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Silibinin modulates UVB-induced apoptosis via mitochondrial proteins, caspases activation, and mitogen-activated protein kinase signaling in human epidermoid carcinoma A431 cells

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Abstract

Several recent studies by us have shown the strong chemopreventive efficacy of silibinin against both ultraviolet B (UVB) radiation and chemical carcinogen-induced tumorigenesis in mouse skin models. The molecular mechanisms underlying silibinin protective efficacy, however, are not completely known. Here, we examined the effect of silibinin on UVB-caused apoptosis in human epidermoid carcinoma A431 cells. Irradiation of cells with different doses of UVB (5–100 mJ/cm^2) and different time periods (0.5–24 h) resulted in a dose- and time-dependent increase in apoptosis ($P < 0.05$–0.001). Silibinin (100–200 μM) pre-treatment, however, resulted in an increase in UVB-induced apoptosis ($P < 0.05$–0.001); interestingly, its post-treatment caused a decrease in UVB-induced apoptosis ($P < 0.05$–0.001). A similar pattern in the activation of caspases-9, -3, and -7 was observed with these silibinin treatments. Further, silibinin treatment prior to or immediately after UVB exposure altered Bcl-2, Bax, Bak, and cytochrome *c* levels in mitochondria and cytosol in favor of or against apoptosis, respectively. Silibinin treatment prior to UVB also increased the activation of mitogen/stress activated protein kinases Erk1/2, JNK, and p38 kinase as compared to its post-treatment. Together, for the first time, our results demonstrate the role of mitochondrial apoptotic machinery and MAPK signaling cascade in silibinin-caused increase as well as protection in UVB-induced apoptosis in A431 cells, and suggest that similar mechanisms might be involved in preventive efficacy of silibinin against UVB-induced skin tumorigenesis.

Keywords: Ultraviolet B radiation; Apoptosis; Caspases; Mitogen-activated protein kinases; Silibinin

Non-melanoma skin cancer (NMSC) is the most common human malignancy. The major cause of NMSC is solar radiation in the ultraviolet (UV) range, most notably the UVB (280–320 nm) part of the spectrum which induces morphologic, biochemical, and genetic damages in human skin keratinocytes causing an early neoplastic progression in this tissue [1,2]. UVB causes photodamage in cells that could be either acute or chronic [3]. Lower doses of UVB cause DNA mutation leading to tumor initiation, whereas higher doses result in irreparable DNA damage causing apoptosis (sunburn) and eventually cell deletion; formation of sunburn cells is linked to the severity of UVB-induced DNA damage [4]. Following UVB exposure of the cells, both intracellular and extracellular signals (apoptotic/survival/mitogenic) are altered that determine if a cell would die or live. Mitochondria play a major role in this monitoring system, through regulating the release of cytochrome *c* [5], which classically shuttles electrons between the bc_1 complex (complex III) and cytochrome oxidase (complex IV) in the electron transport chain, generating ATP. Bcl-2 family proteins are also known to play a crucial role in the modulation of apoptosis by influencing the integrity of mitochondria in response to

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different death stimuli [6,7]. MAPK family proteins are also involved in determining the proliferative or apoptotic response in DNA-damaged cells [8]. These signaling events are potential mechanisms of UVB-induced photodamage and apoptosis, suggesting that they could also be potential molecular targets for the chemoprevention of UVB-induced carcinogenesis.

Chemoprevention of UVB-caused skin damage and/or skin carcinogenesis by the use of natural or synthetic agents is recently being explored as one of the possible and practical approaches in controlling this malignancy [9–14]. It is likely that the agents which could prevent UVB-induced DNA damage and/or accelerate the elimination of severely damaged cells could be of potential importance in preventing UVB-induced skin damages as well as NMSC [14–16]. Silibinin, a naturally occuring flavonoid antioxidant, is isolated from milk thistle (*Silybum marianum*), a member of the aster family, which also includes the artichoke, a close relative [14]. Silibinin is the major active compound in silymarin, which is present in a widely consumed dietary supplement milk thistle extract [17]. Several recent studies by others and us have shown that both silibinin and silymarin are highly effective against photo- as well as chemical-induced skin carcinogenesis, and are also effective against other epithelial cancers in both cell culture and animal models [14,18–24]. Silibinin is also effective in treating a wide range of liver and gall bladder diseases, including hepatitis and cirrhosis, as well as dermatological conditions [17,25]. Non-toxicity is one of the most important properties of this compound, which has been tested in various animal models using different modes of administration [17].

