Actin disruption inhibits hypoxia inducible factor-1α expression via inactivity of Mdm2-mediated p70^{S6K}

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Abstract. The intracellular actin cytoskeleton is a central player in tumor cell migration and adhesion, and interacts with the extracellular matrix during the progression to metastasis. Although recent reports on motility events have revealed that the destabilization of actin affects cancer progression and hypoxia inducible factor-1α (HIF-1α) activity, little is known about the responsive activity of HIF-1α following actin disruption. Here, we demonstrate that the inhibition of actin polymerization or depolymerization attenuates HIF-1α expression independently of proteasomal degradation. The disruption of actin dynamics inactivates HIF-1α translational expression through p70^{S6K} translational signaling; this is independent of p53 activation, suggesting that actin dysfunction-mediated HIF-1α destabilization may lead to the development of novel anticancer chemotherapeutic targets.

Introduction

Actin is the most abundant protein in all eukaryotic cells, and provides a structural and functional framework for cellular processes such as growth, motility, cell division and apoptosis (1). Natural products disrupting the actin dynamics are thought to be potent anticancer agents, and are therefore postulated to control cancer growth and proliferation. Actin polymerization is inhibited by Latrunculin A and B (LA and LB) and Pectenotoxin-2 (PTX-2) with G-actin, in contrast to actin stabilizers such as Jasplakinolide (JSP), Phalloidin and (-)-Doliculide, which disrupt actin depolymerization (2).

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The angiogenic transcriptional factor HIF-1 plays a crucial role in the regulation of oxygen homeostasis, which promotes the cancer angiogenesis-related transcriptional pathway involved in cancer progression, growth, angiogenesis and metastasis. The overexpression of HIF-1α is responsible for metastatic activity in human cancers (3). In the majority of hypoxic tumor cells, accumulated HIF-1α binds to hypoxia response elements in the nucleus, forming active complexes with HIF-1β, by preventing rapid ubiquitination and proteasomal degradation. This transactivated HIF-1α results in the increased expression of numerous genes associated with tumorigenic activity. Additionally, HIF-1α has importance in the development of anticancer drugs, in that it confers chemo- and radio-resistance to cancer cells (4). Under non-hypoxic conditions, however, a variety of cytokines binding to their receptors induce the stability of HIF-1α accumulation through the regulation of phosphatidylinositol 3-kinase (PI3K)/AKT/mammalian target of rapamycin (mTOR) signaling, followed by the phosphorylation of eukaryotic initiation factor 4E (eIF4E) binding protein (4E-BP1) and 70-kDa S6 kinase (p70^{S6K}). Hyper-phosphorylation of 4E-BP1 reduces its affinity for eIF4E factor in the translational process, thereby allowing 4E-BP1 to function as an initiator for the control of translational steps (5,6). Notably, mTOR is involved in the signaling cascade during transcription, proteolysis and actin dynamics; the components mediating these events, including p70^{S6K}, are colocalized with actin fibers (7). p70^{S6K} regulates the phosphorylation and stability of murine double minute 2 (Mdm2), a negative regulator of the p53 tumor suppressor and a positive activator of HIF-1α (8,9), and leads to an increase of HIF-1α expression in Mdm2 protein turnover (10,11).

In this report, we further characterized the effects of several actin-disrupting drugs on human cancer cells. The results contribute to the understanding of HIF-1α regulation, with the aim of developing novel anticancer reagents.

