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Silibinin inhibits expression of HIF-1α through suppression of protein translation in prostate cancer cells

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ABSTRACT

Silibinin is a polyphenolic flavonoid isolated from the milk thistle (*Silybum marianum*) and is reported to exhibit anticancer properties. Recently, it has been reported that silibinin inhibits hypoxia-inducible factor-1α (HIF-1α) expression in cancer cells. However, the precise mechanism by which silibinin decreases HIF-1 expression is not fully understood. In this study, silibinin inhibited basal and hypoxia induced expression levels of HIF-1α protein in LNCaP and PC-3 prostate cancer cells, while the rate of HIF-1α protein degradation and mRNA levels were not affected. We found that the decrease in HIF-1 protein by silibinin correlated with suppression of de novo synthesis of HIF-1α protein. Silibinin inhibited global protein synthesis coincided with reduction of eIF4F complex formation and induction of phosphorylation of the translation initiation factor 2α (eIF-2α) which can cause inhibition of general protein synthesis. These results suggest that silibinin's activity to inhibit HIF-1α protein expression is associated with the suppression of global protein translation.

Introduction

Hypoxia-inducible factor-1 (HIF-1) plays important roles in tumor progression and angiogenesis [1,2]. HIF-1 is a heterodimeric transcription factor composed of an oxygen sensitive HIF-1α subunit and constitutively expressed HIF-1β subunit. The expression level of HIF-1α is regulated through both protein degradation and protein synthesis. Under normoxic conditions, HIF-1α is degraded rapidly through the ubiquitin–proteasome system after protein hydroxylation on Pro-402 and Pro-564 by specific HIF-prolyl hydroxylases, and subsequent binding of von Hippel–Lindau (VHL), a component of an E3 ubiquitin-protein ligase [2,3]. Under hypoxic conditions, HIF-1α hydroxylation is inhibited and results in nuclear accumulation by translocating to the nucleus where it heterodimerizes with HIF-1β and activates the transcription of more than 40 genes important for adaptation and survival under hypoxia [1,2,4]. In addition, HIF-1α expression is also regulated by its rate of de novo synthesis. Oxygen-independent signaling pathways activated by growth factors [insulin-like growth factor-1 (IGF-1), IGF-2, and epidermal growth factor] and cytokines can induce HIF-1α accumulation through enhance of HIF-1α protein synthesis [2,5,6].

Prostate cancer is extremely common in Western nations, representing the second leading cause of cancer death among men in the United States. The significance of angiogenesis in human prostate cancer progression has been established. Several studies showed a significant correlation between microvessel density with Gleason score, pathologic stage, and patient survival [7,8]. Vascular endothelial growth factor (VEGF) levels are significantly higher in prostate tumors relative to normal tissues, and furthermore serum VEGF levels are higher in metastatic prostate cancer patients compared to localized prostate cancer patients [9]. Hypoxia-inducible factor-1, an important mediator of VEGF expression, is highly expressed in prostate tumor tissues, compared to normal and benign prostate tissues [10], suggesting the importance of HIF-1 mediated VEGF expression in prostate tumor.

Silibinin is a polyphenolic flavonoid isolated from the fruits or seeds of milk thistle (*Silybum marianum*). Silibinin has been used clinically for the treatment of various liver diseases, and has been marketed as a dietary supplement [11]. It has been reported that silibinin has anticancer effects against solid tumors, including prostate, skin, and lung, in animal models [12–14]. In prostate cancer cells, silibinin induces cell cycle arrest, apoptosis, reduction of growth factor induced cell proliferation, and differentiation [15–18]. In animal studies, silibinin inhibited growth of DU145 and PC-3 xenografts in nude mice and showed chemopreventive

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efficacy in the transgenic adenocarcinoma of the mouse prostate (TRAMP) model [13,19,20]. It has been suggested that the antiangiogenic effect of silibinin relates to the antineoplastic properties of silibinin [12,21,22]. Recently, several studies have showed silibinin suppressing the expression of HIF-1α [23,24]. But still, the mechanism of inhibitory action of silibinin on HIF-1α expression is unclear. In this study, we investigated the effects of silibinin on the expression of HIF-1α in prostate cancer cells.

