hypoxia-induced drug resistance by the modulation of HIF-1α

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Abstract. Hypoxia-inducible factor-1α (HIF-1α) is a transcriptional factor which is activated by hypoxia and associated with cell survival, proliferation and drug resistance. Recent studies have shown that the down-stream molecules of the epidermal growth factor receptor (EGFR) signal are involved in the hypoxia-dependent or -independent HIF-1α protein accumulation. Thus, we hypothesized that an EGFR-TK inhibitor, gefitinib, might circumvent the hypoxia-induced drug resistance via the regulation of HIF-1α expression. In our data, treatment of gefitinib suppressed induced HIF-1α by hypoxia. This action of gefitinib was caused by reduced protein stability without any change in the level of HIF-1α mRNA. The effect of gefitinib on down-regulation of HIF-1α was reversed by transfection of constitutively active form of Akt. The cellular response to gefitinib was similar in both normoxia and hypoxia condition. However, the response to conventional chemotherapeutic drugs decreased >50% under hypoxia condition and they did not change HIF-1α expression. In addition, the suppression of HIF-1α using siRNA overcame partially hypoxia-induced drug resistance. In conclusion, gefitinib was able to circumvent hypoxia-induced drug resistance suggesting that the effective suppression of HIF-1α by the inhibition of EGFR-Akt pathway may overcome the hypoxia-induced drug resistance.

Introduction

Tissue hypoxia is an important phenomenon in developmental biology, normal physiology and many pathological conditions, such as, vascular, pulmonary diseases and solid tumors (1-4).

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It leads to acidic microenvironments that are low in oxygen and nutrients and can limit tumor growth and metastasis. However, hypoxic cells trigger a transcriptional program that can adapt to hypoxic microenvironment through alterations in cellular metabolism and the stimulation of neovascularization and sequent proliferation and survival (5,6). Moreover, tumor hypoxia can result in more aggressive and metastatic phenotypes, associated with lower sensitivity to chemotherapy and radiotherapy and poor prognosis (7,8). Thus, the suppression of factors that regulate hypoxic adaptation can enhance cancer therapy.

Hypoxia-inducible factor (HIF-1) is one of the key regulators of cellular adaptation to oxygen deprivation (4,9). HIF-1 is a heterodimer composed of HIF-1α and HIF-1ß subunits. HIF-1ß is constitutively expressed in normoxic cells, whereas HIF-1α is degraded by the ubiquitin-proteasome pathway in normoxia and stabilized under hypoxia (10). Under normoxia, HIF-1α is hydroxylated by specific prolyl hydroxylases, which is recognized by the von Hippel-Lindau (VHL) and it results in HIF-1α degradation through the recognition component of the E3 ubiquitin-protein ligase. When oxygen tension is limited, the rates of proline hydroxylation is decreased and becomes stabilized.

Although oxygen tension plays a major role in the process of HIF-1α stabilization and activation, previously, many studies showed that this response can be further modulated by major signal transduction pathways, including the ERK and PI3-K/ Akt cascades (11). These pathways are also associated with HIF-1α activation in normoxia (12,13). In addition, the induction of HIF-1α in normoxia occurred by a variety of growth factors, such as EGF, IGF-1, IGF-2 and insulin (13-16), although the role of HIF-1α in these conditions is unclear.

The epidermal growth factor receptor (EGFR) signaling pathway plays a key role in the regulation of cell proliferation, survival and differentiation (17,18). It has been shown that the level of EGFR is up-regulated in many human tumor tissues including lung cancer (19). The activation of EGFR exerts cellular effects via ERK and PI3-K/Akt pathways. Thus, inhibition of EGFR signaling can lead to the reduction of induced-HIF-1α by hypoxia and suppression of this pathway also may overcome HIF-1α-mediated resistance to chemo-

therapeutic drugs. In this study, we examined whether gefitinib, an EGFR-TK inhibitor, might circumvent the hypoxia-induced drug resistance by the down-regulation of HIF-1α expression in non-small cell lung cancer (NSCLC) cells. In addition, whether the suppression of HIF-1α using siRNA can reverse hypoxia-induced drug resistance was investigated.

