Silibinin inhibits UVB- and epidermal growth factor–induced mitogenic and cell survival signaling involving activator protein-1 and nuclear factor-κB in mouse epidermal JB6 cells

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Abstract

UVB radiation is the major etiologic factor in the development of nonmelanoma skin cancer. In addition to tumor-initiating effect, UVB also causes tumor promotion via mitogenic and survival signaling. Studies have shown strong preventive effects of silibinin against both UVB-induced and chemically induced tumor promotion in mouse skin models; however, mechanisms are not understood completely. Here, we used tumor promoter–sensitive JB6 mouse epithelial cell model and studied the effect of silibinin on two different mitogens [UVB and epidermal growth factor (EGF)] that induce mitogenic and cell survival signaling pathways. UVB (50–800 mJ/cm^2) dose-dependently induced phosphorylation of extracellular signal-regulated kinase 1/2 (ERK1/2), c-Jun-NH$_2$-kinase 1/2 (JNK1/2), and p38 kinase (p38K) as well as Akt, with an optimum response at 400 mJ/cm^2 UVB dose. UVB caused a biphasic phosphorylation of ERK1/2 in a time kinetics study. Silibinin treatment before or immediately after UVB exposure, or both, resulted in a strong decrease in UVB-caused phosphorylation of ERK1/2 and Akt in both dose- and time-dependent manner, without any substantial response on JNK1/2 and p38K. Silibinin also suppressed UVB-induced activator protein-1 (AP-1) and nuclear factor-κB (NF-κB) activation, which are activated by ERK1/2 and Akt. Silibinin treatment under similar conditions also strongly inhibited EGF-induced ERK1/2, JNK1/2, and p38K as well as Akt phosphorylation, and also suppressed EGF-induced AP-1 and NF-κB activation. Because AP-1 and NF-κB are important nuclear transcription factors for tumor promotion, these results suggest that silibinin possibly prevents skin tumor promotion by inhibiting UVB- and EGF-induced mitogenic and cell survival signaling involving both AP-1 and NF-κB. [Mol Cancer Ther 2006;5(5):1145–53]

Introduction

Both experimental and epidemiologic evidences suggest that UVB (280–320 nm) is the key component of solar radiation for skin cancer development. UVB is considered a complete carcinogen because it can initiate and induce cancer growth in the absence of any other carcinogen (1). Over the years, there are substantial evidences to suggest that signal transduction pathways are involved in UVB-induced skin carcinogenesis (2, 3). These signaling events lead to distinct cellular responses, including cell proliferation, transformation, and cell death (4). Transcription factors nuclear factor-κB (NF-κB) and activator protein-1 (AP-1) are stoutly implicated in UVB-mediated cell transformation and tumor promotion (5–7). Further, UVB-mediated signaling events are primarily mediated through mitogen-activated protein kinases (MAPK), including extracellular signal-regulated kinase 1/2 (ERK1/2), c-Jun-NH$_2$-kinase 1/2 (JNK1/2), and p38 kinase (p38K; ref. 8). Once activated, these serine/threonine kinases translocate to the nucleus and phosphorylate target transcription factors, including AP-1 (8–12). Although these kinases could be activated in response to distinct stresses, their activation is not mutually exclusive (13, 14). The activation of MAPKs by tumor-promoting agents plays a major role in tumor promotion and malignant transformation (8, 15, 16). Further, Akt, the downstream serine/threonine kinase of phosphatidylinositol-3-kinase family members, is also critical for UVB-induced tumor promotion (17).

Epidermal growth factor (EGF) receptor, 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA), and tumor necrosis factor-α have been used to study UVB-mediated promotional events in JB6 cells (7, 18). Inhibition of EGF pathway affords resistance to UVB-mediated cell transformation (7). However, EGF- and UVB-mediated responses are considered different because EGF mediates a mitogenic signaling for proliferation, whereas UVB-induced signaling mediates various stress responses, including genotoxic stress (7, 19).

Received 11/18/05; revised 2/22/06; accepted 3/17/06.

Grant support: USPHS grant RO1 CA64514.

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doi:10.1158/1535-7163.MCT-05-0478

Agents that modulate UVB- and EGF-mediated signaling pathways could be of use in the chemoprevention of skin cancer. Silibinin is a milk thistle flavonoid that possesses anticarcinogenic effect against many epithelial cancers. Previously, we have observed the inhibitory effect of silymarin/silibinin against skin carcinogenesis in animal models (20, 21). In a recent study, silibinin modulated UVB-mediated signal transduction pathways for its photoprotective effects in SKH-1 mice (22). Silibinin also inhibited UVB-induced cell proliferation, DNA damage (cyclobutane pyrimidine dimer formation), and induction of apoptosis in skin (23). More recently, we also observed that silibinin inhibits UVB-induced mitogenic and survival signaling in mouse skin (24); however, the transcription factors linked to this effect of silibinin are not yet identified.

Mouse keratinocyte JB6 cells are well suited to study tumor promotion, as these cells are sensitive to tumor promoter–mediated cell transformation and promotion (7). Because silibinin inhibits UVB-induced skin tumorigenesis, in the present study, we analyzed the effect of silibinin on tumor promotion–specific events. We used UVB and EGF as two different tumor promoter agents and assessed the modulatory effects of silibinin on tumor promoter–mediated activation of transcription factors AP-1 and NF-κB, and MAPK family members and Akt. Results obtained in the present study suggested their role in anti–tumor-promoting effect of silibinin.