Materials and methods

Cell lines and reagents. PC-3 and LNCaP cells were cultured in DMEM medium containing 10% fetal bovine serum (Hyclone), penicillin and streptomycin. Silibinin, protein synthesis inhibitor cycloheximide (CHX), cobalt chloride ($CoCl_2$) and trichloroacetic acid (TCA) were obtained from Sigma. Rapamycin and MG132 were purchased from Calbiochem. Prolyl hydroxylase inhibitor *N*-(methoxyoxoacetyl)-glycin methyl ester (DMOG) was obtained from Cayman Chemical.

Immunoblotting. Anti-HIF-1α (BD Biosciences), anti-HIF-1β (Santa Cruz Biotechnology), anti-phospho-p70S6K (Thr421/Ser424) (Cell Signaling Technology), anti-phospho-RPS6 (Ser235/236) (Cell Signaling Technology), anti-phospho-4EBP1 (Thr37/46) (Cell Signaling Technology), and anti-phospho-eIF-2α (Cell Signaling Technology) antibodies were used at a dilution of 1:1000. Anti-actin antibody (Sigma) was used at a dilution of 1:5000. Western blotting was performed as described previously [25]. Immunoblotting was detected by enhanced chemiluminescence (Amersham Biosciences). The membrane was exposed to X-ray film or analyzed with LAS 3000 (Fujifilm Co.) image analyzer using MultiGauge software.

RT-PCR. Total RNA was isolated from cultured cells using Trizol reagent (Invitrogen). RT-PCR reactions were performed as described previously [26]. The RT-PCR products were separated on agarose gels and visualized by ethidium bromide staining under ultraviolet transillumination. The primer sequences were as follows: (forward primer) 5′-CTC AAA GTC GGA CAG CCT CA-3′ and (reverse primer) 5′-CCC TGC AGT AGG TTT CTG CT-3′ for HIF-1α, (forward primer) 5′-CGT CTT CAC CAC CAT GGA GA-3′ and (reverse primer) 5′-CGG CCA TCA CGC CAC AGT TT-3′ for GAPDH.

7-Methyl GTP pull-down assay. For the isolation of eIF4E and associated proteins, cell were lysed in the buffer containing 50 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, pH 7.4, 75 mM NaCl, 10 mM $MgCl_2$, 1 mM dithiothreitol, 8 mM ethylene glycol-bis[*b*-aminoethyl ether]-*N*,*N*,*N*′,*N*′-tetraacetic acid, 10 mM *b*-glycerophosphate, 0.5 mM Na3VO4, 0.5% triton-X-100 and protease inhibitor cocktail. Cell extracts were incubated for 10 min on ice and centrifuged at 12,000*g* for 10 min at 4 °C. The supernatants (300 μg total protein) precleared with Sepharose 4B beads (Sigma) were next incubated with 7-methyl-GTP-Sepharose 4B (Amersham) for 2 h at 4 °C under constant rotating. Pelleted beads were washed