In the present study employing A431 cells, we assessed the effect of silibinin on UVB-caused apoptosis and associated mechanisms. Our findings, for the first time, indicate that alterations in Bcl-2 family members leading to mitochondrial damage, cytochrome *c* release, and activation of caspase cascade, together with modulation of MAPK signaling, are involved in the regulation of UVB-induced apoptosis by silibinin.

Materials and methods

Materials. Human epidermoid carcinoma cell line A431 was obtained from American Type Culture Collection (Manassas, VA). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco-BRL, Grand Island, NY) with 10% fetal bovine serum (Hyclone, Logan, UT) and 1% penicillin–streptomycin (Gibco-BRL) under standard culture conditions (37 °C, 95% humidified air, and 5% CO_2). Silibinin was from Sigma–Aldrich Chemical (St. Louis, MO). Anti-cytochrome *c* antibody was obtained from BD Pharmingen (San Diego, CA). Anti-Bcl-2, anti-Bax, and anti-Bak primary antibodies were from Upstate Biotechnologies (Lake Placid, NY). Antibodies which specifically recognize phospho- and total-MAP kinase family members, including Erk1/2, JNK, and p38 kinase; cleaved-caspases and peroxidase-conjugated anti-rabbit secondary antibody were from Cell Signaling Technology (Beverly, MA). Anti-Bid antibody was from R&D Systems (Minneapolis, MN). ECL system was from Amersham (Piscataway, NJ). Other chemicals were obtained in their commercially available highest purity grade.

Cell culture, silibinin treatment, and UVB irradiation. A431 cells were grown in DMEM under standard culture conditions to near confluency. Silibinin was dissolved in dimethyl sulfoxide (DMSO) as stock solution. The final concentration of DMSO in the culture medium during different treatments did not exceed 0.1% (v/v) and was same in all the treatments including controls. Cells were pre- or post-treated to UVB exposure with various doses of silibinin for different time points. Before UVB irradiation, the medium was removed from the culture plates and cells were washed with phosphate-buffered saline (PBS) twice and then covered with a thin layer of PBS. This was 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, Bryan, OH), which emitted ~80% radiation in the range 280–340 nm with a peak emission at 314 nm, as monitored with a SEL 240 photodetector, 103 filter, and 1008 diffuser attached to an IL1400A Research Radiometer (International Light, Newburyport, MA). The UVB irradiation doses were also calibrated using an IL1400A radiometer. Cells were irradiated with different doses of UVB (5–100 mJ/cm^2) and incubated thereafter at 37 °C for different time periods (0.5–24 h).

Apoptotic cell death assay. For analysis of apoptotic cells, A431 cells were plated in 60 mm dishes, and following various treatments without or with silibinin and UVB exposures, were collected at desired time-periods. The cells were then stained with annexin V and PI (Molecular Probes) following the manufacturer's protocol and apoptotic cells were analyzed immediately by flow cytometry using FACS Analysis Core facility of the University of Colorado Cancer Center, as published recently [21].

Immunoblotting. Following the desired treatments, cell lysates were prepared in non-denaturing lysis buffer (10 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 1 mM EGTA, 0.3 mM phenylmethylsulfonyl fluoride, 0.2 mM sodium orthovanadate, 0.5% NP-40, and 5 U/ml aprotinin) and protein concentration in the lysates was determined using a Bio-Rad DC protein assay kit (Bio-Rad Laboratories, Hercules, CA) [21]. For immunoblot analyses, 40–100 μg of protein lysates per sample was denatured in 2× SDS–PAGE sample buffer and subjected to SDS–PAGE on 12% (for MAPK and Bcl-2 family proteins) or 16% (for caspases and Bid) Tris–glycine gels. The separated proteins were transferred onto nitrocellulose membrane followed by blocking with 5% (w/v) non-fat milk powder in TBS (10 mM Tris, 100 mM NaCl, and 0.1% Tween 20) for 1 h at room temperature or overnight at 4 °C. Membranes were then probed with specific primary antibodies followed by peroxidase-conjugated secondary antibody and visualized with an ECL detection system.

