Silibinin inhibits cytokine-induced signaling cascades and down-regulates inducible nitric oxide synthase in human lung carcinoma A549 cells

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Abstract

Recently, we reported that silibinin inhibits primary lung tumor growth and progression in mice and down-regulates inducible nitric oxide synthase (iNOS) expression in tumors; however, the mechanisms of silibinin action are largely not understood. Also, the activation of signaling pathways inducing various transcription factors are associated with lung carcinogenesis and their inhibition could be an effective strategy to prevent and/or treat lung cancer. Herein, we used human lung epithelial carcinoma A549 cells to explore the potential mechanisms and observed strong iNOS expression by cytokine mixture (containing 100 units/mL IFN-γ + 0.5 ng/mL interleukin-1β + 10 ng/mL tumor necrosis factor-α). We also examined the cytokine mixture–activated signaling cascades, which could potentially up-regulate iNOS expression, and then examined the effect of silibinin (50-200 μmol/L) on these signaling cascades. Silibinin treatment inhibited, albeit to different extent, the cytokine mixture–induced activation of signal transducer and activator of transcription 1 (Tyr^{701}), signal transducer and activator of transcription 3 (Tyr^{705}), activator protein-1 family of transcription factors, and nuclear factor-κB. The results for activator protein-1 were correlated with the decreased nuclear levels of phosphorylated c-Jun, c-Jun, JunB, JunD, phosphorylated c-Fos, and c-Fos. Further, silibinin also strongly decreased cytokine mixture–induced phosphorylation of extracellular signal-regulated kinase 1/2 but only marginally affected JNK1/2 phosphorylation. Silibinin treatment also decreased constitutive p38 phosphorylation in the presence or absence of cytokine mixture. Downstream of these pathways, silibinin strongly decreased cytokine mixture–induced expression of hypoxia-inducible factor-1α without any considerable effect on Akt activation. Cytokine mixture–induced iNOS expression was completely inhibited by silibinin. Overall, these results suggest that silibinin could target multiple cytokine-induced signaling pathways to down-regulate iNOS expression in lung cancer cells and that could contribute to its overall cancer preventive efficacy against lung tumorigenesis. [Mol Cancer Ther 2008;7(7):1817–26]

Introduction

Lung cancer is the leading cause of cancer burden worldwide with >3 million incidences and 1 million deaths annually. In United States, it is the second leading cause of cancer-related incidences and is the leading cause of cancer-related deaths (1). It is estimated that, among men, lung cancer alone will kill (31% of total cancer deaths) more than the next three cancers combined [prostate (9%), colon and rectum (9%), and pancreas (6%; ref. 1)]. Despite extensive research, the overall 5-year survival rate in lung cancer patients is only 8% to 14% and has improved only marginally during the past 25 years (1, 2). These alarming statistics suggest the need for effective preventive measures to lower the burden of this malignancy. Chemoprevention is a potentially important approach to reduce the large number of lung cancer–related deaths. Numerous studies have found that chemoprevention using phytochemicals, especially flavonoids, can prevent variety of cancers including lung cancer (3–5). In this regard, a large clinical study has suggested the presence of inverse association between flavonoid intake and subsequent lung cancer incidences (6).

Silibinin, a flavonoid, is the major bioactive constituent present in silymarin, which is isolated from milk thistle (*Silybum marianum*), and widely used as a hepatoprotective agent and has been marketed as a dietary supplement. *In vitro* and *in vivo* studies have revealed pleiotropic capabilities of silibinin against various epithelial cancers including lung cancer (3, 7–12). Silibinin treatment induces a significant growth inhibition, a moderate cell cycle arrest, and a strong apoptotic death in SHP-77 cells (small cell lung carcinoma cells) and A-549 cells (non-small cell lung carcinoma cells; ref. 13). Dietary silibinin (up to 1%, w/w) strongly retards the urethane-induced lung tumor growth and progression via inhibition of proliferation and angiogenesis (3). Further, we observed that silibinin suppresses the expression of proliferating cell nuclear antigen, cyclin

Received 3/15/08; revised 5/1/08; accepted 5/17/08.

Grant support: National Cancer Institute grant RO1 CA113876.

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doi:10.1158/1535-7163.MCT-08-0256

D1, vascular endothelial growth factor, and cyclooxygenase-2 and, with profound magnitude, inducible nitric oxide synthase (iNOS) and microvessel density in lung tumors (3). Silibinin has been shown to inhibit the invasive capability of human lung cancer cells via decreasing the production of urokinase plasminogen activator, metalloproteinase-2, and by enhancing the expression of tissue inhibitor of metalloproteinase (14). The anti-invasive effect of silibinin in lung cancer cells was related with inhibition of phosphatidylinositol 3-kinase/Akt and mitogen-activated protein kinase (MAPK) signaling pathways (15). In another study, silibinin suppressed the growth of human non-small cell lung carcinoma A549 xenograft and also enhanced the therapeutic potential of doxorubicin (16). In all *in vivo* studies, silibinin was reported to be nontoxic and biologically available and therefore could be useful for chemopreventive approach against lung cancer.

During lung carcinogenesis, various cytokines, secreted by tumor cells itself or cells in its vicinity, are known to maintain a chronic proinflammatory and immunosuppressive condition in the tumor microenvironment (17) and promote cell proliferation, apoptosis resistance, angiogenesis, invasion, and metastasis (18). Therefore, understanding of cytokine-induced signaling is vital to comprehend the process of lung carcinogenesis. Herein, we first examined the effect of a defined cytokine mixture, consisting of IFN-γ, interleukin-1β (IL-1β), and tumor necrosis factor-α (TNF-α), on key cellular signaling molecules [signal transducers and activators of transcription (STAT), nuclear factor-κB (NF-κB), activator protein-1 (AP-1), MAPK, Akt, and hypoxia-inducible factor-1α (HIF-1α)], which regulate the expression of iNOS in human non-small lung cancer A549 cells, followed by the use of this model to assess the effect of silibinin on these activated signaling molecules. The mixture of interferon-γ (IFN-γ), IL-1β, and TNF-α has been used in various studies and has been shown to increase iNOS expression (19–22). Results clearly suggest the efficacy of silibinin in inhibiting the cytokine mixture–induced signaling pathways as well as expression of iNOS. These mechanistic observations for iNOS expression could be highly relevant, as iNOS is known to promote inflammation and tumor angiogenesis, and associated with growth and progression of lung tumors.