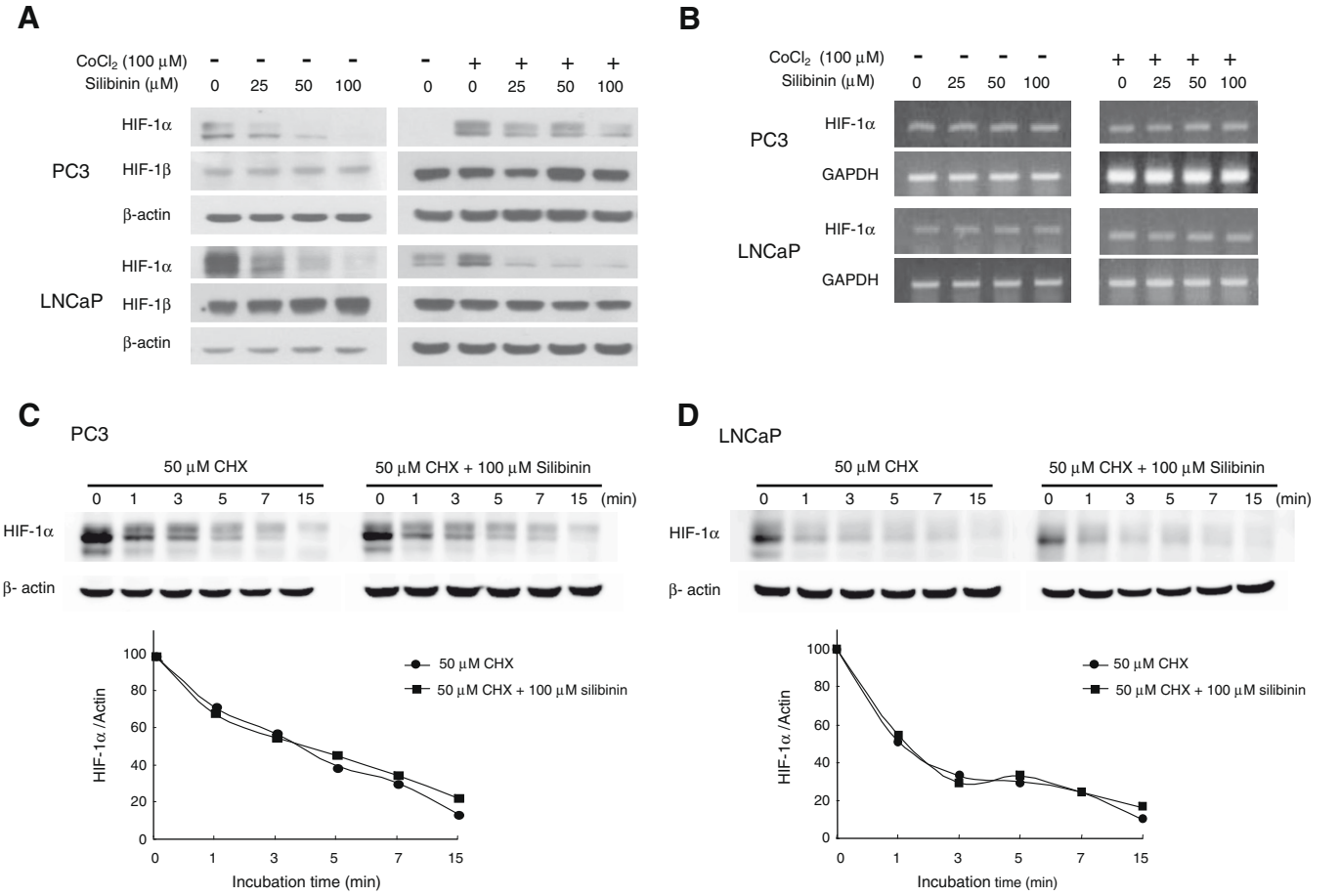

Fig. 1. Silibinin reduces HIF-1α protein expression. (A) PC-3 and LNCaP cells were treated with the indicated concentrations of silibinin in the absence or presence of $CoCl_2$ for 4 h. The cell lysates were subjected to Western blot analysis. (B) PC-3 and LNCaP cells were treated with silibinin in the absence or presence of $CoCl_2$ for 4 h. Total RNAs were isolated and subjected to RT-PCR analysis. (C and D) PC-3 and LNCaP cells were pretreated with 100 μM silibinin for 4 h and cycloheximide (CHX) was added for indicated times. Equal amounts of protein from each sample were analyzed by SDS–PAGE and immunoblotting. Lower panel shows quantification of HIF-1α signal by LAS 3000 image analyzer following normalization by β-actin levels. HIF-1α levels from untreated cells are arbitrarily given the value of 100%.

three times with lysis buffer, and SDS–PAGE sample buffer was added. The samples were boiled for 5 min, and supernatants were analyzed by SDS–PAGE and immunoblotting.

Metabolic labeling, immunoprecipitation, and TCA precipitation. PC-3 and LNCaP cells were seeded in 60 mm culture dishes at a density of 5×10^5 cells/dish and 7×10^5 cells/dish. After overnight incubation, the cells were washed with PBS and treated with silibinin in methionine-free DMEM. After 3 h, ^{35}S-methionine/cystein (PerkinElmer life and analytical sciences) was added to a final concentration of 200 μCi/ml. The cells were incubated for 1 h, and harvested. Equal amount of the extracted protein were subjected to immunoprecipitation using anti-HIF-1α antibody (Santa Cruz Biotechnology). The immunoprecipitates were washed and separated by SDS–PAGE. The gel was dried and exposed to X-ray film. To examine general protein synthesis, radioactivity incorporated into TCA precipitable material in cell lysates extracted from metabolic labeled cells were measured by liquid scintillation analyzer (Packard instrument Co).