Materials and methods

Cell culture, reagents and antibodies. Human cervical cancer (HeLa) and metastatic prostate cancer (PC3M) cells were obtained from the American Type Culture Collection. Cells were grown in Dulbecco's modified Eagle's medium, supple-

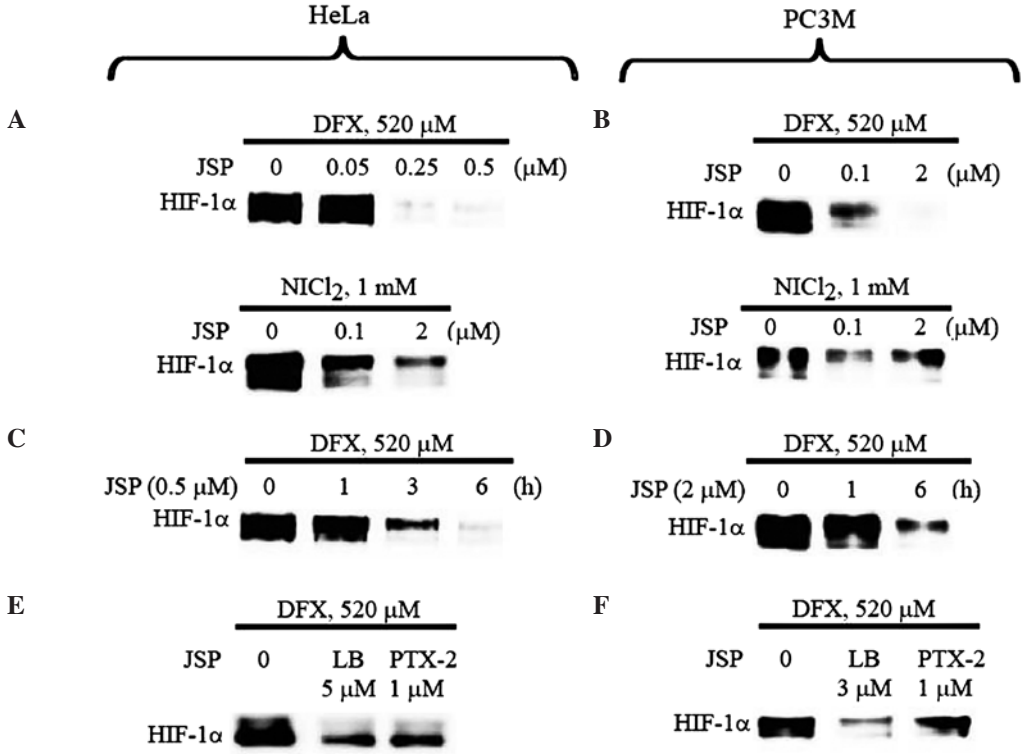

Figure 1. Actin disrupting reagents attenuate HIF-1α protein expression. HeLa and PC3M cells were treated with actin dysfunction chemicals (JSP, LB and PTX-2) for 6 h (A-F) under a mimicked hypoxic state induced by either DFX or $NiCl_2$ in order to analyze the activity pattern of the chemicals under the following conditions: various concentrations (0.05, 0.25 and 0.5 μM on HeLa cells; 0.1 and 2 μM on PC3M cells) of JSP in a dose-dependent manner (A and B) and incubation for up to 6 h with 0.5 or 2 μM of JSP in a time-dependent manner (C and D); disrupted actin dynamics by actin stabilizing (A, B, C and D) or destabilizing (E and F) effect. Ponceau-S staining was applied to confirm the equal loading of proteins in the immunoblotting analysis.

mented with 1% antibiotic-antimycotic (Gibco) and 10% fetal bovine serum (Gibco) and incubated at 37˚C in a humidified atmosphere of 5% CO_2. Chemical reagents and antibodies were from the following suppliers: JSP and LB were from Calbiochem; PTX-2 was a gift from Dr J. Jung (Pusan National University, Korea) (12); proteasome inhibitor MG132 was from Sigma; IgG-specific monoclonal and polyclonal antibodies were from GE Healthcare; anti-mouse HIF-1α monoclonal antibody was from BD Transduction Laboratories; anti-rabbit phospho-AKT, 4E-BP1 and phospho-$p70^{S6K}$ polyclonal antibodies were from Cell Signaling; anti-mouse Mdm2 monoclonal antibody was from Santa Cruz Biotechnology.