Materials and Methods

Reagents and Antibodies

Eagle's MEM, gentamicin, and other culture materials were from Life Technologies (Grand Island, NY). Fetal bovine serum was from Hyclone Laboratories (Logan, UT). Silibinin (Sigma Chemical Co., St. Louis, MO) was dissolved in DMSO. Unless specified otherwise, the final concentration of DMSO in the culture medium during different treatments did not exceed 0.1% (v/v). Primary antibodies against phosphorylated and total ERK1/2, JNK1/2, Akt, and total p38K and peroxidase-conjugated anti-rabbit secondary antibody were from Cell Signaling Technologies (Beverly, MA). Anti-phospho-p38K antibody was from BD PharMingen (San Diego, CA). Anti-phospho-c-Fos antibody was from EMD Biosciences-Calbiochem (San Diego, CA). Other antibodies used were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). [γ-^{32}P]ATP was from Amersham Biosciences (Piscataway, NJ). NF-κB- or AP-1-specific oligonucleotides were from Promega (Madison, WI). The enhanced chemiluminescence detection system and peroxidase-conjugated anti-mouse secondary antibody were from Amersham Corp. (Arlington Heights, IL). Other chemicals were obtained in their highest commercially available purity grade.

Cell Culture and UVB Irradiation

Mouse epidermal keratinocyte JB6 cells were maintained in 5% MEM containing 2 mmol/L L-glutamine and 25 μg/mL gentamicin at standard culture conditions (37°C, 95% humidified air, and 5% CO_2). For all treatments, cells were grown to 80% confluence and then starved in 0.1% fetal bovine serum in MEM at 37°C in a 5% CO_2 incubator. After 48 hours of starvation, the cells were either pretreated or posttreated with silibinin for 6 hours or as indicated in the figures or with DMSO (vehicle) alone. Before UVB irradiation, the medium was removed from culture plates; cells were washed with PBS twice and then covered with a thin layer of PBS followed by UVB irradiation. Control cultures were identically processed but not irradiated. The UVB light source was a bank of four FS24T12-UVB-HO sunlamps equipped with a UVB Spectra 305 Dosimeter (Daavlin Co., Bryan, OH). In studies with EGF, 48 hours serum-starved JB6 cells were treated with silibinin (100 μmol/L) for 6 hours, and then stimulated with EGF (10 ng/mL), and harvested 30 minutes and 12 hours later for whole cell lysate and nuclear extract preparations, respectively. All experiments were repeated at least twice.

Western Blotting

Following desired treatments, cell lysates were prepared in nondenaturing lysis buffer [10 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 1% Triton X-100, 1 mmol/L EDTA, 1 mmol/L EGTA, 0.3 mmol/L phenylmethylsulfonyl fluoride, 0.2 mmol/L sodium orthovanadate, 0.5% NP40, and 5 units/mL aprotinin], and protein concentration in lysates was determined. For immunoblot analyses, 40 to 100 μg protein lysates per sample were denatured in 2× SDS-PAGE sample buffer and subjected to SDS-PAGE on 12% to 16% Tris-glycine gels. The separated proteins were transferred onto nitrocellulose membrane followed by blocking with 5% nonfat milk powder (w/v) in TBS (10 mmol/L Tris, 100 mmol/L NaCl, and 0.1% Tween 20) for 1 hour at room temperature. Membranes were then probed with specific primary antibodies followed by peroxidase-conjugated secondary antibody and visualized by enhanced chemiluminescence detection system.

Preparation of Cytosolic and Nuclear Extracts

Following the desired treatments, cells were scraped from the plates in ice-cold buffer A [10 mmol/L HEPES (pH 7.9), 10 mmol/L KCl, 0.1 mmol/L EDTA, 0.1 mmol/L EGTA, 1 mmol/L DTT, 0.5 mmol/L phenylmethylsulfonyl fluoride, and 10% NP40] and left on ice for 20 minutes. After vigorous vortexing for 10 seconds, homogenates were centrifuged at 14,000 rpm for 30 seconds. The resulting supernatant was collected as cytosolic extract. The pellet was then resuspended in buffer C [20 mmol/L HEPES (pH 7.9), 1 mmol/L EDTA, 1 mmol/L EGTA, 1 mmol/L DTT, and 1 mmol/L phenylmethylsulfonyl fluoride], vigorously rocked at 4°C for 15 minutes, and centrifuged for 5 minutes at 14,000 rpm. The supernatant (nuclear extract) thus obtained was used to assay the DNA-binding activity of AP-1 and NF-κB. Both the extracts were also used for immunoblot analysis.

Electrophoretic Mobility Shift Assay

For electrophoretic mobility shift assay, AP-1- or NF-κB-specific oligonucleotides (3.5 pmol) were end-labeled with [γ-^{32}P]ATP (3,000 Ci/mmol at 10 mCi/mL) using

T_4 polynucleotide kinase in 10× kinase buffer as per the protocol of the manufacturer (Promega). Labeled double-stranded oligo probe was separated from free [γ-^{32}P]ATP using G-25 Sephadex column. The consensus sequences of the oligonucleotide used were 5′-CGCTTG-ATGAGTCAGCCGGAA-3′ and 3′-GCGAACTACTCAG-TCGGCCTT-5′ for AP-1 and 5′-AGTTGAGGGGACTTTC-CCAGGC-3′ and 3′-TCAACTCCCCTGAAAGGGTCCG-5′ for NF-κB. Four and 8 μg protein (for AP-1 and NF-κB, respectively) from nuclear extracts were 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 minutes at 37°C. DNA-protein complex thus formed was resolved on 6% DNA retardation gels (Invitrogen, Gaithersburg, MD). The gels were dried and bands were visualized by autoradiography. Supershifts and competition assays were done using specific antibodies (p65 and p50 for NF-κB; c-Fos and c-Jun for AP-1) and unlabeled probes in the reaction mixtures to check the specificity of the bands, and nonspecific antibodies (p65 antibody in AP-1 electrophoretic mobility shift assay, and c-Fos antibody in NF-κB electrophoretic mobility shift assay) were also included to rule out the nonspecific inhibition of the DNA binding.