Statistical analysis. Data represents mean ± SD from three independent experiments. Statistical analysis was performed by Student's *t*-test at a significance level of $P < 0.05$.

Results

Silibinin inhibits HIF-1α protein expression in prostate cancer cells

We investigated the effects of silibinin on HIF-1α protein expression in PC-3 and LNCaP prostate cancer cells under normoxic and hypoxic conditions. Hypoxic conditions were mimicked by cobalt chloride ($CoCl_2$) treatment. Treatments of silibinin reduced the expression of HIF-1α protein both in PC-3 and LNCaP cells. These reductions were dose-dependent, and were observed under both normoxic and hypoxic conditions (Fig. 1A). Next, in order to evaluate how silibinin inhibits HIF-1α protein expression we examined the changes in HIF-1α mRNA levels after treatment of silibinin (Fig. 1B). Silibinin did not result in significant changes in the expression levels of HIF-1α mRNA, suggesting that silibinin may affect post-transcriptional regulation.

Silibinin reduces synthesis of HIF-1α protein in prostate cancer cells

To determine the effects of silibinin on HIF-1α post-transcriptional regulation, we examined the changes in HIF-1α protein stability after silibinin treatment. To check the changes of HIF-1α protein half-life, PC-3 and LNCaP cells were treated with protein translation inhibitor cycloheximide (CHX) alone or in combination with silibinin. As shown in Fig. 1C and D, silibinin did not significantly change the half-life of HIF-1α protein either in PC-3 and LNCaP cells. These results suggest that silibinin does not affect HIF-1α protein degradation. Next, to check the effects of silibinin on synthesis of HIF-1α protein, we examined the rate of HIF-1α protein accumulation in PC-3 and LNCaP cells treated with silibinin in the presence of prolyl hydroxylase inhibitor DMOG. We expected that if silibinin inhibits HIF-1α protein synthesis, silibinin would reduce the accumulation of HIF-1α protein in the presence of DMOG since prolyl hydroxylation is responsible for HIF-1α protein degradation under normoxic condition. As expected, HIF-1α protein was accumulated in the presence of DMOG under normoxic condition, but the rate of accumulation was significantly lower in silibinin treated PC-3 and LNCaP cells (Fig. 2A and B). To confirm this observation, we examined the effects of silibinin in HIF-1α protein synthesis by metabolic labeling assay. PC-3 and LNCaP cells were labeled with