Nuclear and cytosolic fractionation. Harvested cells were incubated in buffer A (10 mM HEPES, pH 8.0; 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol, 300 mM sucrose, 0.1% NP-40 and 0.5 mM phenylmethylsulfonyl fluoride) on ice for 5 min. After centrifugation for 5 min at 8,500 rpm, the supernatant (cytosolic fraction) was obtained and stored at -70˚C, and then the pellet containing nuclei was resuspended in buffer B (20 mM HEPES, pH 8.0; 20% glycerol, 100 mM KCL, 100 mM NaCl, 0.2 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride and 0.5 mM dithiothreitol) for 20 min on ice. The nuclear fraction was collected by centrifugation at 12,000 rpm for 5 min.

Western blot analysis. Protein concentration was determined by the bicinchoninic acid protein assay (Pierce Chemical Co.) according to the manufacturer's instructions. Total nuclear and cytosolic proteins (20 μg) were resolved by 8 or 12% SDS-PAGE gels and then electrophoretically transferred onto a nitrocellulose transfer membrane (Whatman) by electroblotting. The membranes were soaked in TBS-T buffer [20 mM Tris, pH 7.4; 137 mM NaCl and 0.1% (v/v) Tween-20] containing 5% (w/v) non-fat milk, and were probed with the specific primary antibodies diluted in TBS-T. The proteins were then detected with the respective secondary antibody-linked horseradish peroxidase, followed by enhancement of chemiluminescence (GE Healthcare). Ponceau-S (Sigma) staining of membranes was applied to confirm equal loading of the proteins and successful transfer to the membranes.

Reverse transcriptase-polymerase chain reaction (RT-PCR). Total cellular RNA was extracted from confluent cells grown in 100-mm tissue culture dishes with TRIzol reagent (Invitrogen) according to the manufacturer's recommendations. The first complementary DNA were synthesized from 2 μg of total RNA with random primers (forward; 5'- TCCAGTTACGTTCCTTCGATCA -3', reverse; 5'- TTT GAGGACTTGCGCTTTCA -3') for human HIF-1α and Moloney murine leukemia virus reverse transcriptase (Promega) using a MyCycler personal thermal cycler (BioRad) under the following thermal conditions: denaturation at 94˚C for 30 sec; annealing at 52˚C for 1 min; extension at 72˚C for 1 min. The PCR products were separated on a 1% agarose gel, visualized by ethidium bromide staining and quantified by glycerldehyde-3-phosphate dehydrogenase (GAPDH) loading as the internal control.