Preparation of mitochondrial and cytosolic fractions. After the desired treatments, mitochondrial and cytosolic lysates were prepared as described earlier [21]. Briefly, cells were lysed in S-100 buffer (20 mM Hepes, pH 7.5, 10 mM KCl, 1.9 mM $MgCl_2$, 1 mM EGTA, 1 mM EDTA, and a mixture of protease inhibitors) and left on ice for 20 min. They were then homogenized using a Dounce pestle homogenizer for ~40–45 strokes. After a short centrifugation at 1000*g* for 5 min, the supernatants were again centrifuged at 14,000*g* for 30 min, and the cytosolic supernatants and mitochondrial pellets were collected. The pellets were washed once with extraction buffer and then finally suspended in mitochondrial lysis buffer (150 mM NaCl, 50 mM Tris–HCl, pH 7.4, 1% NP-40, 0.25% sodium deoxycholate, 1 mM EGTA, and protease inhibitors).

Statistical analysis. All experiments were repeated at least twice. The data were analyzed using the Jandel Scientific SigmaStat 2.03 software. For all measurements, as needed, Student's *t* test was employed to assess the statistical significance of difference between different treatment groups. A statistically significant difference was considered to be present at $P < 0.05$.

Results and discussion

Silibinin pre- or post-treatment to UVB irradiation modulates apoptotic death in A431 cells

The most important aspect of any given mechanistic study is its biological significance. To address this issue, first we assessed the apoptotic effect of UVB irradiation alone at different doses and time periods (Figs. 1A and B). An appropriate UVB dose and a time period were selected from this experiment to study the effect of pre- or post-treatment of silibinin to UVB exposure on apoptotic death of A431 cells. As shown in Fig. 1, in apoptotic death assay employing annexin V/PI staining followed by FACS analysis, UVB showed a dose- and time-dependent apoptotic death of A431 cells starting with 15 mJ/cm^2 dose and maximum response at 50 mJ/cm^2 UVB irradiation ($P < 0.05$–0.001). In terms of time for apoptotic response following its irradiation, UVB-caused apoptosis at 50 mJ/cm^2 dose was observed as early as 3 h after exposure with maximum response after 12 h that sustained at 24 h (Fig. 1B). In representative FACS analysis scatter-grams (data not shown), we observed that control cells showed a large viable cell population with a very little staining for apoptotic cells; however, UVB alone treatment (50 mJ/cm^2) significantly ($P < 0.01$) increased apoptotic cell population as compared to control (Fig. 2A). When cells were treated with different doses of silibinin for 12 h prior to UVB irradiation (50 mJ/cm^2), as compared to UVB treatment alone, a significant ($P < 0.01$) additional shift from live cells to early apoptotic cell population was observed (Fig. 2A). Further, we observed that pre-treatment of silibinin (100 μM) for different time periods before UVB exposure (50 mJ/cm^2) showed a time-dependent (3–12 h) induction of apoptosis (37–45%) as compared to UVB alone (35%) ($P < 0.05$ to < 0.001) (Fig. 2B).