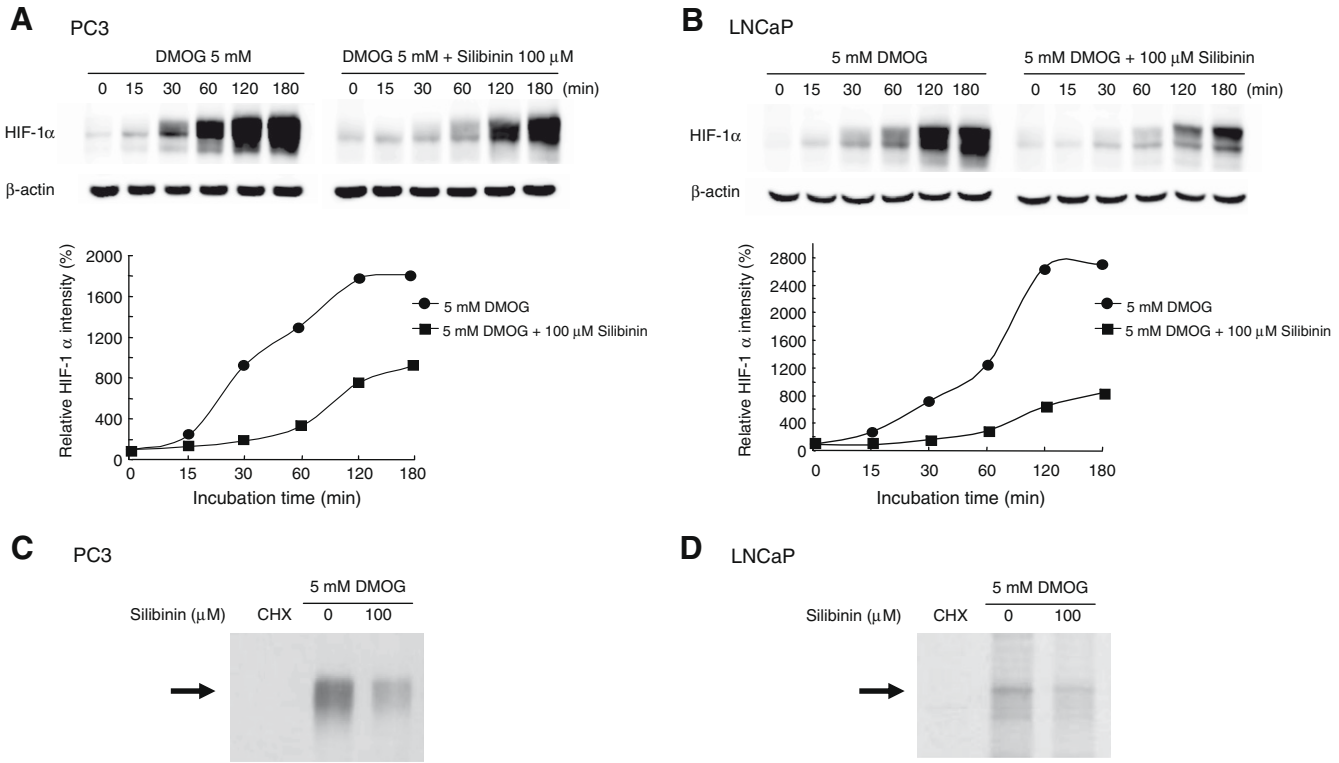

Fig. 2. Silibinin inhibits HIF-1α protein synthesis. (A and B) PC-3 and LNCaP cells were co-treated with 100 μM silibinin and 5 mM DMOG for 1 h. Equal amounts of each protein extract were analyzed by SDS–PAGE and immunoblotting. Lower panel shows quantification of HIF-1α signal by LAS 3000 image analyzer following normalization by β-actin levels. HIF-1α levels from untreated cells are arbitrarily given the value of 100%. (C and D) Metabolic labeling and HIF-1α immunoprecipitation was performed as described in Materials and methods. PC-3 and LNCaP cells were treated with 100 μM silibinin for 3 h in normoxic condition. ^{35}S-methionine/cystein was added to medium for additional 1 h. DMOG (5 mM) or CHX (50 μM) was co-treated with ^{35}S-methionine/cystein at indicated samples.

^{35}S-methionine/cystein in the presence or absence of silibinin for 60 min (Fig. 2C and D). The metabolic labeling assays were done in the presence of DMOG to block degradation of newly synthesized HIF-1α protein. In this assay silibinin significantly inhibited newly synthesized HIF-1α protein accumulation in both PC-3 and LNCaP cells. Taken together with CHX experiments, these results suggest silibinin inhibits HIF-1α protein expression in the prostate cancer cells through the inhibition of HIF-1α protein translation rather than enhancement of its degradation.

Silibinin inhibits global protein synthesis

To determine whether silibinin inhibits HIF-1α protein translation specifically or inhibits general protein synthesis, we examined the effects of silibinin on general protein synthesis. PC-3 and LNCaP cells were metabolic labeled with ^{35}S-methionine/cystein in the presence or absence of silibinin for 60 min, thereafter whole cell lysates were assayed using TCA precipitation and SDS–PAGE electrophoresis (Fig. 3). Silibinin induced significant inhibition of protein synthesis in PC-3 and LNCaP cells. The radioactivity incorporated into TCA precipitable material from cell lysates were significantly reduced in a dose-dependent manner (Fig. 3A and B). When the metabolic labeled cell lysates were electrophoresed in SDS–PAGE gel, similar results were obtained (Fig. 3C and D). These results suggest that silibinin-induced decrease of HIF-1α protein is caused by the silibinin's inhibitory action on general protein synthesis.