Materials and methods

Cell culture and reagents. Human NSCLC, A549 and H460 cells were grown in RPMI-1640 (Invitrogen, Carlsbad, CA) and Calu-1 cells were grown in MacCoy's 5A (Invitrogen). The media were supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin (Invitrogen) at 37°C in atmosphere of 5% CO_2. LY294002, PD98059, MG132, cisplatin and paclitaxel were purchased from Sigma (St. Louis, MO). The gefitinib and gemcitabine were kindly provided by AstraZeneca Korea (Seoul, Korea) and Lilly Korea (Seoul, Korea), respectively.

Hypoxia treatment. Cells were plated in 60-mm dishes, or 96-well plates according to the experiment requirements and cultured at 37°C in a 19% O_2, 5% CO_2 previously. On the day of experiment, the medium was replaced with a fresh medium with 1% FBS. After treatment of indicated chemotherapeutic agents or inhibitors, cells were placed in a humidified airtight anaerobic incubator (Thermo Electron Corp., Waltham, MA) and set the incubator at 37°C, 1% O_2 and 5% CO_2.

MTT assay. Briefly, cells in the logarithmic growth phase were harvested and seeded in 96-well plates (Costar, Cambridge, MA, USA) overnight. The chemotherapeutic agents were added and cells were further incubated for 48 h under normoxia or hypoxia conditions. The viability of cells was determined using the MTT assay according to the method described by Carmichael *et al* (20).

Flow cytometric analysis. The cells were treated with chemotherapeutic agents or inhibitors and exposed to normoxia or hypoxia as indicated. Cells were harvested, washed with PBS containing 5 mM EDTA and fixed in 70% ethanol at -20°C. After 24 h, the cells were collected by centrifugation and resuspended in PBS containing 5 mM EDTA. After digestion with 50 μg/ml RNase A, cells were stained with 50 μg/ml propidium iodide (PI) for at least 30 min before analysis using flow cytometry (Becton-Dickinson FACSort). The extent of cell death was determined by measuring the sub-G_1 population.

Western blot analysis. Cells were washed with PBS and solubilized in lysis buffer (25 mM HEPES, 0.1% SDS, 0.5% deoxycholate, 1% Triton X-100, 10 mM EDTA, 10 mM NaF and 125 mM NaCl). The protein was then collected after centrifugation at 10,000 x g for 10 min at 4°C. The resulting supernatant (40 μg) was separated on 8-12% SDS-PAGE and transferred to nitrocellulose filters and probed with specific antibodies against HIF-1α [(R&D Systems, Minneapolis, MN), 1:1000], ß-actin [AC-74 (Sigma), 1:4000], p-Akt [Ser 473 (Santa Cruz), 1:1000], Akt [C-20 (Santa Cruz), 1:2000],

p-Erk [E-4 (Santa Cruz), 1:1000], or Erk [K-23 (Santa Cruz), 1:2000]. The secondary antibodies used a horseradish peroxidase-conjugated antibodies diluted to 1:2000 and then the membrane were developed using an ECL kit (Amersham Biosciences, Piscataway, NJ).

Quantitative reverse transcription-PCR. Total RNA was isolated from cells according to the protocol of the Purescript RNA purification kit (Gentra Systems, Plymouth, MN). The cDNA was synthesized using Accupower RT mix reagent, according to the manufacturer's protocol (Bioneer Corp., Seoul, Korea). The reaction was incubated at 42°C for 60 min and then inactivated at 95°C for 5 min. After inactivation, the cDNA was stored at -20°C until use. RT-PCR was performed with the following cycling conditions: 30 cycles of 94°C for 45 sec, 60-65°C for 45 sec and 72°C for 1 min for ß-actin and HIF-1α. This was followed by a final extension step at 72°C for 5 min. The specific primer sequences were as follows: forward primer 5'-CTCAAAGTCGGACAGCCTCA-3' and reverse primer 5'-CCCTGCAGTAGGTTTCTGCT-3' for HIF-1α; forward primer 5'-GCGAGAAGATGATGACCCA GATC-3' and reverse primer 5'-CCAGTGGTACGGCCAG AGG-3' for ß-actin. Amplified products (3 μl) were resolved on 5% polyacrylamide gels in TBE (90 mM Tris-Cl, 90 mM boric acid, 1 mM EDTA), stained with ethidium bromide, visualized with a transilluminator and photographed. The level of gene expression was assessed by densitometric measurement of the amount of PCR products on scanned polyacrylamide gels. The units of expression were calculated as the ratio of the amount of PCR product of HIF-1α mRNA to the amount of PCR product of the constitutively expressed housekeeping gene ß-actin, the amount of which was assumed to be expressed constantly in the cell. The measurements were made with a Bio-Rad analysis system.