Results

Dose and Time Kinetics of UVB Irradiation on MAPK and Akt Signaling in JB6 Cells

MAPK family molecules, namely ERK1/2, JNK1/2, and p38K, and Akt are involved in UVB-induced tumor promotion (8). Therefore, first we studied the effects of different doses (50–800 mJ/cm^2) of UVB irradiation on the activation of these signaling molecules in mouse keratinocyte JB6 cells that were serum starved for 48 hours (Fig. 1A). From this study, we selected an effective dose of UVB for time kinetics study (5 minutes–12 hours; Fig. 1B). Then, we selected the most effective UVB dose and treatment time to study the modulatory effect of silibinin on these pathways. In a dose-response study, we observed that increasing UVB doses showed increasing levels of phosphorylation of ERK1/2 and JNK1/2 at lower doses, which reached to the optimum level at the dose of 100 to 400 mJ/cm^2; however, it kept on increasing for p38K and Akt to 800 mJ/cm^2 (Fig. 1A). We used 400 mJ/cm^2 UVB dose for the time kinetics study, which showed a biphasic activation response for ERK1/2 phosphorylation, reaching optimum at 30 minutes and 12 hours postirradiation (Fig. 1B). For JNK1/2, p38K and Akt(Ser^{473}) phosphorylation, optimum response was obtained at 30 minutes, which further decreased with the increase in postirradiation time (Fig. 1B). In both studies, we did not observe any considerable change in the total levels of the measured proteins (Fig. 1).

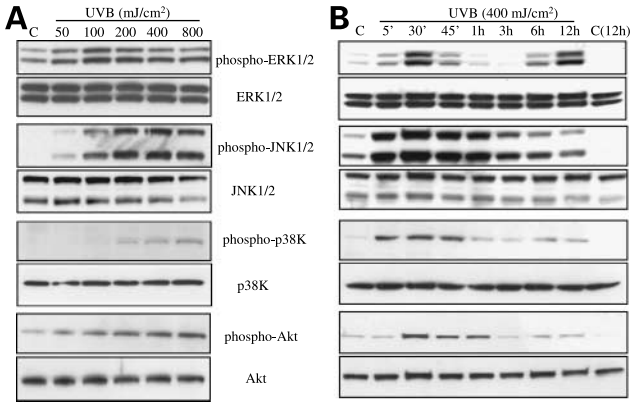

Figure 1. Dose- and time-dependent effect of UVB irradiation on the phosphorylation of MAPKs and Akt in JB6 cells. **A,** cells at ~80% confluence were serum starved for 48 h and then exposed to different doses of UVB (50–800 mJ/cm^2) or sham irradiated and harvested 30 min later. **B,** serum-starved (48 h) cells were exposed to 400 mJ/cm^2 dose of UVB and harvested at different time intervals from 5 min to 12 h as indicated. Cell lysates were prepared at the end of treatments and phosphorylated and total protein levels of ERK1/2, JNK1/2, p38K, and Akt were probed using specific antibodies by Western blotting as mentioned in Materials and Methods.

Effect of Silibinin on UVB-Induced MAPK and Akt Signaling

First, we assessed the effect of 6 hours of silibinin (100 μmol/L) pretreatment on UVB (400 mJ/cm^2)–induced MAPK and Akt activation in 48 hours serum-starved JB6 cells. Cells were harvested 30 minutes post-UVB irradiation. Silibinin pretreatment showed an inhibitory effect on UVB-induced ERK1/2 phosphorylation (Fig. 2A); however, it did not show any effect on JNK1/2 phosphorylation but a slight increase in p38K phosphorylation was observed (Fig. 2B and C). Silibinin pretreatment strongly and completely inhibited UVB-induced Akt(Ser^{473}) phosphorylation (Fig. 2D). To further confirm the inhibitory effect of silibinin on UVB-induced ERK1/2 and Akt activation and to seek a dose-response effect of silibinin, we used a lower dose of UVB (100 mJ/cm^2), which was effective in causing the activation of these two molecules and three doses (25, 50, and 100 μmol/L) of silibinin. The results from this study also showed a strong inhibition of UVB-caused phosphorylation of ERK1/2 and Akt by silibinin where all doses of the agent showed comparable effect on ERK1/2 phosphorylation but a dose-response was evidenced in Akt phosphorylation (Fig. 2E and F).

Effect of Silibinin Pretreatment to UVB on MAPK and Akt Activation

First, we did 1 to 24 hours of silibinin (100 μmol/L) pretreatment to 48 hours serum-starved JB6 cells, to assess the exposure time of silibinin for its maximum inhibitory effect on UVB-induced (400 mJ/cm^2) ERK1/2 and Akt activation, and to further assess whether it has any effect on JNK and p38K activation. Cells were harvested 30 minutes post-UVB irradiation. Silibinin pretreatment for as short as 1 hour inhibited UVB-induced ERK1/2