To evaluate the mechanism by which silibinin inhibits global protein synthesis, we assessed the altered levels of eukaryotic initiation factor (eIF) 4F complex which stimulates cap-dependent translation initiation by 7-methyl-GTP pull-down assay (Fig. 4A). Analysis of the 7-methyl-GTP pull-down eluents showed that silibinin decreased the levels of eIF4E-associated eIF4G and increased the levels of eIF4E-associated 4EBP1 both in PC-3 and LNCaP cells. These results indicate that silibinin reduces the levels of eIF4F complex formation. Rapamycin, a mTOR inhibitor, which is known to inhibit mTOR-mediated phosphorylation of 4EBP1 showed similar results. As several investigators have reported silibinin inhibits Akt/mTOR signaling pathway [12,27], we checked the phosphorylation status of Akt and the direct targets of mTOR, 4EBP1 and p70S6K after silibinin treatment (Fig. 4B). Both in PC-3 and LNCaP cells, silibinin decreased phosphorylation of Akt, 4EBP1, and 70S6K. The downstream target of p70S6K, ribosomal protein S6 (RPS6), was also dephosphorylated. These data suggest that silibinin reduces protein translation through the inhibition of translation initiation in association with suppression of Akt/mTOR signaling. Recently, it has been reported that eIF-2α phosphorylation down-regulated HIF-1α expression causing general protein synthesis repression in prostate cancer cells [28]. We sought to examine whether silibinin induces the phosphorylation of eIF-2α (Fig. 4C). The eIF-2α was dose dependently phosphorylated by silibinin. It is known that phosphorylation of eIF-2α reduces global protein translation [29,30]. These results suggest that silibinin's