Materials and Methods

Cell Line and Reagents

Human epithelial lung carcinoma A549 cells were from the American Type Culture Collection. RPMI 1640 and other cell culture materials were from Invitrogen. Silibinin was from Sigma and dissolved in DMSO as stock solution. TNF-α, IL-1β, and INF-γ were purchased from Biosource. Consensus NF-κB- and AP-1-specific oligonucleotides and the gel shift assay system were from Promega. The primary antibodies for iNOS, phosphorylated c-Jun, c-Jun, JunB, JunD, Fra-1, Fra-2, phosphorylated c-Fos, p65, and p50 were from Santa Cruz Biotechnology. Antibodies for phosphorylated and total extracellular signal-regulated kinase 1/2 (ERK1/2), STAT1, STAT3, JNK1/2, p38, and inhibitor of κBα and goat anti-rabbit immunoglobulin horseradish peroxidase–conjugated secondary antibodies were purchased from Cell Signaling Technology. The rabbit anti-mouse antibody and enhanced chemiluminescence detection system was from Amersham. HIF-1α antibody was from Novus Biologicals. Antibody for β-actin was from Sigma.

Cell Culture and Treatments

A549 cells were cultured in RPMI 1640 containing 10% fetal bovine serum and 1% penicillin/streptomycin. At about 70% confluency, cultures were switched to serum-free medium for 24 h and then treated with different doses of silibinin (50, 100, and 200 μmol/L) in DMSO for 2 h and then stimulated with cytokine mixture (100 units/mL IFN-γ + 0.5 ng/mL IL-1β + 10 ng/mL TNF-α) in serum-free medium from 15 min to 24 h. The concentrations of IFN-γ, IL-1β, and TNF-α used in the present study were based on earlier published work (19, 22). An equal amount of DMSO (vehicle) was present in each treatment, including control; DMSO concentration did not exceed 0.1% (v/v) in any treatment. After desired treatments, medium was aspirated, cells were washed two times with cold PBS, and total cell lysates or cytosolic and nuclear extracts were prepared as described earlier (23, 24).

Immunoblotting

For immunoblotting, cytosolic extracts or nuclear extracts or total cell lysates (40-80 μg protein/sample) were denatured with 2× sample buffer and samples were subjected to SDS-PAGE on 8% or 12% Tris-glycine gel. The separated proteins were transferred onto nitrocellulose membrane followed by blocking with 5% nonfat milk powder (w/v) in TBS [10 mmol/L Tris-HCl (pH 7.5), 100 mmol/L NaCl, 0.1% Tween 20] for 1 h at room temperature. Membranes were probed for the protein levels of desired molecules using specific primary antibodies followed by the appropriate peroxidase-conjugated secondary antibody and visualized by enhanced chemiluminescence detection system. In each case, blots were subjected to multiple exposures on the film to make sure that the band density is in the linear range. The blots were scanned with Adobe Photoshop 6.0 (Adobe Systems), and the mean density of each band was analyzed by the Scion Image program (NIH). Each membrane was stripped and reprobed with anti-β-actin antibody to normalize for differences in protein loading in the densitometric values given below each band.

Electrophoretic Mobility Shift Assay

For electrophoretic mobility shift assay (EMSA), NF-κB- or AP-1-specific oligonucleotides (3.5 pmol) were end-labeled with [γ-^{32}P]ATP (3,000 Ci/mmol at 10 mCi/mL) using T4 polynucleotide kinase in 10× kinase buffer as per the manufacturer's protocol (Promega). Labeled double-stranded oligo probe was separated from free [γ-^{32}P]ATP using a G-25 Sephadex column. The consensus sequences of the oligonucleotides used were 5′-AGTTGAGGGGACTTTCCCAGGC-3′ and 3′-TCAACTCCCCTGAAAGGGTCCG-5′ for NF-κB and 5′-CGCTTGATGAGTCAGCCGGAA-3′ and 3′-GCGAACTACTCAGTCGGCCTT-5′ for

AP-1. For EMSA, 4 or 8 μg protein (for AP-1 and NF-κB, respectively) from nuclear extracts was first incubated with 5× gel shift binding buffer [20% glycerol, 5 mmol/L $MgCl_2$, 2.5 mmol/L EDTA, 2.5 mmol/L DTT, 250 mmol/L NaCl, 50 mmol/L Tris-HCl, and 0.25 mg/mL poly(deoxyinosinic-deoxycytidylic acid)·poly(deoxyinosinic-deoxycytidylic acid)] and then with ^{32}P-end-labeled consensus oligonucleotide for 20 min at 37°C. DNA-protein complexes thus formed were resolved on 6% DNA retardation gels (Invitrogen). In supershift assay, samples were incubated with anti-p65, anti-p50, anti-c-Jun, anti-c-Fos, anti-JunB, anti-JunD, anti-Fra-1, anti-Fra-2, or anti-FosB antibody before the addition of ^{32}P end-labeled NF-κB or AP-1 oligo. DNA-protein or DNA-protein-antibody complexes thus formed were resolved on 6% DNA retardation gels. To check the specificity of DNA binding, only labeled probe sample was also run together with other samples. In each case, the gel was dried and bands were visualized by autoradiography.

Results

Cytokine Mixture Treatment Activates Various Signaling Pathways in A549 Cells

Various studies have shown the role of transcription factors (STAT, AP-1, NF-κB, and HIF-1α), MAPK (ERK1/2, JNK1/2, and p38), and iNOS expression in lung tumorigenesis (25–27). In the present study, we first analyzed the effect of a defined cytokine mixture (containing 100 units/mL IFN-γ + 0.5 ng/mL IL-1β + 10 ng/mL TNF-α) on these key signaling molecules at various time points (30 min to 24 h). Cytokine mixture exposure resulted in increased phosphorylation of STAT1 (Tyr^{701}) and STAT3 (Tyr^{705}) as early as 30 min, after which there was a gradual decrease in the phosphorylation, without having any considerable effect on STAT3 (Ser^{727}) phosphorylation, which was constitutively activated in A549 cells (Fig. 1A). The total level of STAT1 showed an increase starting from 6 h; however, no considerable effect was observed on total STAT3 level (Fig. 1A).