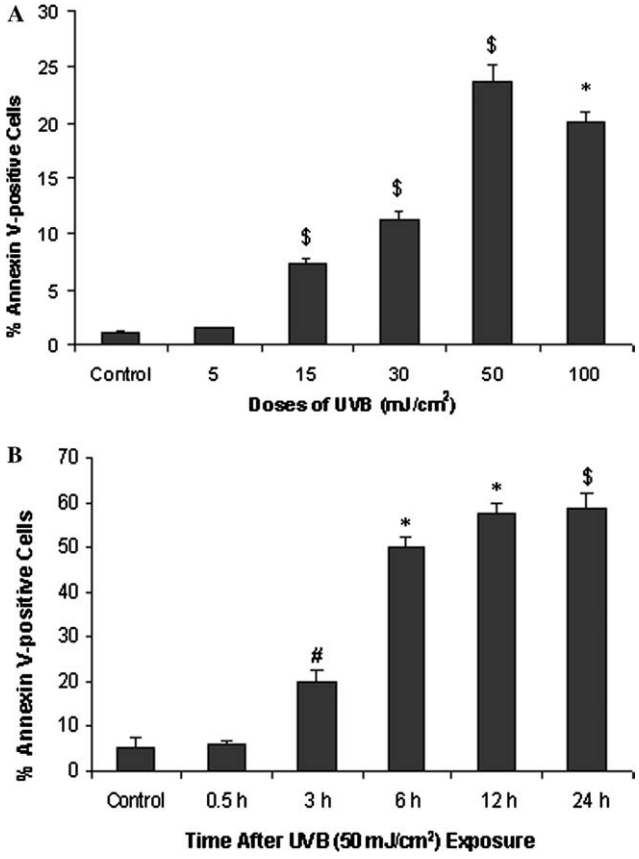

Fig. 1. Dose- and time-dependent apoptotic death of A431 cells by UVB irradiation. (A) Cells were irradiated with different doses of UVB (5–100 mJ/cm^2), harvested after 6 h, stained with annexin V and PI, and analyzed by flow cytometry as detailed in 'Materials and methods.' (B) Cells were irradiated with UVB (50 mJ/cm^2) and incubated thereafter at 37 °C for different time periods (0.5–24 h). At the end of each treatment time, cells were harvested and stained with annexin V and PI, followed by flow cytometric analysis. Quantification of apoptosis was analyzed by only annexin V-positive cells. In each case, the data shown are means ± SE of three samples. $^*p < 0.001$; $^\$p < 0.01$, and $^\#p < 0.05$ as compared to control.

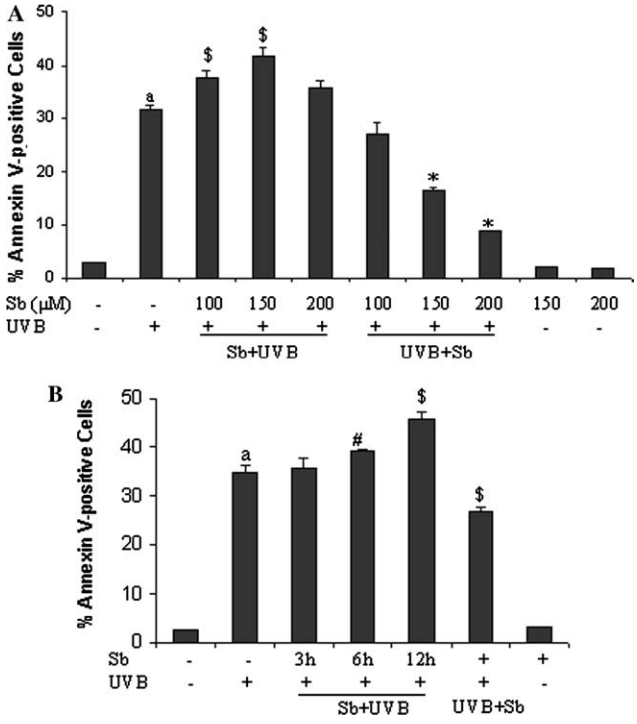

Fig. 2. Effect of pre- or post-treatment of silibinin on UVB-induced apoptotic death of A431 cells. (A) Cells were either pre- or post-treated with indicated doses of silibinin and then irradiated with UVB (50 mJ/cm^2). After 6 h from irradiation, cells were harvested, stained with annexin V and PI, and analyzed by flow cytometry. (B) Cells were pre-treated with silibinin for indicated times or post-treated immediately after UVB (50 mJ/cm^2) irradiation and harvested 6 h post-irradiation followed by annexin V and PI staining and flow cytometric analysis for the quantification of apoptotic cells. In each case, data shown are means ± SE of three samples. $^ap < 0.001$ as compared to control; $^*p < 0.001$; $^\$p < 0.01$; and $^\#p < 0.05$ as compared to UVB treatment alone. Sb, silibinin; Sb + UVB, silibinin pre-treatment to UVB exposure; and UVB + Sb, silibinin post-treatment to UVB exposure.