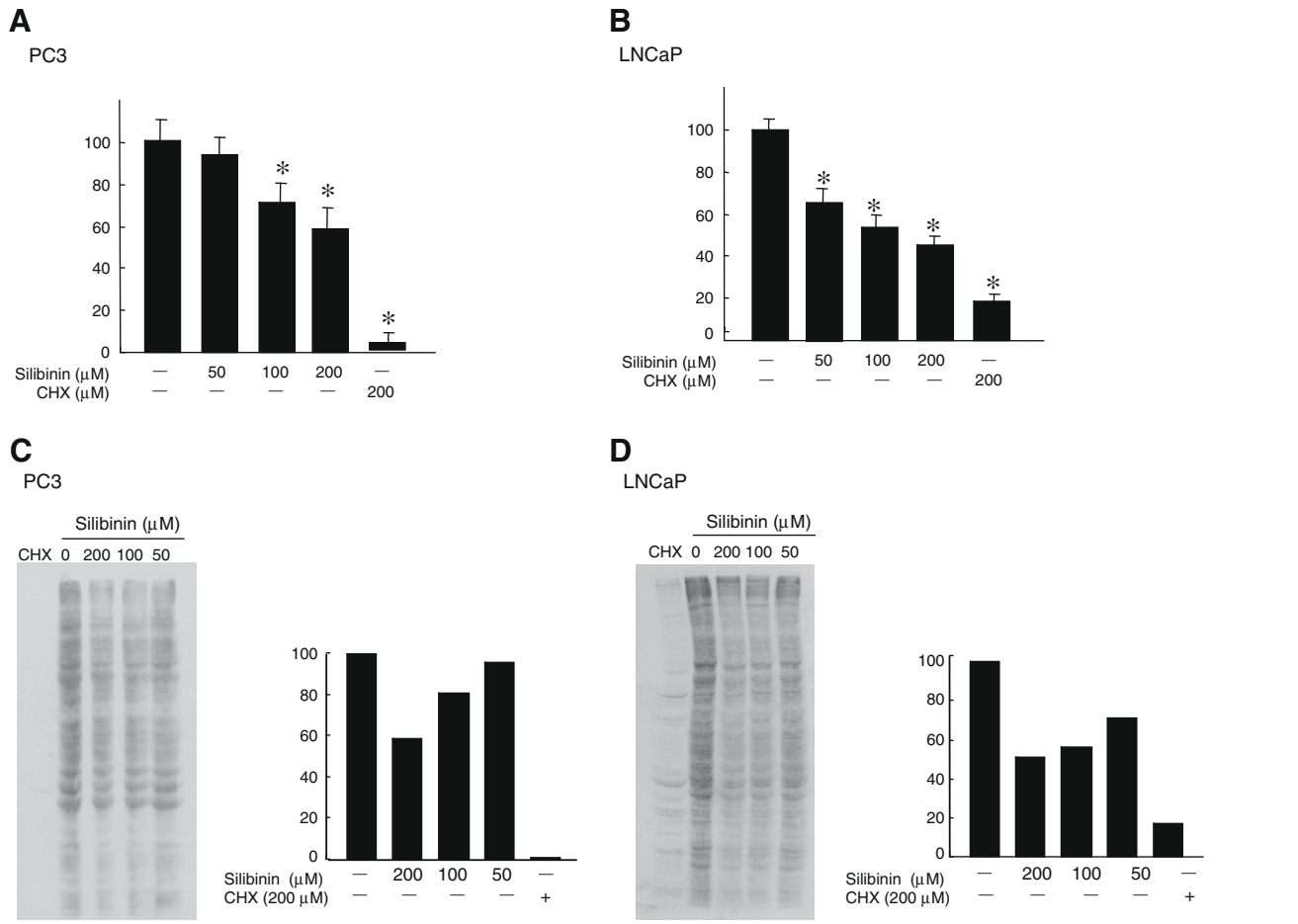

Fig. 3. Silibinin reduces global protein synthesis. PC-3 and LNCaP cells were pretreated with the indicated concentrations of silibinin for 3 h, and ^{35}S-methionine/cystein was added to medium for 1 h. (A and B) Cells were harvested after labeling the radioactivity incorporated into TCA precipitable material was measured as described in Materials and methods. The levels of protein synthesis are shown as a percentage of the value obtained in the absence of silibinin. * indicates a significant difference from vehicle treated group. (C and D) Cells were harvested, and cell lysates were electrophoresed in SDS–PAGE and radioactivity incorporated was detected by autoradiography. Right panel shows quantification of signal intensity of each lane.