We next examined the effect of cytokine mixture on transcription factors AP-1 and NF-κB. EMSA results showed that there was a gradual increase in AP-1 DNA-binding ability and it reaches to a maximum level at 24 h of treatment (Fig. 1B). AP-1 is a dimeric complex of various combination of Jun family (c-Jun, JunB, and JunD) and Fos family members (c-Fos, FosB, Fra-1, and Fra-2). Gel supershift assay revealed the various constituents of AP-1 complex—c-Jun, c-Fos, JunB, JunD, Fra-1, Fra-2, and FosB (Fig. 1C); among these constituents, c-Jun, JunD, and Fra-2 were identified as key constituents in cytokine mixture–induced active AP-1 complex (Fig. 1C). Cytokine mixture also increased the NF-κB activation maximally after 30 min of treatment and then gradually decreased nearly to the basal level after 24 h of treatment (Fig. 1D). We also observed increased nuclear localization of p65 and inhibitor of κB phosphorylation (Ser^{32}) and degradation as early as 15 min of cytokine mixture treatment (data not shown). Gel supershift assay identified the p65 and p50 as the constituents of NF-κB (Fig. 1D).

Cytokine mixture treatment also increased the phosphorylation of ERK1/2 and JNK1/2 maximally by 30 min (Fig. 2A). ERK1/2 phosphorylation declined in a biphasic manner, whereas JNK1/2 phosphorylation declined steadily to the basal level by 24 h (Fig. 2A). p38 was constitutively activated in A549 cells; cytokine mixture exposure decreased its phosphorylation, and by 12 h, it was undetectable, although there was no change in the total p38 levels (Fig. 2A). Cytokine mixture also increased the Akt phosphorylation after 30 min, which remained phosphorylated even after 24 h of treatment, but there was a decline in the total Akt levels (Fig. 2A). Under similar treatment conditions, cytokine mixture treatment increased the expression of transcriptional factor HIF-1α, with maximal expression after 24 h of treatment (Fig. 2B). Further, cytokine mixture strongly increased the iNOS expression as early as 30 min, with the maximum level after 24 h of treatment (Fig. 2C). These results suggested that most of the transcription factors and signaling molecules are activated after 30 min of cytokine mixture treatment, which was, next, used to examine the effect of silibinin pretreatment (2 h before cytokine mixture exposure) on the cytokine mixture–induced signaling cascades in A549 cells.

Effect of Silibinin on Cytokine Mixture–Induced Activation of STAT1 and STAT3 in A549 Cells

STAT is broadly known as oncogene, which regulates broadly diverse biological processes, including cell proliferation, transformation, apoptosis, differentiation, angiogenesis, inflammation, and immune response (26, 28, 29). Herein, we analyzed the effect of silibinin pretreatment (2 h) on the increased STAT phosphorylation caused by 30 min exposure to cytokine mixture in A549 cells. Silibinin pretreatment (50-200 μmol/L) strongly reduced the cytokine mixture–induced phosphorylation of STAT1 (Tyr^{701}) in the total cell lysates. Silibinin treatment also decreased the total STAT1 level compared with cytokine mixture treatment alone (Fig. 3A). Further, silibinin pretreatment strongly inhibited the phosphorylation of uninduced as well as cytokine mixture–induced STAT3 at Tyr^{705} site but moderately inhibited the phosphorylation at Ser^{727} site. Silibinin treatment moderately decreased the cytokine mixture–induced level of total STAT3 (Fig. 3A). Because nuclear localization of STAT is necessary for its transcriptional function, we next analyzed the effect of silibinin on STAT phosphorylation and total STAT levels in nuclear and cytoplasmic fractions. Similar as in total cell extract, the inhibitory effect of silibinin on STAT activation was also evidenced in nuclear (Fig. 3B) and cytosolic extracts (Fig. 3C).

Effect of Silibinin on Cytokine Mixture–Induced Activation of AP-1 in A549 Cells

AP-1 transcription factor is crucial for regulating the cell proliferation, death, transformation, inflammation, and innate immunity response (30–32). In the present study, silibinin pretreatment (2 h) at 100 and 200 μmol/L doses