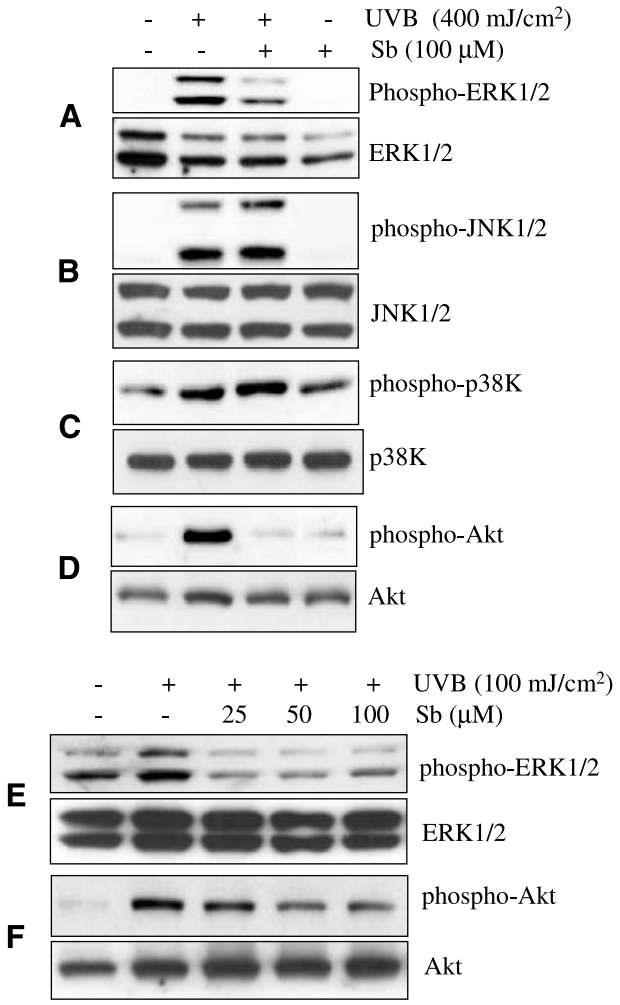

Figure 2. Effect of silibinin pretreatment on UVB-caused ERK1/2, JNK1/2, p38K, and Akt phosphorylation in JB6 cells. Cells at ~80% confluence were serum starved for 48 h, and during the last 6 h of starvation were treated with indicated doses of silibinin (*Sb*). Thereafter, cells were exposed to 400 mJ/cm^2 (**A**–**D**) or 100 mJ/cm^2 (**E** and **F**) dose of UVB; 30 min thereafter, lysates were prepared and Western blotting was carried out to probe phosphorylated and total protein levels of ERK1/2 (**A** and **E**), JNK1/2 (**B**), p38K (**C**), and Akt (**D** and **F**) as mentioned in Materials and Methods.

activation; however, the maximum inhibitory effect was at 12 hours pretreatment time (Fig. 3A). There was no observable effect on UVB-induced JNK1/2 phosphorylation by silibinin (Fig. 3B); however, a slight increase in p38K phosphorylation was evident from 1 to 12 hours of silibinin pretreatments (Fig. 3C). Only 6 and 24 hours of silibinin pretreatments showed inhibition of UVB-induced Akt phosphorylation (Fig. 3D).

Effect of Silibinin Pretreatment and Posttreatment to UVB on MAPK and Akt Activation

We further assessed the effect of silibinin presence in the culture medium both before and after UVB irradiation, and only during post-UVB irradiation. Serum-starved cells were pretreated with silibinin for 6 hours and after UVB irradiation, cells were again exposed to silibinin for 30 minutes, 6 hours, and 12 hours, and then harvested. These silibinin treatments again strongly inhibited UVB-induced ERK1/2 activation (Fig. 4A). For JNK1/2, there was no noticeable change in UVB-caused phosphorylation, except for slight increase in post-UVB 30-minute silibinin treatment (Fig. 4B). No considerable effect was observed for p38K activation (Fig. 4C); however, UVB-induced Akt phosphorylation was decreased by the presence of silibinin for all the treatment times (Fig. 4D).

In the post-UVB experiment, serum-starved cells were treated with silibinin only post-UVB irradiation for 30 minutes, 6 hours, and 12 hours, and analyzed for the similar molecular events as described above (Fig. 5). Consistent with the above results, these silibinin treatments strongly inhibited UVB-induced ERK1/2 phosphorylation (Fig. 5A), with a marginal inhibitory effect on JNK1/2 and p38K at 6 and 12 hours of treatments (Fig. 5B and C). Post-UVB silibinin treatments for 6 and 12 hours also showed strong inhibitory effect on Akt phosphorylation (Fig. 5D). Overall, these results suggested the strong inhibitory effect of silibinin on UVB-induced ERK1/2 and Akt signaling in JB6 cells.

Silibinin Inhibits EGF-Induced Mitogenic and Survival Signaling

MAPK signaling cascades are essential transducers of EGF-induced signaling and are indispensable for EGF-mediated cell transformation of JB6 cells. Also, EGF is a

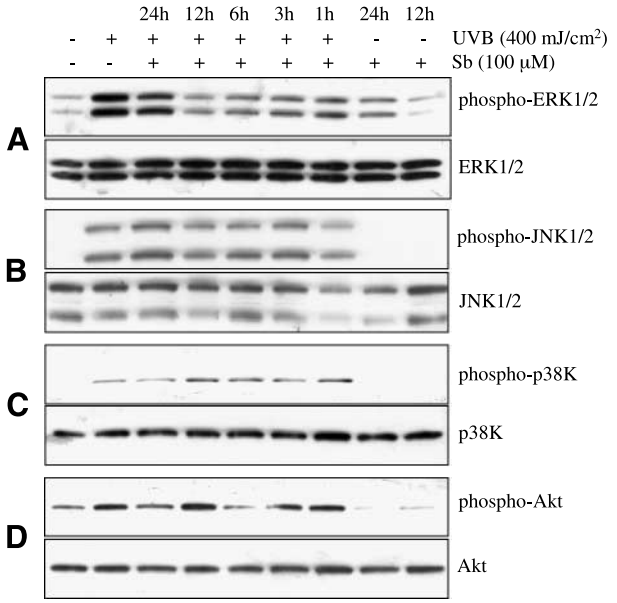

Figure 3. Effect of silibinin pretreatment times on UVB-caused MAPK and Akt phosphorylation in JB6 cells. Cells at 80% confluence were serum starved for 48 h and then either irradiated with UVB (400 mJ/cm^2) alone or pretreated with silibinin (100 μmol/L) for 1, 3, 6, 12, and 24 h, or treated with silibinin alone for 12 and 24 h. At the end of these treatments and 30 min after UVB exposure, cell lysates were prepared and Western blotting was carried out to probe phosphorylated and total protein levels of ERK1/2 (**A**), JNK1/2 (**B**), p38K (**C**), and Akt (**D**) as mentioned in Materials and Methods.