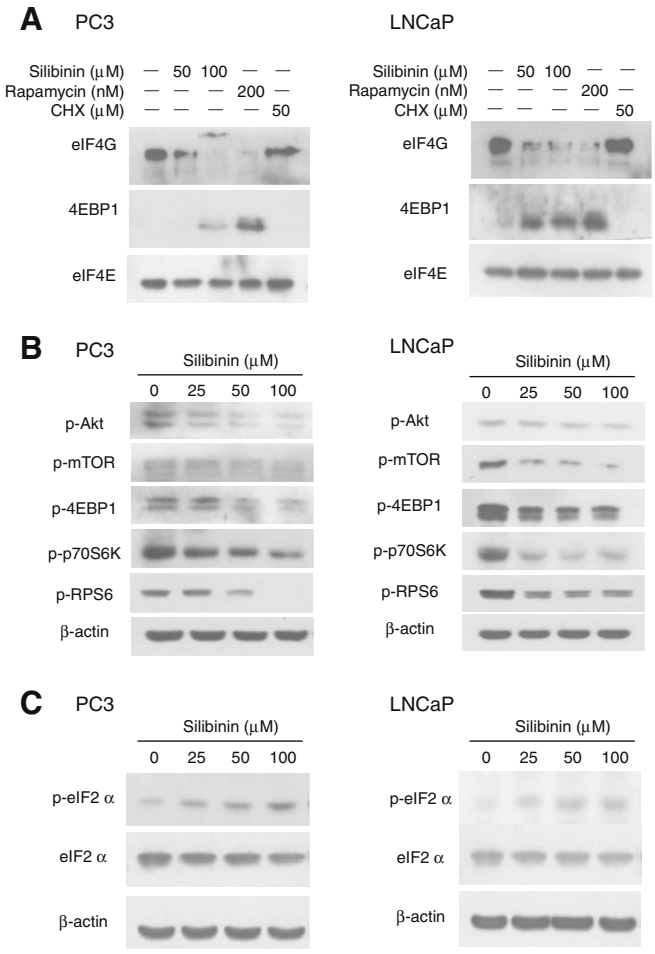

Fig. 4. (A) Silibinin reduces eIF4F complex formation. The effect of silibinin on eIF4F complex formation was examined by 7-methyl-GTP-Sepharose pull-down assay. PC-3 and LNCaP cells were treated with indicated concentration of silibinin for 4 h. The cell lysates were prepared and proteins bound to 7-methyl-GTP-Sepharose 4B were analyzed by SDS–PAGE, and Western blotting with indicated antibodies as described in Materials and methods. (B) Effects of silibinin on mTOR signaling. PC-3 and LNCaP cells were treated with indicated concentrations of silibinin for 4 h and cell lysates were analyzed by Western blotting. (C) Silibinin induces eIF-2α phosphorylation. PC-3 and LNCaP cells were treated with silibinin for 4 h. Cell lysates were analyzed by Western blotting.

inhibitory activity on global protein synthesis is associated with phosphorylation of eIF-2α. Taken together, these data suggest that silibinin may suppress global protein translation through modulation of multiple translation factors including eIF4F and eIF-2α.

Discussion

Overexpression of HIF-1α has been demonstrated in many human cancers and their metastases compared to their normal adjacent tissues, including prostate, breast, lung and head and neck cancers [10]. The expression of HIF-1α correlates with increased vascularity, resistance to chemotherapy and radiotherapy, and poor prognosis [4]. Hence, the development of cancer therapeutics targeting HIF-1 appears to be attractive. In this study, we showed that silibinin down-regulated HIF-1α expression through the inhibition of HIF-1α translation in association with suppression of global protein synthesis.

The steady state level of HIF-1α is controlled by the balance of degradation and synthesis of the protein. We found in this study, silibinin decreases HIF-1α levels through the inhibition of its translation in PC-3 and LNCaP prostate cancer cells, without affecting HIF-1α protein degradation. This result is consistent with the recent report of Garcia-Maceira and Mateo [24] which shows that silibinin inhibits HIF-1α expression at the translational level in association with inhibition of mTOR/p70S6K/4EBP1 signaling pathway in HeLa and Hep3B cells. mTOR signaling pathway is known to regulate HIF-1α protein translation [6,30]. mTOR controls protein translation by the phosphorylation of the downstream effectors p70S6K and 4EBP1 [31]. Dephosphorylation of 4EBP1 inhibits eIF4F complex formation which interacts with the mRNA cap structure and stimulates cap-dependent protein translation [32]. In this study silibinin inhibited 4EBP1 phosphorylation correlating with reduced eIF4F complex formation. In consistent with our data, recently Lin et al. [33] reported silibinin reduced eIF4F complex levels in MCF-7 breast cancer cells. Therefore silibinin's activity to reduce HIF-1α could be caused by the inhibition of protein translation in association with the inhibition of mTOR/4EBP1 signaling.

In addition, we found that silibinin induced eIF-2α phosphorylation. It is reported that eIF-2α is phosphorylated in response to cellular stress and the phosphorylation of eIF-2α inhibits global protein translation through suppressing the formation of ternary complex (eIF2-GTP-Met-tRNA) in translation initiation [29,30]. Moreover, it is reported that HIF-1α expression could be suppressed by eIF-2α phosphorylation-mediated translation repression [28]. Thus, phosphorylation of eIF-2α by silibinin could play an important role in the suppression of HIF-1α protein translation. Additional studies are required to elucidate the molecular mechanism of silibinin-induced phophorylation of eIF-2α.

In this report, silibinin showed significant inhibition of global protein translation in correlation with reduced formation of eIF4F complex and phosphorylation of eIF-2α. Deregulation of protein translation has emerged as important event in oncogenesis. Most cancers are caused by deregulation of signaling pathways which affect translation. Previously, many investigators have reported the anticancer effects of silibinin. Therefore our results suggest silibinin's anticancer activity may be caused by its ability to reduce protein synthesis.

In summary, we report that silibinin inhibits HIF-1α expression by suppressing global protein translation. We also show silibinin's action on protein translation inhibition is associated with the reduction of eIF4F complex formation and induction of eIF-2α phosphorylation.

Acknowledgments

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