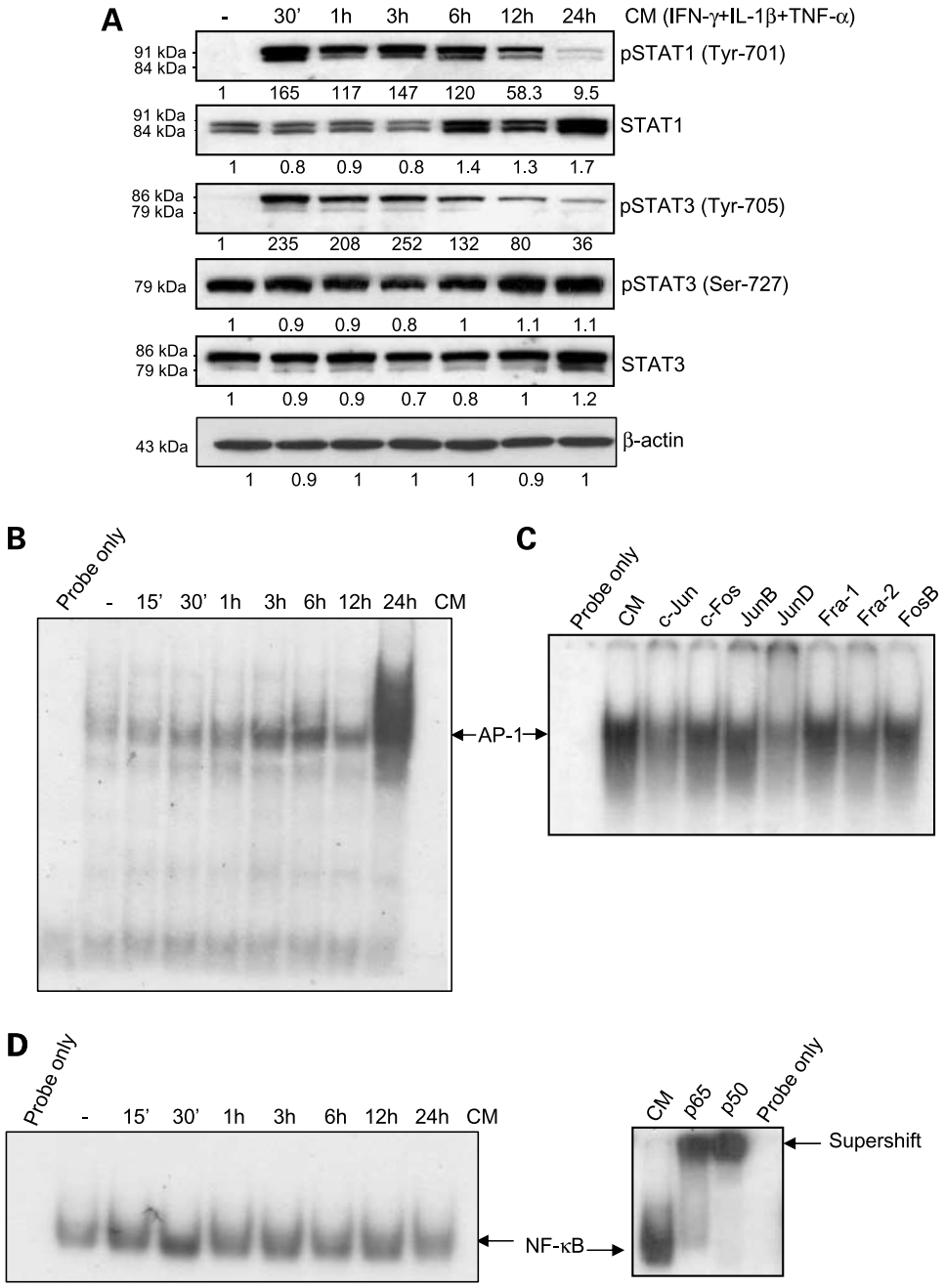

Figure 1. Time-course effect of cytokine mixture on the activation of STAT, AP-1, and NF-κB in human lung epithelial A549 cells. **A,** time-course (30 min to 24 h) expression of phosphorylated STAT1 (Tyr^{701}), STAT1, phosphorylated STAT3 (Tyr^{705}), phosphorylated STAT3 (Ser^{727}), and STAT3 with cytokine mixture treatment in total cell lysate as described in Materials and Methods. *Lane 1,* control (without cytokine mixture). In all cases, membranes were stripped and reprobed with anti-β-actin antibody for protein loading correction. The densitometry data presented below the bands are ''fold change'' compared with control after normalization with respective loading control value. **B,** DNA binding of AP-1 at various time points with cytokine mixture treatment as measured by EMSA. **C,** gel supershift assay was done to examine the specific constituents of AP-1 after cytokine mixture treatment for 24 h. **D,** DNA binding of NF-κB at various time points with cytokine mixture treatment as measured by EMSA. Gel supershift assay was done to examine the constituents of NF-κB after 30 min treatment with cytokine mixture. The data shown are representative of at least two independent experiments.

inhibited the DNA-binding activity of AP-1, which was induced by 30 min exposure to cytokine mixture in A549 cells (Fig. 4A). Constitutively active AP-1 was also decreased by silibinin treatment (maximum inhibition at 100 μmol/L dose; Fig. 4A). Because AP-1 is composed of either homodimers or heterodimers between members of Jun and Fos families, and the expression of AP-1 subunits is differentially regulated in response to various stimuli, we next analyzed the effects of silibinin on the AP-1 subunits. The levels of Jun and Fos family members were measured by Western blot analysis in the nuclear lysates. As shown in Fig. 4B, silibinin pretreatment (2 h), 30 min before exposure to cytokine mixture, resulted in a marginal to strong decrease in the cytokine mixture–induced expression of phosphorylated c-Jun and c-Fos as well as total levels of c-Jun, JunB, JunD, and c-Fos. However, under similar treatment conditions, silibinin did not affect the Fra-1 and Fra-2 levels in the nuclear lysates (data not shown).

Effect of Silibinin on Cytokine Mixture–Induced NF-κB Activation in A549 Cells

NF-κB has been shown to regulate the expression of several genes whose products are involved in tumorigenesis

(33). In the present study, cytokine mixture treatment for 30 min caused a strong activation of constitutively active NF-κB as evidenced by EMSA results, which was moderately inhibited by pretreatment with the highest dose of silibinin (Fig. 5A). Western blot analysis of nuclear extracts revealed that silibinin at 200 μmol/L dose moderately inhibited the cytokine mixture–activated p50 nuclear level but only marginally affected the p65 level (Fig. 5B). Silibinin treatment also resulted in a moderate to marginal decrease in the expression of p50 and p65 in the presence or absence of cytokine mixture in the cytosol (Fig. 5C). Thus, the moderate inhibitory effect of silibinin on cytokine mixture–induced NF-κB activation was mediated via an overall decrease in the levels of its constituents (mainly the levels of p50).

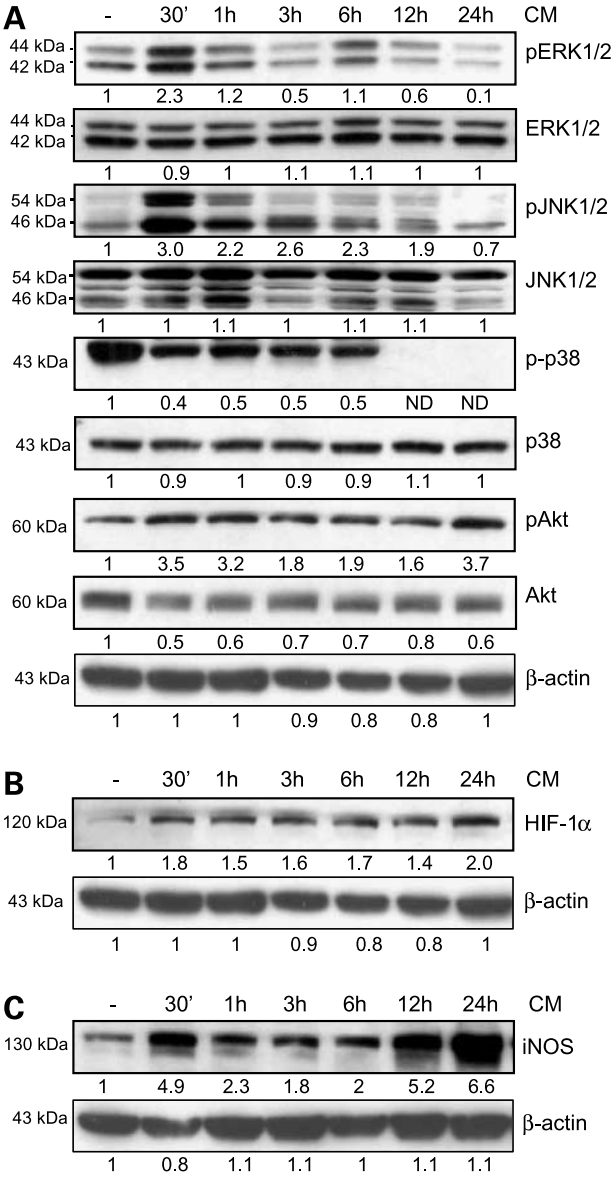

Figure 2. Time-course effect of cytokine mixture on the activation of MAPK and Akt and expression of HIF-1α and iNOS in A549 cells. **A** to **C**, time-course (30 min to 24 h) expression of phosphorylated ERK1/2, ERK1/2, phosphorylated JNK1/2, JNK1/2, phosphorylated p38, p38, phosphorylated Akt, Akt, HIF-1α, and iNOS with cytokine mixture treatment in total cell lysate as described in Materials and Methods. In all cases, membranes were stripped and reprobed with anti-β-actin antibody for protein loading correction. The densitometry data presented below the bands are ''fold change'' compared with control after normalization with respective loading control value. The data shown are representative of at least two independent experiments. *ND,* not detectable.