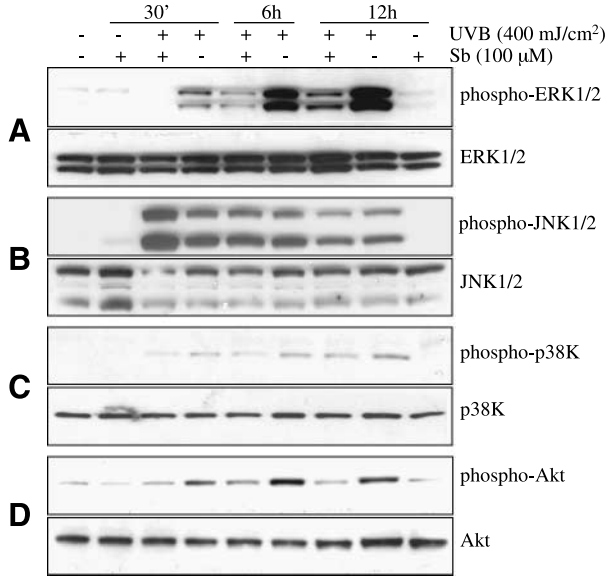

Figure 4. Effect of silibinin before and after treatment on UVB-caused MAPK and Akt phosphorylation. JB6 cells at 80% confluence were serum starved for 48 h and pretreated with DMSO or silibinin (100 μmol/L) for 6 h and then irradiated with UVB (400 mJ/cm^2). The cultures were then posttreated with DMSO or silibinin (100 μmol/L) for 30 min, 6 h, and 12 h. Cells were harvested at indicated time after UVB irradiation, lysates were prepared, and Western blotting was carried out to probe phosphorylated and total protein levels of ERK1/2 (**A**), JNK1/2 (**B**), p38K (**C**), and Akt (**D**) as mentioned in Materials and Methods.

well-known mitogen for the tumor growth and progression. To address the issue whether silibinin effects on signaling cascades are specific to those activated by UVB, we next assessed the effect of silibinin (100 μmol/L) pretreatment on EGF (10 ng/mL)–induced mitogenic and survival signaling in JB6 cells. Similar to UVB, EGF also strongly up-regulated the phosphorylation of ERK1/2, JNK1/2, p38K, and Akt (Fig. 6), and silibinin pretreatment decreased EGF-induced phosphorylation of ERK1/2, JNK1/2, p38K, and Akt (Fig. 6). These results suggest that silibinin also inhibits EGF-induced mitogenic and survival signaling in JB6 cells.

Silibinin Inhibits UVB- and EGF-Induced AP-1 and NF-κB DNA-Binding Activity

Activation of AP-1 and NF-κB transcription factors has been shown to be critical for UVB-induced tumor promotion (7). Increased activity of these transcription factors is directly linked to the activation of mitogenic and survival signaling for tumor promotion as well as progression responses (7, 8). Because we observed that silibinin down-regulated both UVB- and EGF-induced mitogenic and survival signaling, we next assessed the effect of silibinin on UVB- and EGF-induced AP-1 and NF-κB DNA-binding activity. UVB exposure (400 mJ/cm^2) of cells resulted in an increased DNA-binding activity of AP-1 (Fig. 7A) and NF-κB (Fig. 7B), compared with sham-irradiated controls. Silibinin (100 μmol/L) pretreatment for 6 hours showed a strong inhibition of UVB-induced AP-1 and NF-κB DNA-binding activity (Fig. 7A and B). Similar to UVB, EGF treatment showed an increased AP-1 (Fig. 7C) and NF-κB (Fig. 7D) DNA-binding activity, which were also inhibited by silibinin treatment (Fig. 7C and D). Treatment with silibinin alone did not show any observable effect on AP-1 and NF-κB DNA binding (Fig. 7A–D). Supershift and competition assays were done to confirm the specificity of DNA-protein complexes formed with AP-1 (Fig. 7E) and NF-κB (Fig. 7F). We did not observe supershifted band with anti-c-Jun and anti-c-Fos antibodies incubation; however, they reduced the AP-1 DNA-binding activity, suggesting the partial presence of c-Fos and c-Jun in AP-1-DNA complex (Fig. 7E). Anti-p50 showed a shift in NF-κB-DNA complex, whereas both anti-p50 and anti-p65 antibodies strongly reduced NF-κB DNA-binding activity, suggesting the presence of p50 and p65 subunits in NF-κB-DNA complex (Fig. 7F). In both these experiments, the higher DNA-binding activity in control samples compared with other experiments are due to the longer exposure time. Overall, these results suggested that silibinin inhibits both UVB- and EGF-induced transcriptional activation of AP-1 and NF-κB in tumor promoter–sensitive JB6 cells.

Further, we assessed the effect of silibinin on UVB- and EGF-induced phosphorylation and total protein levels of c-Fos and c-Jun in cytosolic and nuclear extracts. In cytosolic fraction, silibinin decreased both UVB- and EGF-induced total protein levels of c-Fos and c-Jun with

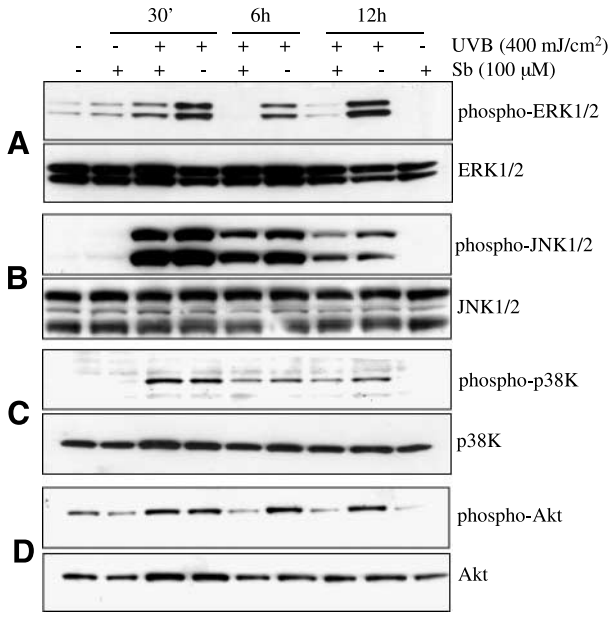

Figure 5. Effect of silibinin posttreatment times to UVB exposure on MAPK and Akt activation. JB6 cells at ~80% confluence were serum starved for 48 h and then irradiated with UVB alone and/or posttreated with silibinin (100 μmol/L). Cells were harvested at the indicated time, lysates were prepared, and Western blotting was carried out to probe phosphorylated and total protein levels of ERK1/2 (**A**), JNK1/2 (**B**), p38K (**C**), and Akt (**D**) as mentioned in Materials and Methods.