Transient transfection. pUSEamp(+)/Akt K179M and pUSEamp(+)/myr Akt were purchased by Upstate Biotechnology (Charlottesville, VA, USA). Transfections were performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Cells were treated with or without indicated gefitinib after transfection and then were incubated in normoxia and hypoxia conditions. Cells were incubated for indicated time and Western blot analysis was conducted, as described above. Small interfering Silencer validated small interfering RNA (siRNA) for HIF-1α was obtained from Santa Cruz. Transfection of siRNA was performed with Lipofectamine as described before. After transfection, cells were treated with anti-cancer drugs and incubated in normoxia and hypoxia conditions. Harvested cells conducted the flow cytometric analysis for determining cell death.

Results

Gefitinib inhibits the accumulation of HIF-1α under hypoxia by decreasing its stability. We investigated whether gefitinib could suppress the induction of HIF-1α by hypoxia in A549 cells. As shown in Fig. 1A, the level of HIF-1α peaked at 6 h in cells exposed to hypoxia, however gefitinib treatment effectively inhibited induction of HIF-1α under hypoxia. This
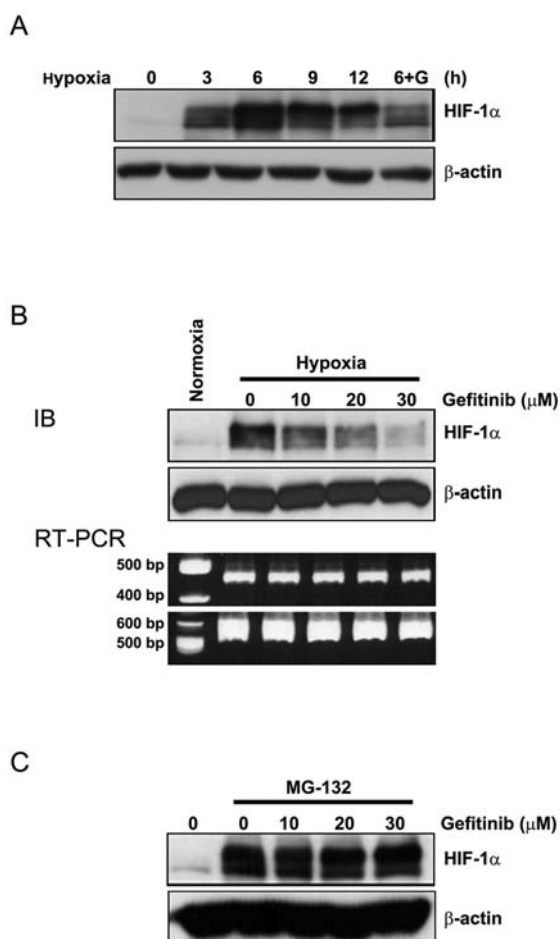
Figure 1. Gefitinib suppressed HIF-1α under normoxia and hypoxia conditions. (A) A549 cells incubated for indicated times under hypoxia following a 3 h preincubation with or without 20 μM gefitinib. HIF-1α was analyzed by Western blotting. (B) Cells were treated with the indicated doses of gefitinib for 3 h and then incubated for 3 h under hypoxia. Cells were harvested and Western blotting and quantitative reverse transcription-PCR was done. (C) Cells were pretreated with 10 μM MG132, proteasomal inhibitor. Six hours later, cells were treated with gefitinib for 3 h and Western blotting was done. G, gefitinib.