Effect of Silibinin on Cytokine Mixture–Modulated Levels of MAPK in A549 Cells

MAPK cascade, consisting of ERK1/2, JNK1/2, and p38 kinases, plays a critical role in lung tumorigenesis (30, 34). Several studies have elucidated the functional role of MAPK in the regulation of cytokine production and activation of inflammatory cells (35, 36). In the present study, cytokine mixture treatment for 30 min increased the phosphorylation of ERK1/2 and silibinin pretreatment for 2 h, even at the lowest dose (50 μmol/L), inhibited this activation without any change in the total ERK1/2 levels (Fig. 6A). We next assessed the effect of silibinin on the JNK1/2 and p38 under serum-starved condition as well as in the presence of cytokine mixture. Silibinin pretreatment only marginally decreased the cytokine mixture–induced JNK1/2 phosphorylation, and there was no change in the total JNK1/2 levels under similar treatment conditions (data not shown). As mentioned earlier, cytokine mixture treatment decreased the constitutive phosphorylation of p38, which was strongly decreased by silibinin pretreatment for 2 h, at the highest used dose (200 μmol/L), without any noticeable changes in total p38 levels (Fig. 6A). Silibinin treatment did not cause any noticeable change in the cytokine mixture–induced phosphorylation of Akt (Ser^{473}; data not shown).

Effect of Silibinin on Cytokine Mixture–Induced HIF-1α Levels in A549 Cells

HIF-1α is known to get activated in response to hypoxia and helps to restore oxygen homeostasis in cells by inducing glycolysis, erythropoiesis, and angiogenesis, promoting cell proliferation and inhibiting apoptosis (37). In the present study, we observed that the level of HIF-1α was increased with cytokine mixture stimulation for 30 min and this could be probably linked with activation of MAPK and Akt, which are known to regulate the HIF-1α expression at transcriptional level (37). Silibinin pretreatment (50-200 μmol/L) dose-dependently decreased the cytokine mixture–induced HIF-1α expression in A549 cells (Fig. 6B).

Effect of Silibinin on Cytokine Mixture–Induced iNOS Levels in A549 Cells

iNOS is an enzyme involved in production of NO via catalyzing the conversion L-arginine to citrulline in the presence of NADPH and oxygen. NO plays a key role in many physiologic as well as pathologic processes, including inflammation, angiogenesis, and neoplasia (25). As mentioned earlier, cytokine mixture treatment for 30 min resulted in a strong activation of iNOS; next, we examined the effect of silibinin pretreatment on the cytokine mixture–induced iNOS expression. As shown in Fig. 6C, silibinin pretreatment (50-200 μmol/L) completely

inhibited the cytokine mixture–induced expression of iNOS in A549 cells.

Discussion

Lung cancer has the highest mortality worldwide, which is in part due to its high potential of progression, local invasion, and metastasis to distant organs. Lung cancer is also highly resistant to conventional cancer therapies; although survival rates have been improved slightly in recent years, >90% of patients diagnosed with lung cancer die from their disease (1, 2). Therefore, identification of the mechanisms preventing or controlling the development