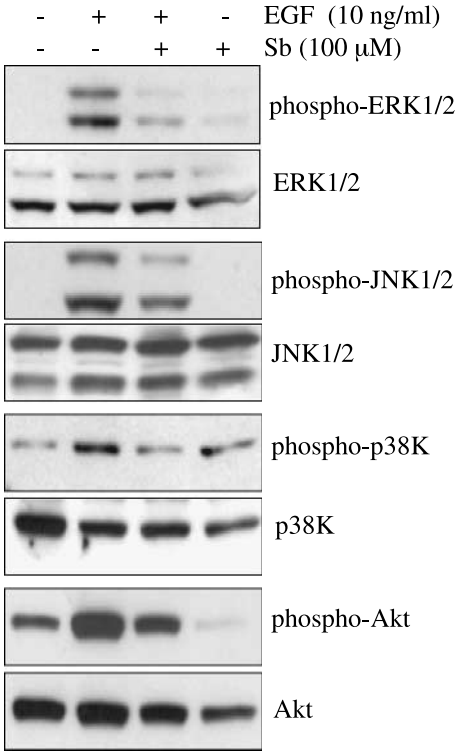

Figure 6. Effect of silibinin on EGF-induced MAPK and Akt activation. JB6 cells at ~80% confluence were serum starved for 48 h and then either pretreated with silibinin (100 μmol/L) for 6 h followed by exposure to EGF (10 ng/mL) or left untreated or exposed to EGF alone. Cells were harvested 30 min thereafter, lysates were prepared, and Western blotting was carried out to probe phosphorylated and total protein levels of MAPKs and Akt as mentioned in Materials and Methods.

a moderate effect on their phosphorylation (Fig. 8A and B). Silibinin did not show any considerable effect on UVB-induced c-Jun phosphorylation or its total level in the nuclear extract; however, a moderate decrease in the total c-Fos nuclear level was observed (Fig. 8A), indicating its partial role in the inhibition of AP-1 activation. Silibinin inhibited EGF-induced phosphorylation of both c-Fos and c-Jun with a strong decrease in the total c-Fos level in the nuclear extract (Fig. 8B). These results suggest the prominent inhibitory effect of silibinin on UVB- and EGF-induced expression of c-Fos and c-Jun.

Discussion

Our previous *in vivo* studies suggest that silibinin inhibits both UVB-induced as well as TPA-induced skin tumor promotion (1, 25); however, its molecular mechanisms are not completely understood. This study was undertaken to investigate the effect of silibinin on molecular events involved in tumor promotion in tumor promoter–sensitive mouse keratinocyte JB6 cells. Overall, the central finding of the present study is that silibinin inhibits both mitogenic

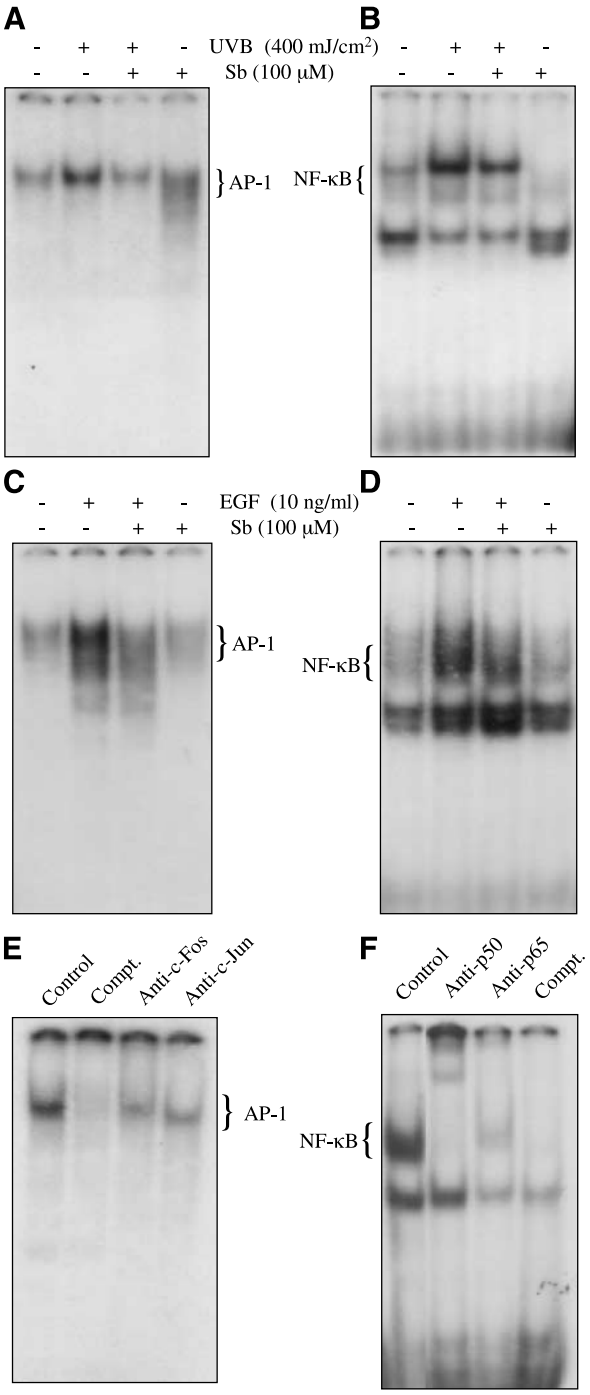

Figure 7. Silibinin down-regulates UVB- and EGF-induced AP-1 and NF-κB activation. **A** and **B,** JB6 cells at ~80% confluence were serum starved for 48 h and then either pretreated with silibinin (100 μmol/L) for 6 h followed by irradiation with UVB (400 mJ/cm^2) or left untreated or irradiated with UVB alone, and incubated for another 12 h and then harvested. **C** and **D,** in similar culture conditions and silibinin treatment, instead of UVB, cells were treated with EGF (10 ng/mL) and harvested 12 h later. Nuclear extracts were prepared and analyzed for AP-1 and NF-κB DNA-binding activity by electrophoretic mobility gel shift assay as described in Materials and Methods. Supershift and competition assays were done to confirm the specificity of AP-1 (**E**) and NF-κB (**F**) binding in nuclear extract.