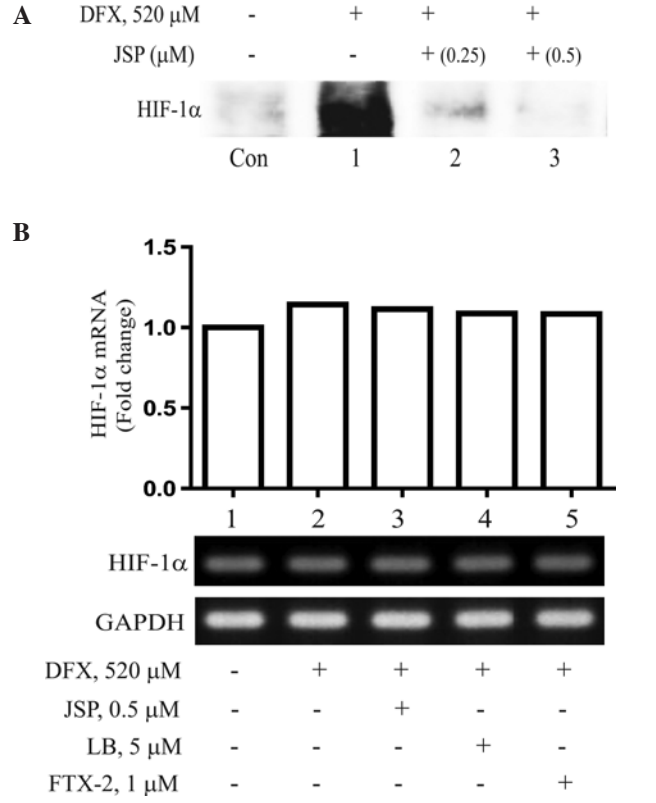

Figure 2. Proteasome inhibitor does not affect the expression of HIF-1α mRNA and heat shock proteins Hsp70 and Hsp90 in HeLa cells. Blocking HIF-1α expression is independent of degradation pattern by proteasome. Cells were treated with either 20 μM of MG132 (A) or 520 μM of DFX (C) and JSP at the indicated concentrations for 6 h, and then nuclear proteins (A) or cytosolic fractionation (C) were subjected to Western blot analysis. Ponceau-S staining was utilized for the loading control. (B) Cells were incubated with JSP, LB or PTX-2 for 6 h, and then total cellular RNA was isolated and HIF-1α mRNA expression was analyzed by RT-PCR. GAPDH was used as an internal standard.

Results

Actin-disrupting reagents attenuate HIF-1α expression in a dose- and time-dependent manner. To determine whether actin dynamics affected the expression of the HIF-1α protein, three actin-disrupting agents were applied under a mimetic hypoxic state induced by 1 mM of the transition metal $NiCl_2$ and 520 μM of the iron chelator desferrioxamine (DFX) for 6 h. The actin-stabilizing agent JSP gradually attenuated the accumulation of stabilized HIF-1α by 6 h in a time- and dose-dependent manner in HeLa and PC3M cells (Fig. 1A-D). In addition, the actin-destabilizing agents LB and PTX-2 also decreased HIF-1α protein expression (Fig. 1E and F). These observations show that disruption of the actin network decreases the accumulation of HIF-1α protein responsible for cancer progression in human cancer cell lines.

Inhibition of HIF-1α accumulation by actin disruption is independent of proteasomal degradation. After HeLa cells were pre-treated with 20 μM of the proteasome inhibitor MG132 for 1 h prior to treatment with JSP for 6 h, HIF-1α protein did not show any accumulation, and no obvious morphological changes of the cells in the normoxic state were observed. Despite the proteasomal inhibition, JSP reduced the accumulation of HIF-1α in a dose-dependent manner through an independent proteasomal degradation pathway (Fig. 2A). No significant changes in the mRNA levels of HIF-1α after treatment of cells with the actin stabilizer or destabilizer were reported (Fig. 2B). These results are consistent with previously published reports (13,14), and suggest that the regulation of HIF-1α expression is independent of proteasomal action. This raises the possibility that other factors may affect this pathway.

Actin disruption inactivates the translational pathway of HIF-1α via 4E-BP1/p70^{S6K} translational signaling. The PI3K/AKT/mTOR signaling pathway is involved in the translational regulation of HIF-1α irrespective of the presence of oxygen in the system (15). To test whether actin disruption played a role in mTOR signaling in the inhibition of HIF-1α protein translation during the hypoxic state, both HeLa and PC-3M cell lines were treated with DFX for 6 h. Cytoplasmic 4E-BP1 was found to be hypophosphorylated by actin disruption. Moreover, inhibition of actin dynamics by either stabilizing or destabilizing agents caused the activity of 4E-BP1 or p70^{S6K} to disrupt the translational machinery (Fig. 3A and B). Basically, the cytoplasmic kinase p70^{S6K} phosphorylated the 40S ribosomal protein S6 for HIF-1α protein synthesis. Actin disruption also inhibited the activity of AKT, an upstream signaling target of mTOR. This was consistent with the hypoxic induction of HIF-1α causing a malfunction in the translational machinery.

Mdm2, but not its target p53, is dependent on p70^{S6K} in HIF-1α expression of actin dynamics. By further testing to investigate the insight signaling of HIF-1α, we observed that concurrent attenuation of Mdm2 and p70^{S6K} expression under hypoxic stress was not associated with the expression levels of p53 in the HeLa (p53+/+) and PC-3M (p53-/-) cell lines (Fig. 4A and B). These results indicate that the attenuation of HIF-1α accumulation is required for the inactivity of Mdm2-mediated p70^{S6K}, independent of p53 expression.