Figure 2. Hypoxia-induced HIF-1α required the activation of Akt. (A) A549 cells were treated with indicated inhibitor (50 μM LY, 20 μM PD, 20 μM gefitinib) for 3 h and then exposed to hypoxia for 3 h. (B) A549 cells were transfected by mock (control vector), Akt-Myr (constitutively active form), or Akt-K179A (dominant-negative form) and treated with or without 20 μM gefitinib for 3 h before exposed to hypoxia for 3 h. Harvested cells performed Western blotting.

action of gefitinib was similar in other NSCLC cells, including H460 and Calu-1 (data not shown).

To determine the mechanism how gefitinib decreases induction of HIF-1α under hypoxia, we observed the expression of HIF-1α mRNA and protein stability. Although gefitinib suppressed HIF-1α dose-dependently, the level of HIF-1α mRNA did not change (Fig. 1B). However, gefitinib did not affect the expresson of HIF-1α, when MG-132, a proteasomal inhibitor was added before the treatment of gefitinib (Fig. 1C) suggesting that gefitinib exerts its effect on the modulation of HIF-1α by decreasing the protein stability, not by affecting the protein synthesis.

The PI-3K/Akt pathway is involved in regulation of HIF-1α by gefitinib. Gefitinib has been known that it can inhibit ERK1/2 and Akt signaling pathways effectively. Therefore, we examined how these pathways were related with the regulation of HIF-1α by gefitinib. We used LY294002 and PD98059 as the specific inhibitor of PI-3K/Akt and ERK pathways, respectively. The inhibitory effect on hypoxia-
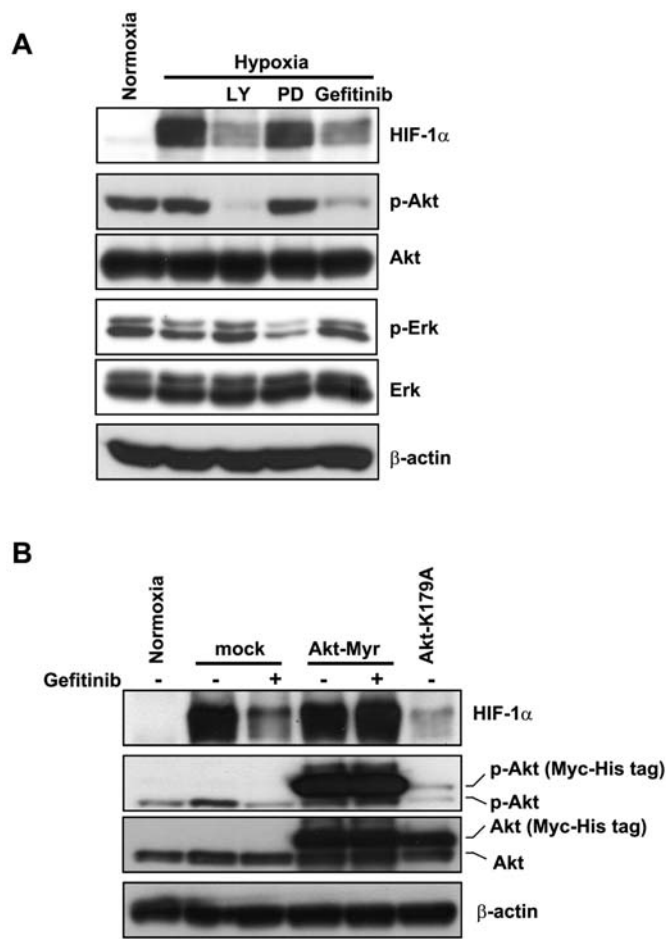
induced HIF-1α expression was only found when cells were treated with LY294002 suggesting that PI-3K/Akt pathway was involved in hypoxia-induced HIF-1α (Fig. 2A). Supporting this, as shown in Fig. 2B, the suppression of HIF-1α was not detected in cells transfected with constitutively active Akt. Moreover, inhibition of Akt using dominant-negative Akt blocked hypoxia-induced HIF-1α. Taken together, these results indicated that Akt pathway is required for hypoxia-induced HIF-1α expression and suppression of HIF-1α by gefitinib was mediated by inhibition of Akt pathway.