and survival signaling as well as activation of AP-1 and NF-κB transcription factors in response to UVB and EGF in JB6 cells. Because JB6 cell line has been established as model to study the mechanisms of skin tumor promotion, our results suggest that down-regulation of MAPK/Akt–AP-1/NF-κB pathways by silibinin could be important molecular mechanisms for its antitumor promotion activity in skin carcinogenesis. In contrast to the observation made here, we recently have shown that when JB6 cells are exposed to UVB under serum condition, silibinin treatment causes DNA-PK-mediated p53 activation followed by strong apoptotic death. This DNA-damage stress response in turn also led to an increase in ERK1/2 and Akt phosphorylation, without any effect on P38K and JNK1/2 phosphorylation (26).

JB6 cells have been used to identify the tumor-promoting activity of various classes of xenobiotic compounds (27). Among these, UV radiation, EGF, TPA, and tumor necrosis factor-α are known to transform JB6 cells, leading to anchorage-independent growth and tumorigenic phenotypes (7). The first two agents, used in the present study, have different molecular mechanisms to induce mitogenic and survival signaling. UVB is shown to activate ERK1/2 and p38K but not JNK1 via phosphatidylinositol-3-kinase, and further downstream MSK1 (mitogen and stress-activated protein kinase-1) is activated to phosphorylate Akt (17). UVB is reported to induce AP-1 via Akt-GSK-3β, ERK1/2, and p38K-MAPK-activated protein kinase-2 pathway (28). UVB exposure generates H_2O_2, which activates EGF receptor and ERK1/2 without having any effect on p38K in human keratinocytes (29, 30). Because silibinin is a proven antioxidant, its effect on UVB-induced mitogenic and survival signaling might also involve its antioxidant activity.

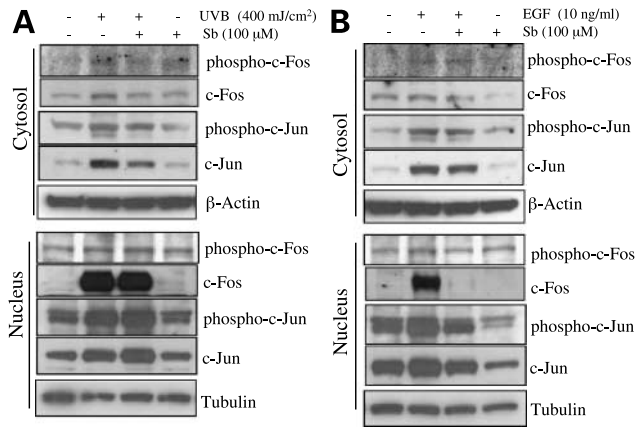

Figure 8. Effect of silibinin on UVB- and EGF-induced phosphorylation and expression of c-Fos and c-Jun. **A,** JB6 cells at ~80% confluence were serum-starved for 48 h and then either pretreated with silibinin (100 μmol/L) for 6 h followed by irradiation with UVB (400 mJ/cm^2) or left untreated or irradiated with UVB alone, and incubated for another 12 h and then harvested. **B,** in similar culture conditions and silibinin treatment, instead of UVB, cells were treated with EGF (10 ng/mL) and harvested 12 h later. Cytosolic and nuclear extracts were prepared and immunoblotting was carried out as mentioned in Materials and Methods.

It has been observed that many tumor promoters activate ERK1/2-AP-1 as well as NF-κB in JB6 cells for the tumorigenic transformation of the cell (7, 8, 31–33). A variant cell line of JB6, represented as JB6(P−), has been found resistant to transformation by tumor promoters (6, 16). JB6(P−) cells are defective in AP-1 activation, which is largely due to the lack of ERK1/2 activation (6). These reports suggest that ERK1/2-AP-1 pathway plays a major role in tumor promotion. However, it has been also observed that some agents could activate AP-1 via phosphatidylinositol-3-kinase–Akt pathway in JB6 cells (32). It is also reported that ERK1/2 and NF-κB are the major determinants for the transformation sensitive phenotype in JB6 cells (6, 19). Furthermore, ERK1/2 and Akt activation by many tumor-promoting agents are known to activate NF-κB in many cell lines, including JB6 cells (7, 31, 34). UVB-induced phosphatidylinositol-3-kinase–Akt pathway is reported to induce AP-1 activation (28). Studies have also shown that both these transcription factors are required for maintaining the transformed phenotype of JB6 cells; therefore, inhibition of ERK1/2 and Akt as well as AP-1 and NF-κB activation in response to tumor promoters could be an underlying mechanism for the antitumor promotion activity of many chemopreventive agents, as observed with silibinin in the present study.