Surprisingly, silibinin treatment immediately after UVB exposure significantly protected the cells from undergoing apoptosis (Figs. 2A and B). Overall, these findings suggest that silibinin pre-treatment sensitizes the cells for UVB-induced apoptosis, whereas silibinin post-treatment protects cells from UVB-induced apoptosis that could possibly involve cell cycle arrest for DNA damage repair [26]. More studies, however, are needed in future to investigate this anticipation.

Effect of silibinin on UVB-induced caspases activation in A431 cells

Caspases activation is regarded as hallmark of apoptosis [27], which was analyzed to assess the effect of silibinin on UVB-induced apoptosis. A431 cells were either exposed to UVB alone or pre-treated with silibinin followed by UVB exposure, or exposed to UVB and immediately thereafter treated with silibinin. In general, caspases are known to be involved in apoptosis induced by different stimuli [27]. In this regard, UVB-caused apoptosis in A431 cells is caspase-dependent and expression of dominant negative caspase-9 has been shown to block UVB-induced apoptosis [28]. In Western blot analysis employing a specific antibody that recognizes only activated caspase-9 (the cleaved product of procaspase-9), silibinin treatment prior to UVB exposure resulted in a further increase in the level of cleaved caspase-9 as compared to UVB alone, which, however, decreased when cells were treated with silibinin after UVB exposure (Fig. 3A). Under identical experimental conditions, we assessed the levels of cleaved caspase-3

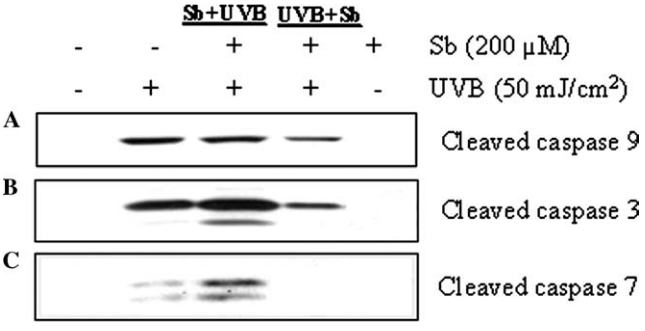

Fig. 3. Effect of silibinin on UVB-induced caspase-9, -3, and -7 cleavage in A431 cells. Sub-confluent A431 cell cultures in 100 mm dishes were exposed to UVB (50 mJ/cm^2) or pre- or post-treated with 200 μM dose of silibinin to UVB irradiation or treated with silibinin alone, as detailed in 'Materials and methods.' Six hours after UVB exposure, cell lysates were prepared with lysis buffer. Equal amount of protein was separated on polyacrylamide–SDS gels, transferred on to membrane, and probed with (A) cleaved caspase-9, (B) cleaved caspase-3, and (C) cleaved caspase-7 specific antibodies followed by peroxidase-conjugated secondary antibody and visualization with ECL detection system. Sb, silibinin; Sb + UVB, silibinin pre-treatment to UVB exposure; and UVB + Sb, silibinin post-treatment to UVB exposure.

and -7, which are the executioner caspases associated with the cleavage of many proteins triggering apoptotic cell death [27]. Consistent with the annexin V staining results for apoptosis, we observed strong levels of cleaved caspase-3 and -7 following UVB exposure, which were further increased when cells were pre-treated with silibinin (Figs. 3B and C). Silibinin post-treatment, however, produced a similar reversal in cleaved caspase-3 and -7 levels as observed for apoptosis. Silibinin alone treatment did not show any detectable levels of cleaved caspases, which was consistent with its lack of apoptotic response. A wide number of studies in recent years have examined and established the molecular mechanism of apoptosis induction via caspases pathway [27–31]. Consistent with these reports, the results in the present study suggest that caspases activation plays a major role in UVB-induced apoptosis as well as in enhancing this response in silibinin pre-treatment-caused further increase in UVB-induced apoptotic death of A431 cells.