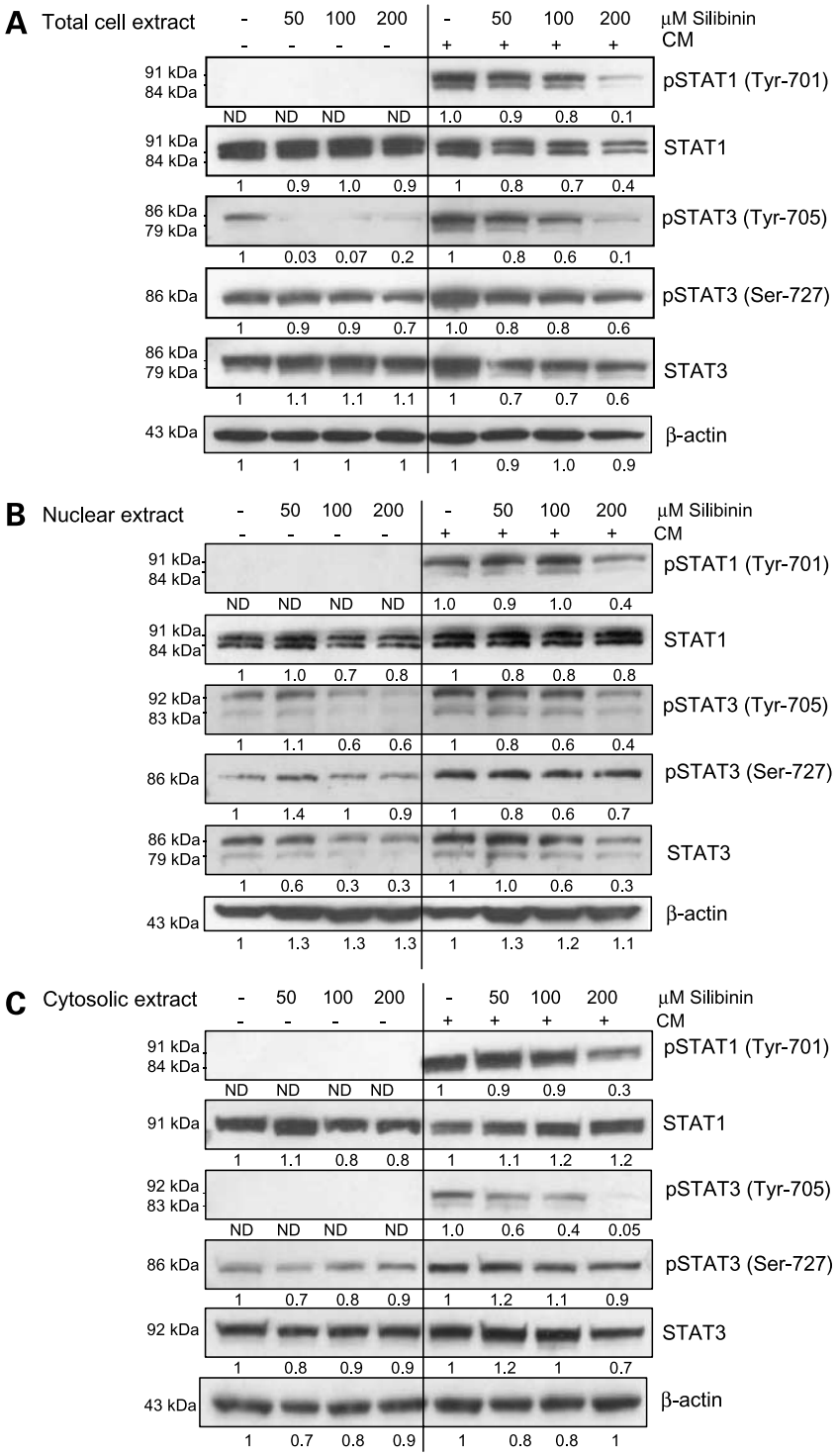

Figure 3. Effect of silibinin on cytokine mixture–induced STAT activation in human lung epithelial A549 cells. **A** to **C**, effect of silibinin pretreatment for 2 h on the expression of phosphorylated STAT1 (Tyr^{701}), STAT1, phosphorylated STAT3 (Tyr^{705}), phosphorylated STAT3 (Tyr^{727}), and STAT3 in the presence (for 30 min) or absence of cytokine mixture in total, nuclear, and cytoplasmic lysates. In all cases, membranes were stripped and reprobed with anti-β-actin antibody for protein loading correction. The densitometry data presented below the bands are ''fold change'' compared with control after normalization with respective loading control value. The data shown are representative of at least two independent experiments. The line in the middle of the blots is to separate the effect of silibinin on constitutive (*left*) or cytokine mixture–modulated (*right*) expression of the molecules.

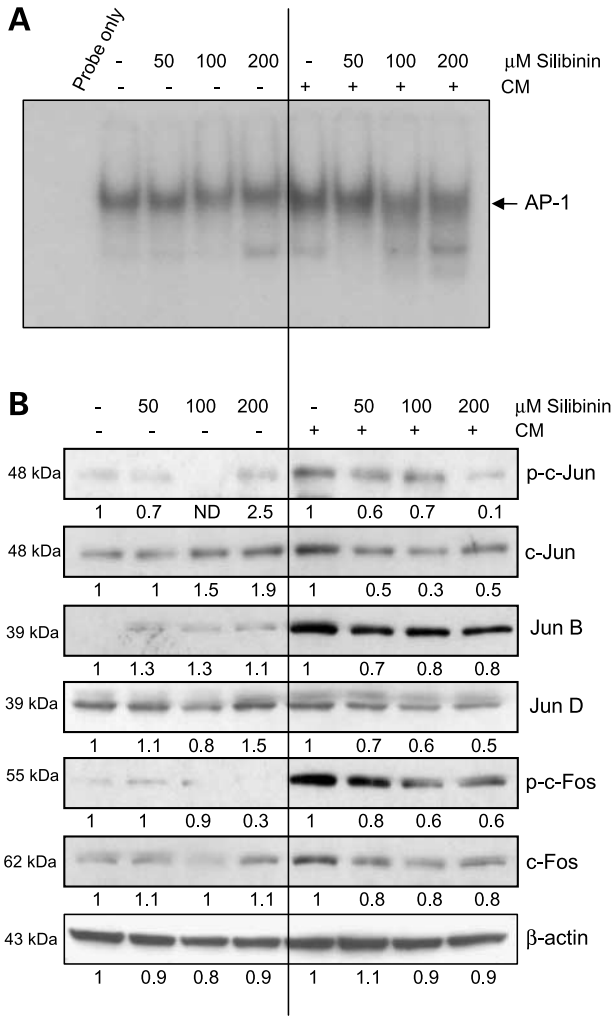

Figure 4. Effect of silibinin on cytokine mixture–induced AP-1 activation and expression of AP-1 constituents in human lung epithelial A549 cells. **A**, EMSA was done in the nuclear extract to analyze the effect of silibinin pretreatment for 2 h on AP-1 activation in the presence (for 30 min) or absence of cytokine mixture as described in Materials and Methods. **B**, nuclear extract was also analyzed for the expression of phosphorylated c-Jun, c-Jun, JunB, JunD, phosphorylated c-Fos, and c-Fos. The densitometry data presented below the bands are ''fold change'' compared with control after normalization with respective loading control value. The data shown are representative of at least two independent experiments. The line in the middle of the blots is to separate the effect of silibinin on constitutive (*left*) or cytokine mixture–modulated (*right*) expression of the molecules.

and progression of this deadly disease is urgently required to devise more effective lung cancer management strategies. In this study, we showed that cytokine mixture consisting of TNF-α, IL-1β, and IFN-γ activates various cellular signaling cascades known to be involved in the lung cancer growth and progression, and silibinin pretreatment differentially inhibited (marginal to strong) these signaling cascades. Although the cytokine mixture has been used earlier for various studies, the present work is perhaps the first approach to examine the effect of a define mixture of cytokines on such a repertoire of cellular signaling molecules. Importantly, this model could be used to study the efficacy of other cancer chemopreventive drugs.

A large volume of evidence has established that STAT activation occurs frequently in malignant tumors including lung cancer (28), and blocking constitutively activated STAT signaling leads to apoptosis of tumor cells but not in normal cells (26). Moreover, STAT signaling inhibition might increase the effectiveness of conventional treatment modalities (chemotherapy and radiotherapy), as their failure/resistance might be ascribed to the presence of constitutively activated STAT proteins (26). In the present study, silibinin treatment strongly inhibited the cytokine mixture–induced STAT1 and STAT3 phosphorylation and also decreased their expression levels in lung cancer A549 cells; thus, it could, in part, account for the antitumor activity of silibinin against lung cancer (3, 16).