Gefitinib can overcome hypoxia-induced resistance. To assess the change of response to chemotherapeutic drugs by hypoxic environment in NSCLC cells, we used MTT assay and flow cytometry for determining growth-inhibitory effect and apoptosis. The cells were treated with chemotherapeutic drugs for 48 h under normoxia or hypoxia condition. The IC_{50} and IC_{80} values of chemotherapeutic drugs in NSCLC cells are shown in Table I. While the cells were more resistant to cisplatin, paclitaxel and gemcitabine in hypoxic condition, the response to gefitinib was similar in both normoxic and hypoxic conditions. The results of the flow cytometry were almost

Table I. Drug response in NSCLC cells under normoxia and hypoxia conditions.

| Drug | A549 | | | | H460 | | | | Calu-1 | | | |
| | Normoxia | | Hypoxia | | Normoxia | | Hypoxia | | Normoxia | | Hypoxia | |
	Mean (SD) IC_{50}	Mean (SD) IC_{80}	Mean (SD) IC_{50}	Mean (SD) IC_{80}	Mean (SD) IC_{50}	Mean (SD) IC_{80}	Mean (SD) IC_{50}	Mean (SD) IC_{80}	Mean (SD) IC_{50}	Mean (SD) IC_{80}	Mean (SD) IC_{50}	Mean (SD) IC_{80}
Gefitinib	7 (0.5)	13 (1.5)	6 (1.2)	12.2 (1.8)	14 (3.2)	30 (5.1)	13.5 (2.1)	32 (4.8)	15 (3)	34 (4)	14 (2)	36 (4)
Cisplatin	15 (2)	35 (4)	37 (5)	71 (2.6)	12 (2.6)	35.4 (6)	24.4 (3.6)	60 (4)	22 (4)	80 (2)	50 (5)	140 (15)
Gemcitabine	50 (7)	150 (20)	160 (20)	310 (20)	5.1 (1.3)	58 (7.9)	5 (1.8)	63 (3.9)	100 (10)	>1000	>1000	>1000
Paclitaxel	1.8 (0.2)	1 (0.8)	2.5 (0.4)	4.8 (0.2)	0.4 (0.1)	2.8 (0.6)	2.1 (0.6)	3.5 (0.3)	5 (0.2)	9 (1)	8 (0.5)	12 (1.5)

Cells were treated with the indicated drug, and were analyzed after 48 h incubation under normoxia or hypoxia conditions. The drug concentration (µM) is that responsible for 50 or 80% growth inhibition as determined in the MTT assay at 48 h. Data represent the mean (SD) from at least three independent experiments. >1000, above 1 mM.

same to those of MTT assay (Fig. 3A) showing that hypoxia rendered cells to acquire the resistance to all tested chemotherapeutic drugs except gefitinib. Expectedly, these drugs did not lead to any change of HIF-1α expression (Fig. 3B).

The suppression of HIF-1α by the transfection of siRNA enhances chemosensitivity under hypoxia. To verify that HIF-1α is really important in hypoxia-induced drug resistance in NSCLC cells we observed the sensitivity to chemotherapeutic drugs after the transfection of HIF-1α siRNA. The level of HIF-1α mRNA was reduced by ~60% compared to that of cells treated with control siRNA in hypoxia (data not shown). Under identical conditions, Western blot analysis showed that HIF-1α protein was detected at all time within the studied time period from 3 to 48 h of hypoxic treatment in cells treated with control siRNA; however, HIF-1α was markedly reduced at early time (from 3 to 6 h) and disappeared at 12 h in cells treated with HIF-1α-siRNA (Fig. 4A). The suppression of HIF-1α by the HIF-1α siRNA led to partially overcome the hypoxia-induced drug resistance. The proportion of sub-G_1 in flow cytometry increased from 22 to 30% with cisplatin, 16 to 29% with paclitaxel and 28 to 37% with gemcitabine (Fig. 4B). Although the response was not fully recovered as in normoxia, these findings indicate that the inhibition of HIF-1α can lead to circumvent hypoxia-induced resistance in NSCLC.