Completed studies show that UVB acts as tumor initiator, tumor promoter, as well as complete carcinogen (1). TPA is a known and widely used skin tumor promoter in animal studies. The tumor promoter activity of UVB and TPA are also influenced by the generation of oxidative stress, which in turn activates ERK1/2 and Akt and downstream AP-1/NF-κB transcription factors (7, 19). Our completed animal studies with silibinin/silymarin using UVB and TPA have shown strong inhibitory effect on tumor promotion and tumorigenesis (1, 20, 22–25). Further, silibinin/silymarin is also shown to inhibit carcinogen-induced cell transformation in rat tracheal cells (35). However, the mechanisms of such efficacy of silibinin are not completely understood. The results in the present study suggest that anti–tumor-promoting activity of silibinin could be mediated, in part, via inhibiting ERK1/2 and Akt activation as well as their downstream effectors AP-1 and NF-κB transcription factors (Fig. 9).

Activation of mitogenic signaling specifically through ERK1/2 as well as survival signaling via Akt pathway is involved in cell transformation as well as clonal expansion of the initiated cell (8, 16, 17). However, activation of these pathways is implicated in a variety of biological responses, including growth, differentiation, and survival/apoptosis (36, 37). In the present study, exposure to UVB strongly activated ERK1/2, JNK1/2, and Akt, and moderately activated p38K. Activation of these molecules has been shown to be a tumor-promotion response, which has been also observed *in vivo* skin carcinogenesis studies (34). Level of ERK1/2 activation is one of the critical factors that determine the sensitivity of JB6 cells to promoter-induced cell transformation (6). EGF, a potent mitogen for cell growth and proliferation, elicited similar effect to that of

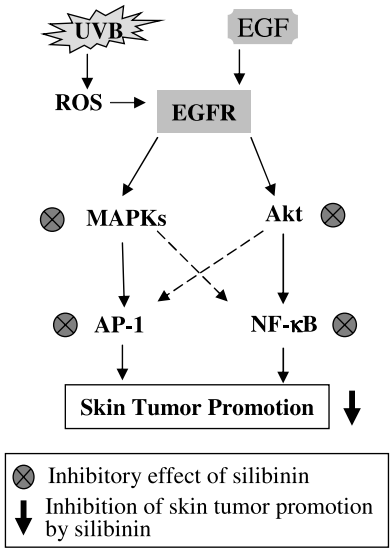

Figure 9. Proposed hypothesis for the inhibitory effect of silibinin on skin tumor promotion. UVB and EGF activate EGF receptor, via reactive oxygen species (*ROS*) and direct interaction, respectively, and transmit signal downstream through MAPKs and Akt. Silibinin down-regulates these mitogenic and survival signaling, in part, via inhibition of MAPK and Akt signaling and further inhibition of transcriptional activation of AP-1 and NF-κB. *Dotted arrows,* possible links of signaling events that might have contributed in overall inhibitory effect of silibinin on AP-1 and NF-κB activation.

UVB on these molecular events, further suggesting the role of these pathways in the sensitivity of JB6 cells for growth and proliferation. As silibinin strongly inhibited UVB- and EGF-induced ERK1/2 and Akt activation in JB6 cells, inhibiting these mitogenic and survival signaling pathways possibly initiates its anti–tumor-promoting effects.

NF-κB and AP-1 transcription factors are activated to transcribe many genes regulating cell growth, differentiation, transformation, survival, and apoptosis (38, 39). Although both UVB and EGF were able to induce NF-κB and AP-1 DNA-binding activity, these transcription factors are activated by distinct signal transducers in response to various kinds of stimuli (38, 39). In this regard, activation of MAPKs and Akt has been implicated in the activation of NF-κB and AP-1 (reviewed in refs. 36–39). Some studies have shown a direct link between the activation of these signaling molecules and transcription factors (36–39). In JB6 cells, ERK2 has been shown to increase UV-induced AP-1 activity (6). Other studies have also shown the role of ERK1/2 activation in AP-1 and NF-κB activation (36). Similarly, Akt signaling is known to activate both NF-κB and AP-1 (37). In the present study, silibinin showed an inhibitory effect on both UVB- and EGF-induced activation of NF-κB and AP-1. As discussed above, silibinin also inhibited ERK1/2 and Akt phosphorylation in response to both these mitogens, suggesting that inhibition of ERK1/2 and Akt signaling by silibinin could play a role in the inhibition of NF-κB and AP-1 activation.

Further, UVB also induced JNK1/2 and p38K phosphorylation, which are also known to activate NF-κB and AP-1, and has been shown to induce proliferation/apoptosis (34, 36). UVB-induced activation of JNK1/2 and p38K are mediated via the generation of reactive oxygen species and subsequently the activation of EGF receptor and downstream signaling molecules (30). EGF directly binds to EGF receptor and transmit mitogenic signal downstream preferably via ERK1/2 and p38K compared with JNK1/2; however, JNK-deficient mice show marked reduction in EGF production and EGF receptor function (40, 41). Silibinin did not show any considerable effect on UVB-induced JNK1/2 and p38K phosphorylation. However, EGF-induced JNK1/2 and p38K phosphorylation were inhibited by silibinin. These results further indicate that both stimuli have different mechanisms for the activation of JNK1/2 and p38K in JB6 cells, in which silibinin targets those that are activated only by EGF. Consistent with this, we also observed that silibinin decreases EGF-induced total protein levels of c-Fos and c-Jun with a moderate effect on their phosphorylation.

In summary, silibinin inhibited UVB-induced ERK1/2 and Akt activation as well as transcriptional activation of NF-κB and AP-1 in tumor promoter–sensitive mouse keratinocyte JB6 cells. Similarly, these pathways were also down-regulated by silibinin in response to EGF. The p.o. feeding of silibinin up to 2 g/kg dose has shown up to 165 μmol/L concentration of silibinin in mouse plasma, without any toxicity (42). Therefore, concentrations of silibinin used in the present study are achievable *in vivo* and should have biological significance. Overall, these findings suggest that silibinin inhibits UVB- and EGF-induced NF-κB and AP-1 activation by modulation of MAPK molecules and Akt; and these could be the underlying mechanisms for the preventive effect of silibinin against both UVB- and chemical tumor promoter–caused tumor promotion in mouse skin.

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Mol Cancer Ther 2006;5:1145-1153.