Silibinin inhibits ultraviolet B radiation-induced mitogenic and survival signaling, and associated biological responses in SKH-1 mouse skin

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Ultraviolet B (UVB) radiation is a complete skin carcinogen causing DNA damage as a tumor-initiating event and activating signaling cascades that play a critical role in its tumor-promoting potential. Recently we reported that a naturally occurring flavonoid, silibinin, protects UVB-induced skin damages and prevents photocarcinogenesis. Here we examined silibinin efficacy on acute and chronic UVB-caused mitogen-activated protein kinases (MAPKs) and AKT activation and associated biological responses in SKH-1 hairless mouse skin. A single UVB exposure at 180 mJ/cm² dose resulted in varying degrees of ERK1/2, in SKH-1 hairless mouse skin. A single UVB exposure at 180 mJ/cm² dose resulted in varying degrees of ERK1/2, JNK1/2, MAPK/p38 and AKT phosphorylation at various time-points in mouse skin; however, topical application of silibinin prior to or immediately after UVB exposure, or its dietary feeding strongly inhibited the activation of these molecules at all the time-points examined. Stronger effects of silibinin towards inhibition of UVB-caused phosphorylation of MAPKs and AKT were also observed in a chronic UVB (180 mJ/cm²/day for 5 days) exposure protocol. Immunohistochemical analysis of chronically exposed skin sections showed that silibinin treatment in all three protocols increases UVB-induced p53-positive cells and decreases UVB-caused cell proliferation, apoptotic and sunburn cells. These findings suggest that silibinin inhibits UVB-induced MAPK and AKT signaling and increases p53 in mouse skin, and that these effects of silibinin possibly lead to a decrease in UVB-caused proliferation and apoptosis, which might, in part, be responsible for its overall efficacy against photocarcinogenesis.

Introduction

The incidence of non-melanoma skin cancer (NMSC) is directly associated with exposure to solar ultraviolet (UV) radiation (1). In the USA alone, more than one million new cases of NMSCs are diagnosed annually in the Caucasians and this number is rising steadily (2). The use of sunscreens as the only measure to prevent the incidence of NMSC has proven to be insufficient enhancing the need for the development of additional and more effective measures in preventing this malignancy (3,4). In this regard, chemoprevention of skin cancer using natural agents is suggested as a promising approach and has generated enormous research efforts in recent times (5). Silimarin, extracted from milk thistle plant, is one such natural compound that has shown strong chemopreventive properties against UVB as well as chemical-induced skin carcinogenesis (6). Silibinin, the active principle of silimarin has also been shown to protect SKH-1 mice from single UVB irradiation-caused cell proliferation and apoptosis by upregulation of p53/p21 pathway (7). More recently, we also reported that topical treatment of silibinin prior to or immediately after UVB irradiation or its dietary feeding strongly prevents photocarcinogenesis in SKH-1 hairless mice (8). Several of our completed studies and those of others have also shown that silibinin is non-toxic even at high doses and longer treatment durations and prevents different epithelial cancers such as prostate, colon and bladder (9–13).

Besides causing the characteristic DNA damage and tumor initiation, UVB radiation also leads to tumor promotion where UVB-induced oxidative stress and activation of various signaling cascades, including receptor and non-receptor tyrosine and serine/threonine kinases, play a major role in cell growth and proliferation leading to clonal expansion of UVB-initiated cells into skin tumors (14–18). MAPK family members are one such class of kinases that have pleiotropic functions, including cell survival, apoptosis, proliferation, transformation and tumor promotion (19–21). Most importantly, MAPK/ERK1/2 has been implicated to mediate cell transformation and tumor promotion caused by diverse tumor promoters, including UV and hydrogen peroxide (22). Activation of other MAPK family members, SAPK/JNK and MAPK/p38, is mainly implicated in response to various stresses such as UV, heat and osmotic shock (23), which activate a number of transcription factors such as AP-1, myc, NFAT4, smad 3, ATFs, Stat1 and CREB (24). JNK plays diverse roles in response to various stimuli and has been shown to induce apoptosis as well as promote tumorigenesis (25,26). With regard to p38, there are recent reports suggesting its crucial role in inflammation, where it induces the production of interleukin-1, tumor necrosis factor-alpha and other cytokines in mediating inflammatory responses (27); p38 activation also contributes to tumor promotion by the epidermal growth factor (28).