Effect of UVB and silibinin on modulation and translocation of Bcl-2 family members and cytochrome c

To determine the role of Bcl-2 family members in silibinin-mediated sensitization to or protection against UVB-caused apoptosis, we prepared mitochondrial and cytosolic fractions and assessed the levels as well as translocation of Bcl-2 family members. As shown in Fig. 4A, a moderate decrease was observed in Bcl-2 level in mitochondrial fraction in UVB exposed or silibinin pre-treated UVB exposed cells as compared to silibinin post-treatment to UVB. In cytosolic fraction, silibinin pre-treatment followed by UVB exposure showed a strong decrease in Bcl-2 level as compared to all other treatments (Fig. 4E). Overall, silibinin treatment prior to UVB exposure decreased the total level of Bcl-2. UVB irradiation with/without silibinin did not show much effect on the pro-apoptotic Bax and Bak protein levels in mitochondria (Figs. 4B and C), whereas treatment with silibinin prior to UVB down-regulated Bax and Bak protein levels in cytosol as compared to silibinin post-treatment (Figs. 4F and G). Together, these results suggest that at least part of the sensitizing effect of silibinin pre-treatment to UVB for apoptosis is mediated by a decrease in Bcl-2 level in mitochondria and a decrease in total Bcl-2, Bax, and Bak protein levels as observed in cytosol together with mitochondrial fraction. Further, protective effect of silibinin could be mediated via an increase in cytosolic levels of Bax and Bak when compared with UVB alone exposed samples.

Cytochrome *c* release is directly associated with apoptosis [5,7,27–30] and has been observed in UV irradiated cells [28]. In this regard, we also observed that UVB decreased the level of mitochondrial cytochrome *c*, which was further decreased by silibinin pre-treatment with a concomitant increase in its cytosolic level (Figs.