MAPK signaling involving ERK, JNK, and p38 is known to play central role in tumor progression via promoting cell proliferation and antiapoptotic action (30). These MAPK respond differently depending on the nature and extent of stimulus but generally converge and stimulate the activation of transcriptional factor AP-1. AP-1 plays a key role in various processes related to carcinogenesis. In human lung carcinoma cell line, a transient transfection assay has shown that AP-1 activation is important for the induction of iNOS transcription (38). In the present study, we observed a strong increase in AP-1 activation with cytokine mixture treatment. The cytokine mixture treatment also resulted in increased phosphorylation of ERK1/2 and JNK1/2 (maximally after 30 min) but with a consistent decrease in p38 phosphorylation level, suggesting the differential effect of cytokine mixture on MAPK. However, further studies are needed to understand the significance of these differential effects of cytokine mixture on MAPK. Nevertheless, silibinin treatment significantly decreased the cytokine mixture–induced phosphorylation of ERK1/2 as well as AP-1 but only marginally decreased the constitutively active and cytokine mixture–induced levels of JNK1/2 (data not shown). Silibinin was also shown to inhibit the phosphorylation of p38 in the presence (at 200 μmol/L dose) or absence (at 50, 100, and 200 μmol/L doses) of cytokine mixture. These results suggest the differential inhibitory effect of silibinin on the activation of MAPK members in lung cancer A549 cells and that may, in part, account for its antiproliferative and antitumor effects against lung tumorigenesis (3, 16).

NF-κB is an important link between inflammation and cancer development and has role in the malignant conversion and progression of cancer (39). NF-κB signaling pathway gets activated by various proinflammatory cytokines and regulate the expression of many genes whose products can suppress tumor cell death, stimulate tumor cell cycle progression, and provide newly emerging tumors with an inflammatory microenvironment that supports their progression, invasion of surrounding tissues, angiogenesis, and metastasis (33, 39, 40). In earlier studies, we observed that doxorubicin treatment

resulted in activation of NF-κB in A549 cells and silibinin treatment increased the therapeutic potential of doxorubicin by inhibiting the NF-κB activation (16). In the present work, cytokine mixture activated the NF-κB and silibinin treatment moderately inhibited it at 200 μmol/L dose, which might result in reduced expression of various proinflammatory, antiapoptotic, or survival factors. Further studies are warranted to understand these aspects of silibinin action.

HIF-1α is known to be activated in response to hypoxic conditions in the tumors and in initiating the process of angiogenesis by inducing the expression of several angiogenesis-related genes, including iNOS, vascular endothelial growth factor, etc. (37). In addition to hypoxia, HIF-1α expression is regulated by many growth factors, such as endothelial growth factor, insulin-like growth factor, insulin, and platelet-derived growth factor involving endothelial growth factor receptor, MAPK, and phosphatidylinositol 3-kinase pathways (37). In the present study, we observed that cytokine mixture exposure resulted in increased levels of HIF-1α in A549 cells. To our knowledge, this is the first study suggesting that cytokines treatment could increase the expression of HIF-1α levels in lung cancer cells. Further studies are needed to understand the mechanism of HIF-1α increase by cytokine mixture, but results clearly suggest that silibinin treatment significantly inhibits the cytokine mixture–induced HIF-1α levels.

The levels of NOS protein and/or NOS activity has been positively correlated with the degree of malignancy in number of human cancers, including lung cancer (38, 41). iNOS-mediated production of NO facilitates neovascularization and therefore could be a potential target to control tumor angiogenesis (25). It has been observed that iNOS expression/activity in tumors is higher than the surrounding normal tissues and that it shows a positive correlation with angiogenic state and metastatic potential of the tumor (25). Furthermore, nitric oxide promotes tumor invasiveness by altering the balance between expression of matrix metalloproteinase-2 and its inhibitors tissue inhibitor of metalloproteinase 2 and 3 (41). A recent study showed that carcinogen-induced lung tumorigenesis is inhibited in

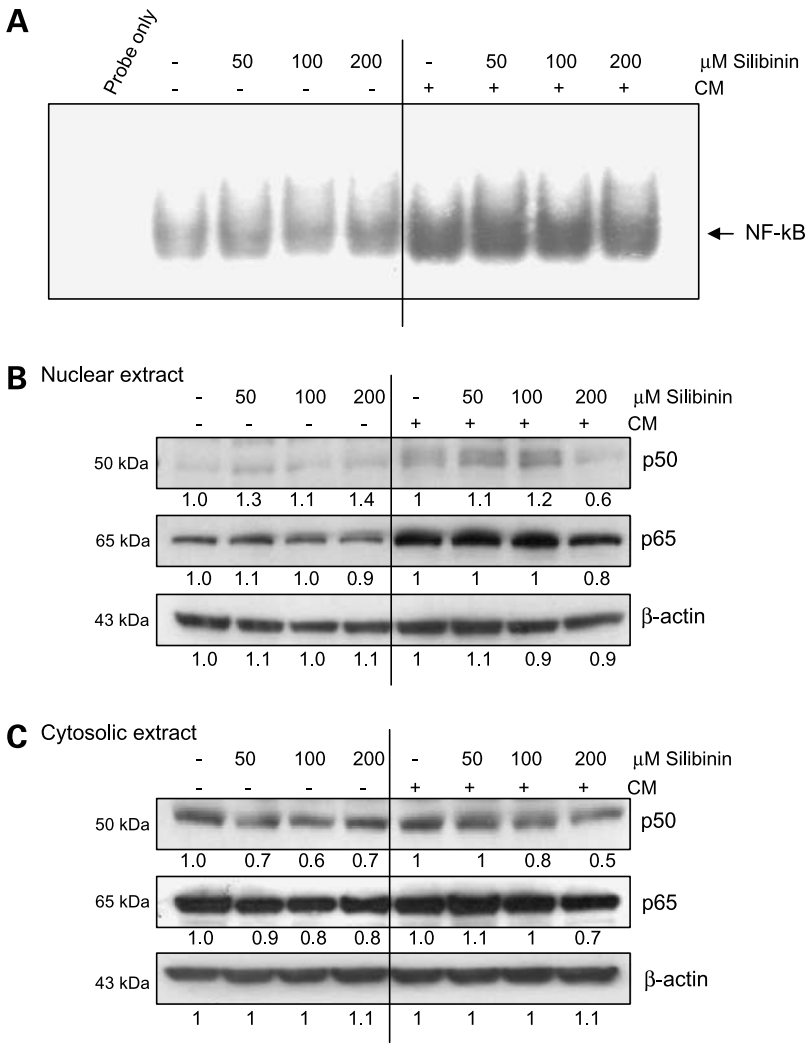

Figure 5. Effect of silibinin on cytokine mixture–induced NF-κB activation and expression of NF-κB constituents in human lung epithelial A549 cells. **A,** EMSA was done in the nuclear extracts to analyze the effect of silibinin pretreatment for 2 h on NF-κB activation in the presence (for 30 min) or absence of cytokine mixture as described in Materials and Methods. **B** and **C,** nuclear and cytoplasmic extracts were also analyzed for the expression of p50 and p65. The densitometry data presented below the bands are ''fold change'' compared with control after normalization with respective loading control value. The data shown are representative of at least two independent experiments. The line in the middle of the blots is to separate the effect of silibinin on constitutive (*left*) or cytokine mixture–modulated (*right*) expression of the molecules.