Discussion

HIF-1α protein plays a key role in transactivating the expression of numerous genes, including vascular endothelial growth factor and erythropoietin, which are responsible for tumor growth and angiogenesis in human cancer. For this reason, the emergence of small targets inactivating HIF-1α in cancer therapy has been developed, with a number of clinical trials still to be conducted. In particular, HIF-1α inhibition by actin filament-targeting provides better insight into the mechanisms of HIF-1α regulation, with the aim of developing novel cancer therapeutic treatments. Actin molecules are responsible for cell division, the signaling cascade and intracellular trafficking events in tumor progression. Therefore, actin-targeting linked to HIF-1α inhibition may prove to be beneficial in treating a variety of tumor types. All actin disrupting agents, including stabilizers or destabilizers such as JSP, LB and PTX-2 used in this study, are isolated from natural products. In contrast to LB inhibiting G-actin polymerization by its binding to the nucleotide cleft of the G-actin molecule and PTX-2 sequestering the G-actin structure, JSP potentially stabilizes the polymerization of actin to achieve the same effect (16-18).

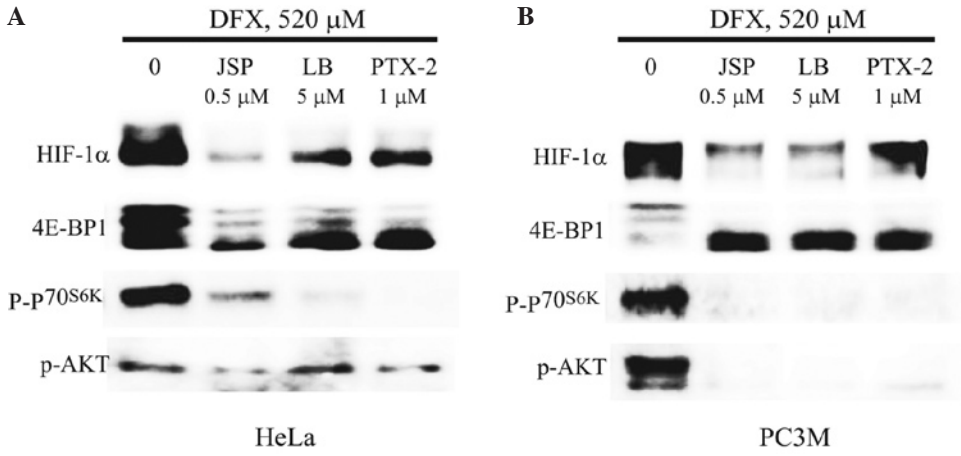

Figure 3. Disruption of actin filaments is associated with HIF-1α translational expression independent of p53. Treatment with or without actin-disrupting agents of the HeLa and PC3M cell lines for 6 h under a hypoxic state inactivated the translational signal pathway of HIF-1α, including the activity of AKT, 4E-BP1 and p70^{S6K} (A and B). Ponceau-S staining was conducted to confirm the equivalent loading of the proteins.