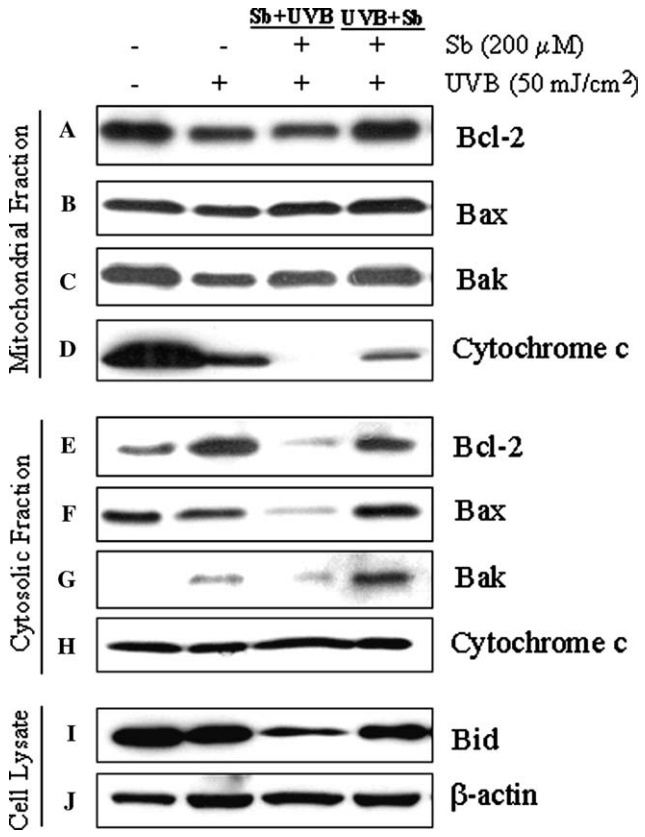

Fig. 4. Effect of silibinin pre- or post-treatment to UVB exposure on Bcl-2 family proteins and cytochrome *c* release. A431 cells were either irradiated with UVB alone or pre- or post-treated with silibinin (200 μM) to UVB irradiation, similar to that described in Fig. 3. Mitochondrial and cytosolic fractions were prepared as described in 'Materials and methods,' and equal protein per sample was separated on polyacrylamide–SDS gels to analyze the levels of (A) Bcl-2, (B) Bax, (C) Bak, and (D) cytochrome *c* in (A–D) mitochondrial and (E–H) cytosolic fractions. In similar treatment, cell lysates were prepared and Western blotting was done to analyze the level of (I) BID. (J) Membrane was stripped and re-probed for β-actin as a loading control. Sb, silibinin; Sb + UVB, silibinin pre-treatment to UVB exposure; and UVB + Sb, silibinin post-treatment to UVB exposure.

4D and H). In silibinin post-treatment, the level of mitochondrial cytochrome *c* increased with a decrease in its cytosolic levels when compared with silibinin pre-treatment (Figs. 4D and H). Further, we assessed the BID cleavage in whole cell lysates, which is known to be cleaved and help in disrupting mitochondrial integrity accompanied by cytochrome *c* release and caspases activation [6]. Consistent with this, we observed a strong decrease in total BID level in silibinin pre-treatment to UVB, suggesting BID cleavage could be another molecular target in sensitizing the apoptotic effect of silibinin to UVB (Fig. 4I). The membrane was re-probed with anti-β-actin that ensured equal loading (Fig. 4J).

The Bcl-2 family consists of both apoptotic and anti-apoptotic proteins and the balance between these proteins is critical to turning on and off the cellular apoptotic machinery [4,6,32]. Whereas the precise mechanisms by which Bcl-2 family members act remain unclear, it has been established that they play a key role in the mitochondrial apoptotic pathway [6]. Bcl-2 is an anti-apoptotic protein whereas Bax and Bak are pro-apoptotic [6]. Homo- and/or hetero-dimerization of anti- and pro-apoptotic proteins of the Bcl-2 family can decide the fate of the cell, whether it will follow the apoptotic or survival pathway [6]. Homodimers of Bcl-2 are known to block cytochrome *c* release from mitochondria, thereby preventing the initiation of caspases activation [6,32]. Homodimers of Bax or Bak are proapoptotic and their heterodimers with Bcl-2 favor apoptosis as compared to Bcl-2 homodimers [6,33]. Based on these reports and the observations in the present study, it could be suggested that silibinin modulates the protein levels as well as alters the translocation of Bcl-2 family proteins in response to UVB stress in deciding its pro-apoptotic or anti-apoptotic effect in A431 cells.

Effect of UVB and silibinin on activation of MAPKs and its association with apoptosis

It has been shown that MAPK signaling plays an important role in response to mitogen/stress to determine cell proliferation/apoptosis (reviewed in [8]). In many recent studies, MAPK pathways have been implicated as mediators of apoptosis in response to a variety of external stimuli [8,34,35]. Classically, the role of ERK1/2 pathway is suggested as protective, whereas JNK and p38 are mostly shown to be activated by stress stimuli for pro-apoptotic response [6,36]. In addition, it has been also reported that the extent and duration of these signaling is crucial for the biological outcome, where ERK1/2 activation could also lead to apoptotic cell death [37]. Furthermore, it has been shown that MAPK family members have a role in activating caspase cascade [6,38].