Discussion

The EGFR overexpression was frequently found in non-small cell lung cancer and associated with poor prognosis (21,22). Previously, many studies have reported the role of EGFR signaling in HIF-1α stabilization and activation under normoxia and hypoxia condition (11,14,23,24), although the expression of HIF-1α under hypoxia condition is regulated mainly by ubiquitination and proteasomal degradation (10). We found that the treatment with EGF increased HIF-1α expression under normoxia condition (data not shown). Both EGF- and hypoxia-induced HIF-1α expression was reduced by the treatment with gefitinib suggesting that the control of HIF-1α, regardless of its induction mechanisms, could be achievable through the inactivation of EGFR signaling.

The action of gefitinib within cell masks ATP binding site of EGFR tyrosine kinase and, in consequence, it blocks effectively ERK and PI-3/Akt pathway (25,26). In our data, hypoxia-induced HIF-1α was repressed by the genetic and chemical inhibition of the PI-3/Akt pathway, but not by the inhibition of MAPK pathway. In addition, the ability of geifitnib to reduce HIF-1α expression was compensated by the restoration of a constitutively active Akt. These results indicate that hypoxia-induced HIF-1α expression requires the activation of Akt and inhibition of HIF-1α expression by gefitinib result from the block of Akt activity among the EGFR signaling pathways. Consistent with our data, many studies have showed that induction of HIF-1α under hypoxia is dependent on a PI-3/Akt pathway (14,27,28); however, the mechanisms of HIF-1α regulation via Akt remain unclear. Some studies suggested that Akt pathway induced HIF-1α stabilization (29)
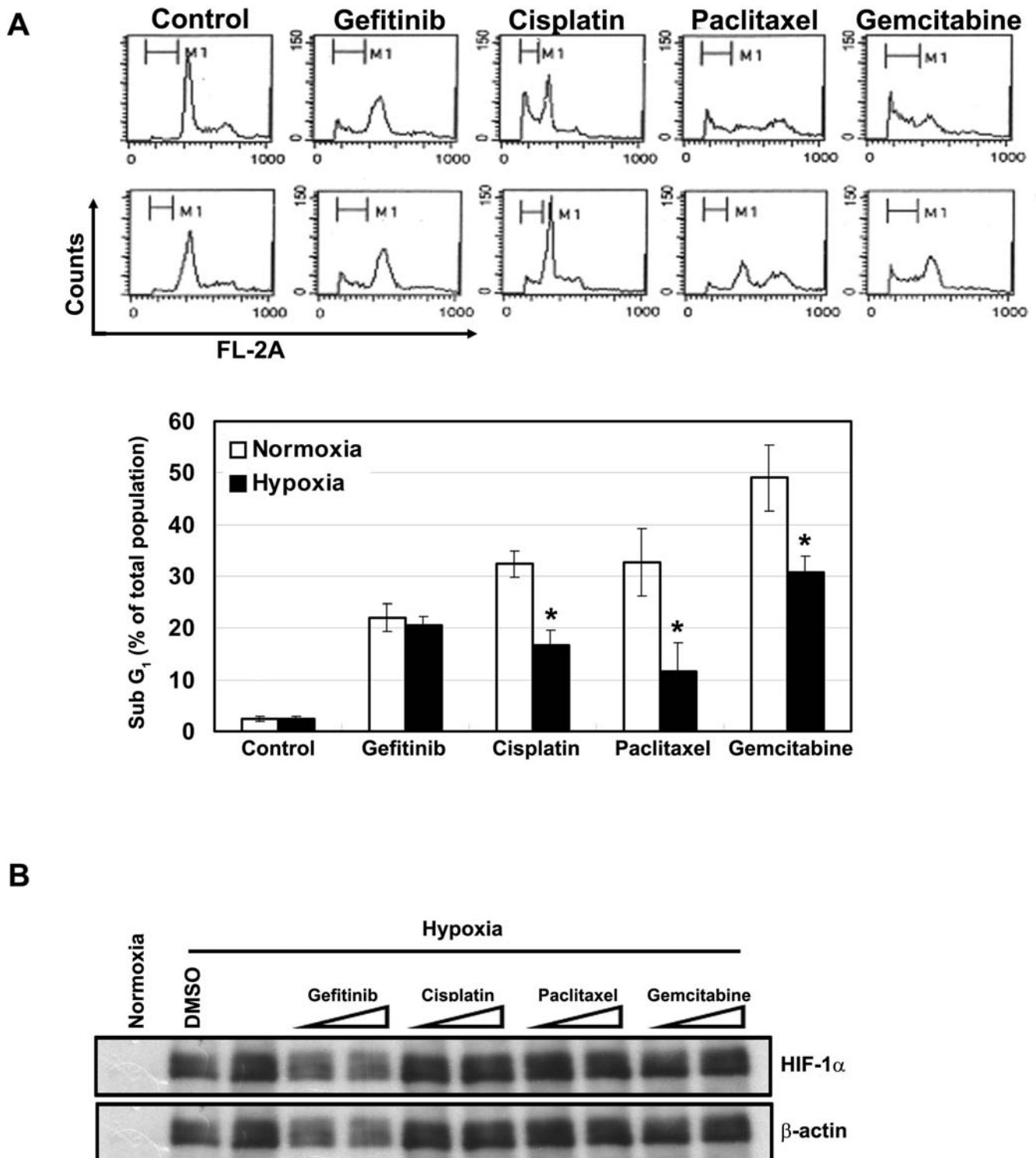
Figure 3. Drug-induced cell death under normoxia and hypoxia condition in A549 cells. (A) A549 cells were treated with or without the indicated drug of IC$_{80}$ values (15 μM gefitinib, 35 μM cisplatin, 120 μM gemcitabine and 3 μM paclitaxel) for 48 h under normoxia or hypoxia condition. Cells were harvested, stained with propidium iodide (PI) and analyzed by flow cytometry. Diagrams of PI flow cytometry in a representative experiment are presented above the graphs. Cell death was determined from three experiments and the bars represent SD. (B) Cells were treated with the indicated drug (gefitinib, 10 and 20 μM; cisplatin, 25 and 35 μM; paclitaxel, 1 and 3 μM; gemcitabine, 100 and 150 μM) or control solvent (DMSO) for 3 h before incubation under hypoxia conditions for 3 h. Cell lysates were blotted with HIF-1α antibodies. P-values were calculated to assess the difference of drug-response according to the hypoxia condition. *P<0.05.