The other crucial cell survival and anti-apoptotic pathway is PI3K–AKT almost universally activated in response to diverse cell survival as well as anti-apoptotic stimuli (29,30). The role of AKT in tumor promotion and progression has been reported in a number of in vitro and in vivo experiments (31,32). Both MAPK and AKT signals converge downstream in activating transcription factors such as NF-κB and AP-1, which are involved in tumor promotion (30,32). In response to UV, these signaling molecules are activated differentially as a function...
of dose and time and are crucial in mediating different cellular responses leading to skin inflammation, photaging as well as skin tumor promotion (33), suggesting that an impairment of UVB-caused activation of these signaling pathways could be useful in protecting the skin against the deleterious effects of UVB radiation and associated tumor promotion as well as photocarcinogenesis. Consistent with this suggestion, in the present study, we assessed the time-course of the activation of MAPK/ERK1/2, SAPK/JNK, p38 kinase and AKT in SKH-1 hairless mouse skin following UVB irradiation and the modulatory effect of silibinin on these events. Furthermore, in order to establish a biological significance of the observed effects of silibinin on UVB-activated signaling cascades, mouse skin was immunohistochemically analyzed for proliferation, apoptosis and p53-positive cells. In all the studies, silibinin treatment was done by: (i) dietary feeding; (ii) topical application prior to UVB exposure; or (iii) topical application immediately following UVB exposure of mice skin; this approach was selected to define the efficacy of silibinin at the molecular level from that of the sunscreen effect, if any.

Materials and methods

Animals and UVB light source

Female SKH-1 hairless mice (5 weeks old), from Charles River Laboratories (Wilmington, MA), were fed diet and water ad libitum and maintained under standard conditions in an animal house facility at the University of Colorado Health Sciences Center a week before the start of the experiment. Mice were exposed to UVB irradiation as reported earlier (8). The UVB light source was a bank of four FS-40-T-12-UVB sunlamps equipped with a UVB Spectra 305 Dosimeter (Duavlin Bryan, OH), which emitted ~80% radiation in the range of 280–340 nm with a peak emission at 314 nm as monitored with an 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.

Experimental design

The experiment was designed to assess the protective effect of silibinin on acute and chronic UVB-exposure-caused mitogenic and survival signaling and associated biologic responses in mouse skin. In the case of the acute study protocol, animals were exposed to a single 180 mJ/cm² UVB dose and killed at different time points (1–24 h) thereafter. In chronic UVB exposure experiment, animals were irradiated with 180 mJ/cm² UVB dose/day for 5 days and killed 24 h after the last exposure. Different treatment groups, each with five mice, were: (i) unirradiated and untreated control, (ii) animals fed with 1% silibinin in diet, (iii) animals topically applied with 9 mg silibinin in 200 µl acetone/mouse, (iv) animals irradiated with 180 mJ/cm² UVB, (v) animals fed with 1% silibinin in diet and irradiated with the same dose of UVB, (vi) animals topically applied with 9 mg silibinin in 200 µl acetone/mouse 30 min prior to UVB exposure and (vii) animals topically applied with 9 mg silibinin in 200 µl acetone/mouse 30 min prior to UVB exposure and 60 min prior to silibinin administration. Silibinin diet (1%, w/w) was prepared by Dyets (Bethlehem, PA) and its feeding was started 2 weeks prior to UVB exposure to establish a stable silibinin level in the diet. The diet was fed to the animals during the whole experiment and the body weight of the animals was monitored daily. Silibinin diet was switched to the control diet after silibinin administration. The animals were re-fed with control diet immediately after silibinin administration. The different treatment groups, each with five mice, were: (i) dietary feeding; (ii) topical application prior to UVB exposure; or (iii) topical application immediately following UVB exposure of mice skin; this approach was selected to define the efficacy of silibinin at the molecular level from that of the sunscreen effect, if any.

Preparation of tissue homogenates and western blotting

Skin samples were scrapped off dermis and underlying fat tissues, and epidermis samples thus obtained were homogenized in lysis buffer (Tris 20 mM (pH 7.5) containing EDTA, EGTA, Triton X-100 and sodium orthovanadate) using a polytron homogenizer for 3 min at setting 4 and then centrifuged at 14 000 r.p.m. as reported recently (8). Supernatants were collected and protein was estimated. For each sample, 50 µg protein was resolved on 12% Tris–glycine gel, transferred onto nitrocellulose membranes and blocked for 1 h at room temperature with 5% non-fat dry milk. The membranes were then incubated with the required primary antibody (phosphorylated and total ERK1/2, JNK1/2, MAPK/p38 and AKT antibodies from Cell Signaling Technologies, San Diego, CA) overnight at 4°C and then with appropriate secondary antibody. Protein was visualized by enhanced chemiluminescence detection system.

Preparation of serial sections for immunohistochemical and H&E analyses

Skin tissues were fixed in 10% phosphate-buffered formalin for 8–10 h at 4°C and dehydrated in ascending concentration of ethanol, cleared in xylene and embedded in Polylite (Triangle Biomedical Sciences, NC). Serial sections of 4 µm were cut and processed for immunohistochemical and H&E staining. In all immunohistochemical stainings, to rule out the non-specific stainings allowing better interpretation at the antigenic site, negative staining controls were employed, where, as required, the sections were incubated with N-Universal Negative Control-mouse or rabbit antibody (Dako, Denmark) under identical conditions.