Based on these reports, we next assessed the effects of silibinin pre- or post-treatment with UVB on ERK1/2, JNK, and p38 activation for their possible role in the modulation of apoptosis. Phospho-specific antibodies were used to selectively probe the activated form of ERK1/2, JNK, and p38, and same membranes were stripped to probe the total levels of these MAPKs. UVB exposure of cells showed strong levels of phospho-ERK1/2, JNK, and p38 as a stress response, which were in accord with its apoptotic effect (Figs. 5A–C). When cells were pre-treated with silibinin to UVB exposure, a further increase in the activation of ERK1/2, JNK, and p38 was observed (Figs. 5A–C), which also corroborated with the increased effect of silibinin pre-treatment on UVB-induced apoptosis in these cells. Further, when silibinin treatment followed immediately after UVB exposure, the activation of these MAPKs diminished as compared to silibinin pre-treatment, however, the activation levels did not fall below that of UVB treatment

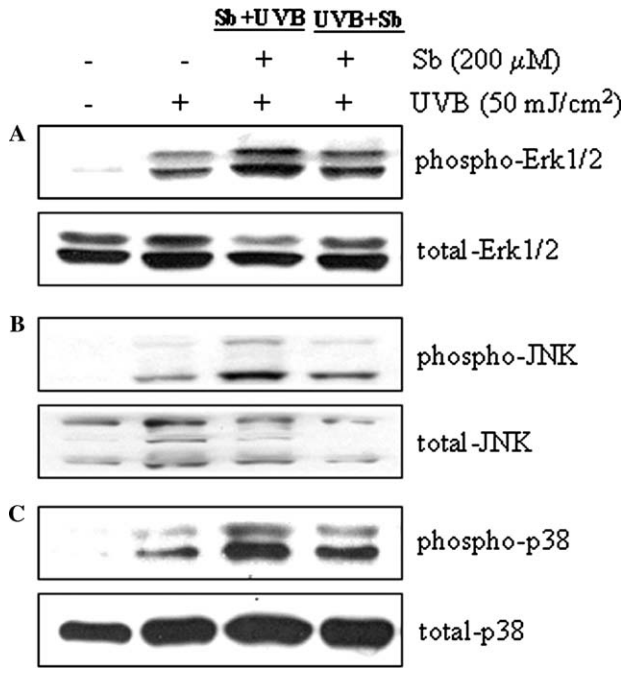

Fig. 5. Effect of silibinin pre- or post-treatment to UVB exposure on MAPK signaling. A431 cells were either irradiated with UVB alone or pre- or post-treated with silibinin (200 μM) to UVB irradiation as mentioned in Fig. 3, and cell lysates were prepared as described in 'Materials and methods.' The cell lysates were separated on polyacrylamide–SDS gels, transferred onto membrane, and probed with phospho-specific and total antibodies against (A) Erk1/2, (B) JNK, and (C) p38 kinase, followed by incubation with appropriate secondary antibody and visualization by ECL detection system. Sb, silibinin; Sb + UVB, silibinin pre-treatment to UVB exposure; and UVB + Sb, silibinin post-treatment to UVB exposure.

alone (Figs. 5A–C). Total ERK1/2, JNK, and p38 protein levels remained comparable in all the treatments (Figs. 5A–C). Overall, these results suggest that silibinin pre-treatment causes a prominent increase in activity of all these MAPKs in A431 cells, which could be associated with its increased apoptotic effect in UVB exposed cells. However, in silibinin-post-treatment, protective effect against UVB-induced apoptosis involves the molecular events which could be, in part, influenced by the down-regulation by these MAPK signaling cascades. Further, the activation/inhibition of these MAPK signaling cascades was in accord with the observed changes in caspases activation and apoptosis, as observed in different treatments.

In summary, our results for the first time show that silibinin treatment prior to UVB irradiation causes a further increase in UVB-induced apoptosis in A431 cells, whereas its post-treatment protects against UVB-induced apoptosis. Mechanistic studies show that the differential effects of silibinin on UVB-induced apoptosis in A431 cells involve the modulation of Bcl-2 family members, cytochrome *c*, and caspases activation together with MAPK signaling. This dual efficacy of silibinin might be of considerable importance to humans that are exposed to sunlight in their daily life, especially since the dose(s) that we used in this study falls within the physiological range of UVB exposure [61]. Together, these findings encourage further mechanistic and in vivo studies to investigate chemopreventive and/or chemotherapeutic efficacy of silibinin against UVB-induced skin tumorigenesis.

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