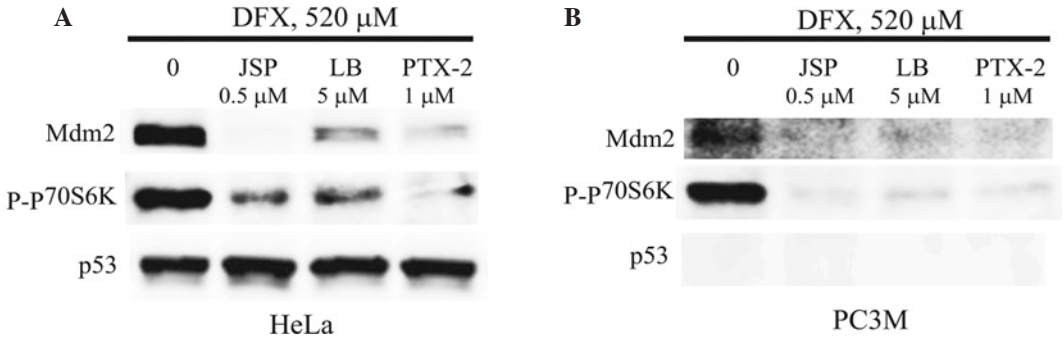

Figure 4. Actin disruption suppresses the expression of the PI3K/AKT pathway regulating p70^{S6K} and 4E-BP1. PC3M cells were treated with either insulin (20 μg/ml) or LB (3 μM) (A), and the result of blocking HIF-1α accumulation was reinforced by another treatment of cells with DFX (520 μM), LY294002 (10 μM) or rapamycin (4 μM) (B) for 18 h. Samples were harvested, and then the expression analysis of nuclear fractions (HIF-1α) and cytosolic proteins (AKT and p70^{S6K}) was performed by Western blot analysis using equivalent amounts of protein (A and B). Ponceau-S staining was conducted to confirm the equivalent loading of the proteins.

Transition metals such as $CoCl_2$ and $NiCl_2$ as well as the iron chelator DFX inhibit the degradation of HIF-1α and stabilize its expression, stimulating the transcription of several genes associated with hypoxia (19,20). In general, HIF-1α is rapidly degraded in the presence of oxygen and is mainly regulated by a proteasomal pathway during oxygen homeostasis. The proteasome inhibitor MG132 (Z-Leu-Leu-Leu-al) disrupts the proteasome and leads to the accumulation of HIF-1α protein in the normoxic state (21% O_2). We found that the inhibition of HIF-1α accumulation by actin disruption was not dependent on the proteasomal pathway, and observed no suppression at the HIF-1α mRNA level in HeLa cells, indicating that the control of HIF-1α protein was through another pathway (Fig. 2). Although normoxia supports the conditions for the ubiquitin-dependent degradation of HIF-1α, it does not affect the translational rate of HIF-1α protein (21), followed by regulation of mTOR signaling, to maintain the status of HIF-1α (22). Though mTOR directly phosphorylates its substrate, p70^{S6K}, and 4E-BP1 is implicated in HIF-1α protein translation, we examined whether the mTOR signaling pathway is involved in the inhibition of the HIF-1α protein by actin dysfunction. Hypophosphorylated 4E-BP1 successfully bound eIF4E and blocked its cap-dependent translation; sequentially, the phosphorylation of 4E-BP1 caused the elimination of eIF4E from the complex following the process of translation (Fig. 3). Under our experimental conditions, cytoplasmic 4E-BP1 and p70^{S6K} were inactivated by actin disruption, irrespective of the application of any actin inhibition or cell types. These results suggest that actin disruption inactivated HIF-1α translation through the mTOR/4E-BP1/p70^{S6K} signaling pathway.

The interaction of HIF-1α and p53 is undisputed; however, the precise mechanisms of this interaction remain unclear (23,24). The expression of p53 is activated by hypoxia or DNA damage, and is negatively regulated by Mdm2 (25). Based on the fact that Mdm2 is a downstream effecter of p70^{S6K} signaling and a positive activator of HIF-1α, this study investigated the interactions between Mdm2 and p70^{S6K} related to p53 in a state of actin disruption (Fig. 4). The results of this study were consistent with previous observations that the instability of actin by LB inhibits HIF-1α accumulation. Thus, this is the first report showing that the inhibition of HIF-1α expression is through actin disruption.

Taken together, blocking the actin dynamics by inhibition of either polymerization or depolymerization attenuates HIF-1α protein expression via the mTOR/p70^{S6K}/Mdm2 signaling pathway in a p53-independent manner. This information may

contribute to the development of a novel chemotherapeutic agent against cancer progression.

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