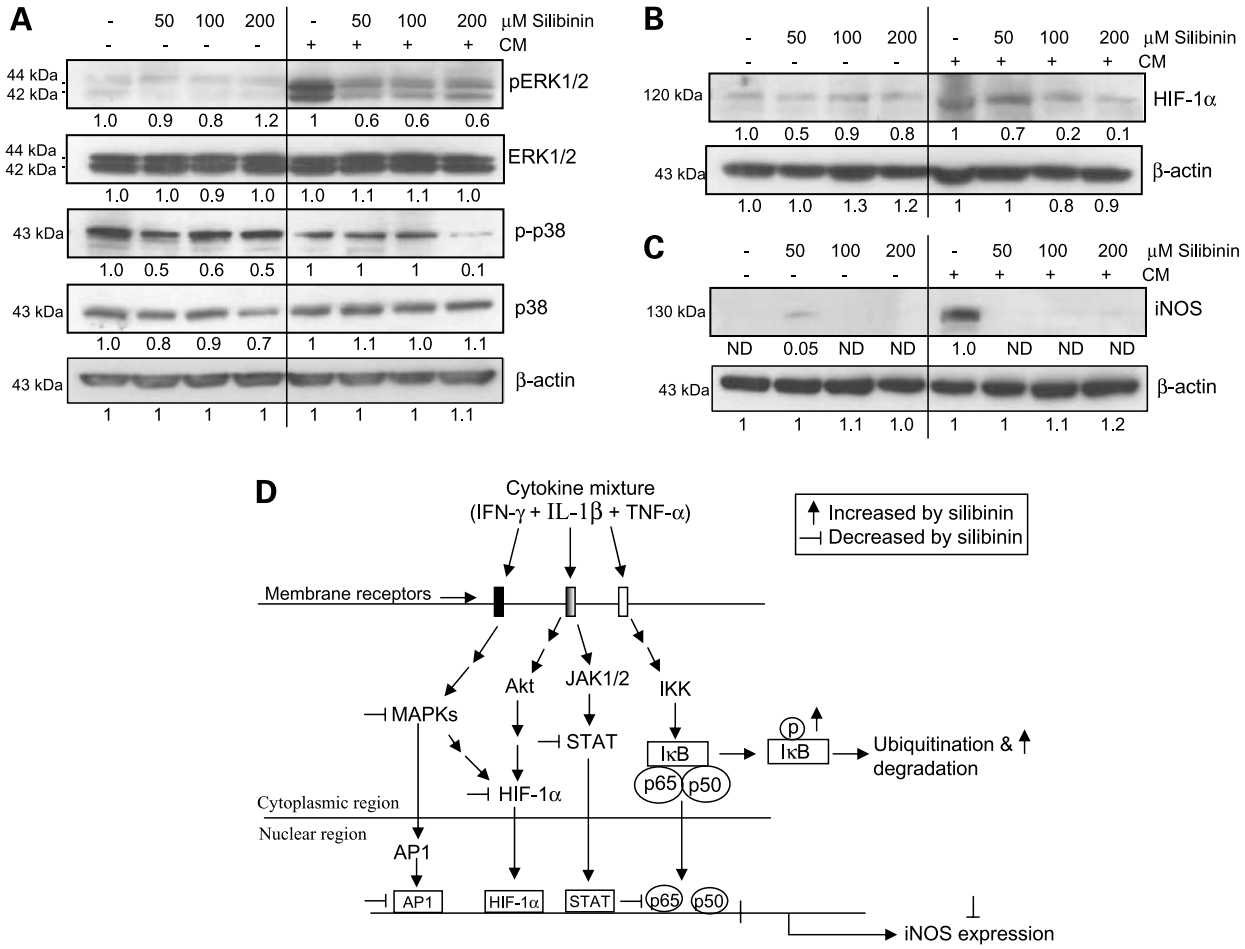

Figure 6. Inhibitory effect of silibinin on cytokine mixture – modulated ERK1/2, p38, HIF-1α, and iNOS expression in A549 cells. **A** to **C,** effect of silibinin pretreatment for 2 h on the expression of phosphorylated ERK1/2, ERK1/2, phosphorylated p38, p38, HIF-1α, and iNOS in the presence (for 30 min) or absence of cytokine mixture. The densitometry data presented below the bands are ''fold change'' compared with control after normalization with respective loading control value. The data shown are representative of at least two independent experiments. The line in the middle of the blots is to separate the effect of silibinin on constitutive (*left*) or cytokine mixture – modulated (*right*) expression of the molecules. **D,** proposed mechanism for the inhibitory effects of silibinin on cytokine-induced signaling cascades regulating iNOS expression in A459 cells. *JAK,* Janus-activated kinase; *IKK,* inhibitor of κB kinase; *IκB,* inhibitor of κB.

iNOS-null mice, which also showed decreased levels of vascular endothelial growth factor (42). Therefore, the inhibition of iNOS has been shown to have significant antitumor and antimetastatic effects. In this regard, we have reported that iNOS is the important target for the angiopreventive efficacy of silibinin in urethane-induced lung tumor progression in mice (3). In the present work, silibinin treatment completely inhibited the cytokine-induced iNOS expression, further suggesting iNOS as potential target of silibinin in angioprevention of lung cancer (Fig. 6D). More importantly, iNOS gene promoter has binding sites for STAT, AP-1, NF-κB, and HIF-1α transcription factors, which may cooperate for its maximum expression (Fig. 6D). Because the activation of all these four transcription factors by cytokines was inhibited by silibinin, it may account for the most likely mechanism of the down-regulation of iNOS expression observed in lung cancer cells (Fig. 6D).

The significance of the present study lies in the context that cytokines secreted by tumor cells and its microenvironment are the important determinants of cancer progression. The present work highlights that an anticancer agent such as silibinin can inhibit cytokine-induced survival, mitogenic, inflammatory, and angiogenic signaling, which could account for its chemopreventive action against lung cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Mol Cancer Ther 2008;7:1817-1826.