and enhanced the translation (30,31). Supporting this, the modulation of HIF-1α by gefitinib was mediated by degradation through proteasomal pathway in our study. Together, we believe that gefitinib modulates HIF-1α

expression by the inhibition of Akt affecting the HIF-1α protein stability.

HIF-1α plays an important role in modulating the response to chemotherapeutic drugs (7,32,33). Indeed, several studies
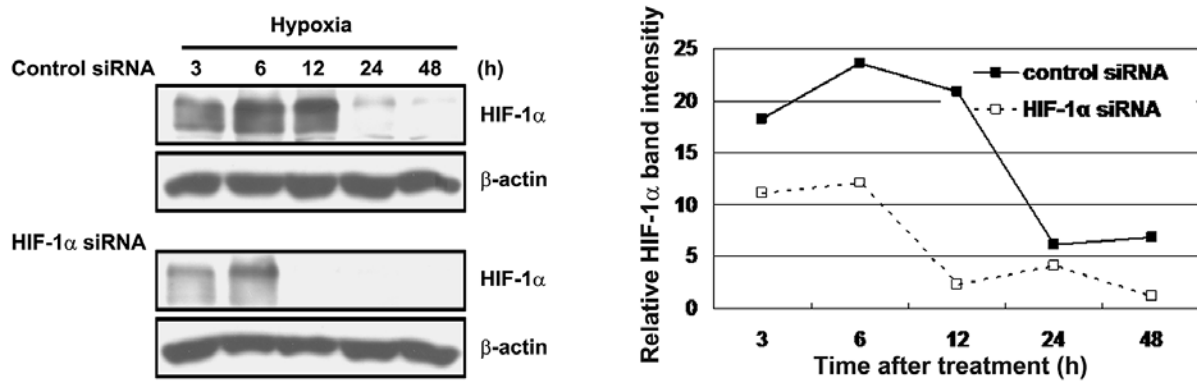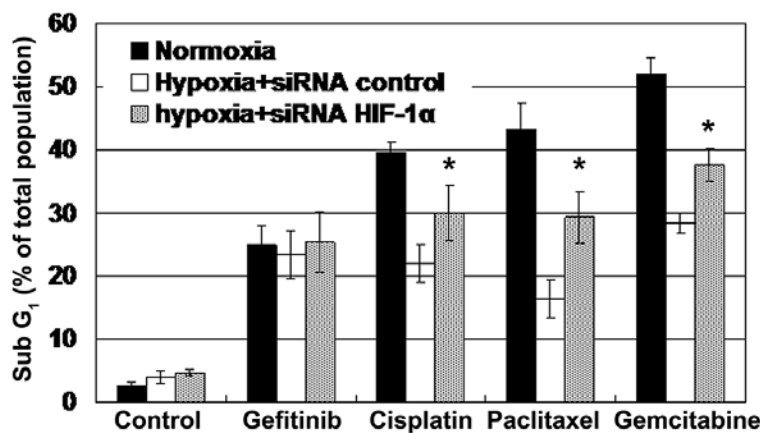
Figure 4. The suppression of HIF-1α by siRNA enhanced chemosensitivity under hypoxia. (A) A549 cells were transfected by control siRNA (100 nM) or HIF-1α siRNA (100 nM) and cultured for 24 h under normoxia condition. After transfection, cells cultured for the given time periods under hypoxia conditions. HIF-1α was analyzed by Western blotting. The graph shows the densitomeric quantification of HIF-1α Western blot analysis. (B) Cells were transfected with control siRNA or HIF-1α siRNA for 24 h and then treated with or without the indicated drug (15 μM gefitinib, 35 μM cisplatin, 120 μM gemcitabine and 3 μM paclitaxel) for 48 h under normoxia or hypoxia conditions. Cells were harvested, stained with propidium iodide (PI) and analyzed by flow cytometry. Cell death was determined from three experiments and the bars represent SD. P-values were calculated to assess the difference of drug-response according to the suppression of HIF-1α. *P<0.05.

showed that the elevated level of HIF-1α was correlated with radio-resistance and chemo-resistance (32,34-36), which was associated with poor prognosis (37,38). Therefore, HIF-1α targeted therapy is potential for enhancement of therapeutic efficacy in many tumor cells. In our study, we showed that the response to conventional chemotherapeutic drugs in NSCLC cells was significantly decreased under hypoxic condition and the down-regulation of HIF-1α by the transfection of siRNA could cause to partially restore the drug sensitivity supporting the role of HIF-1α on the therapeutic resistance. We hypothesized that gefitinib could circumvent hypoxia-induced drug resistance considering that the EGFR signal pathway is related with the HIF-1α expression and gefitinib can effectively control it. As expected, we found that HIF-1α was suppressed by gefitinib and the response to gefitinib was similar both in hypoxic and normoxic conditions.

It is still unclear whether the down-regulation of HIF-1α is the key factor rendering gefitinib to circumvent hypoxia-induced chemoresistance as EGFR signal-related effectors also can be involved in the response to drugs. Indeed, some studies have demonstrated that hypoxic conditions can activate down-stream proteins (Akt and Erk) of the EGFR pathway and activation of these proteins are associated with chemo-resistance (39,40). Nevertheless, we believe that the capability of gefitinib to overcome hypoxia-induced drug resistance is dependent on the regulation of HIF-1α expression, based on previous studies and the results of this study showing the relationship between HIF-1α and drug response.

In summary, we showed that gefitinib inhibited hypoxia-induced HIF-1α expression via modulation of Akt in NSCLC cells and could circumvent hypoxia-induced drug resistance suggesting the EGFR inhibitors might be useful to overcome HIF-1α-related problems in cancer treatment.

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