Immunostaining for p53 and PCNA

Tissue sections were deparaffinized, rehydrated and treated with 0.1 M sodium citrate buffer (pH 6.0) in a microwave for 5 min at full power for antigen retrieval. Sections were then quenched of endogenous peroxidase activity by immersing in 5% hydrogen peroxide for 5 min at room temperature. The sections were either incubated with anti-p53 antibody (cat. no. NCL-p53-CM5p, Novocastra Laboratories, Newcastle upon Tyne, UK) at 1:200 dilution in PBS for 2 h at room temperature in a humidity chamber followed by overnight incubation at 4°C or with mouse monoclonal anti-PCNA antibody (Dako, Denmark) at 1:200 dilution in PBS for 2 h at 37°C in a humidity chamber. Then the sections were incubated with biotinylated secondary antibody for 45 min at room temperature for p53 immunostaining or incubated with biotinylated rabbit anti-mouse antibody IgG (Dako) at 1:300 dilution in 10% normal rabbit serum for 60 min at room temperature for PCNA immunostaining followed by 45 min incubation with conjugated horseradish peroxidase streptavidin. Color development was achieved by incubation with DAB (3,3′-diaminobenzidine) for 10 min at room temperature. The sections were counterstained with Harris hematoxylin, dehydrated and mounted.

TUNEL staining for apoptotic cells

Apoptotic cells were detected using the DeadEnd Colorimetric TUNEL system (Promega, WI) following manufacturer’s protocol, with some modifications. In brief, tissue sections after deparaffinization and rehydration, were permeabilized with proteinase K (30 µg/ml) for 1 h at 37°C. Thereafter, the sections were quenched of endogenous peroxidase activity using 3% hydrogen peroxide for 10 min. After thorough washing with 1× PBS, sections were incubated with equilibration buffer for 10 min, and TdT reaction mixture was added to the sections, except for the negative control, and incubated at 37°C for 1 h. The reaction was stopped by immersing the sections in 2× saline–sodium citrate buffer (Sigma) for 15 min. Sections were then treated with conjugated horseradish peroxidase streptavidin (1:500) for 30 min at room temperature, and after repeated washing, they were incubated with substrate DAB until color development (~10 min). The sections were then mounted after dehydration, and observed under 400× for TUNEL-positive cells.

Measurement of apoptotic sunburn cells

Skin sections were stained conventionally with hematoxylin and eosin (H&E), as mentioned earlier (7), for the identification of sunburn cells. Apoptotic cells are morphologically distinct due to cell shrinkage and nuclear condensation that stain darker by H&E. Dark stained cells were scored in 5 random fields/sample and the percentage/field was calculated.

Immunohistochemical and statistical analyses

All the microscopic immunohistochemical analyses were done using Zeiss Axioscop 2 microscope (Carl Zeiss Jena, Germany). Pictures were taken by a Kodak DC290 camera under 400× magnification and processed by Kodak Microscopy Documentation System 290 (Eastman Kodak Company, Rochester, NY). All samples were coded and evaluated by two investigators in a blinded manner and the mean ± SE values were obtained from the evaluation of multiple fields in each group. For each mouse 5–10 representative fields were counted at 400× magnification, and the data represent the results from at least five mice in each group. For statistical significance of the difference, the data were analyzed using the SigmaStat 2.03 software. The statistical significance of difference between UVB alone versus all other groups was determined by the one-way analysis of variance (one-way ANOVA) followed by the Bonferroni t-test for multiple comparisons. P < 0.05 was considered statistically significant.

Results

UVB irradiation of SKH1 hairless mice causes MAPKs and AKT phosphorylation in skin epidermis

Compared with sham irradiated controls, a single UVB exposure of mice resulted in a strong phosphorylation of
MAPKs ERK1/2, JNK1/2 and p38 as well as AKT in skin epidermis, albeit at different levels, depending on the time after UVB irradiation and the molecule analyzed; representative data are shown at 12 h following UVB exposure in each case (Figure 1A). In all other western immunoblot analyses, sham irradiated controls and both topical and skin epidermal samples treated with only dietary silibinin did not show considerable phosphorylation of MAPKs and AKT as compared with UVB irradiation, but these data are not shown in any figures. In addition, the immunoblot results shown for duplicate samples in each treatment protocol are representative of all samples analyzed in each case.

Fig. 1. UVB irradiation causes MAPKs and AKT phosphorylation, and inhibitory effect of silibinin on UVB-induced ERK1/2 phosphorylation in SKH-1 hairless mouse skin epidermis. (A) Mice were exposed to a single UVB dose of 180 mJ/cm² or sham irradiated, and 12 h thereafter, skin epidermal lysates were prepared and subjected to SDS-PAGE and immunoblotting followed by phospho- and total ERK1/2, JNK1/2, p38 and AKT detection as described in Materials and methods. Membranes were also probed with actin antibody for protein loading. (B) Skin samples collected after 8, 12 and 24 h post-UVB exposure were homogenized in lysis buffer, and lysates were subjected to SDS-PAGE followed by western blotting, as described in Materials and methods. The membranes were probed with phosphorylated and total ERK1/2 antibodies followed by peroxidase-conjugated appropriate secondary antibody as detailed in Materials and methods. Proteins were visualized with ECL detection system. In these treatments, silibinin was given topically prior to UVB (Sb + UVB) or after UVB (UVB + Sb) or in diet (Sbf + UVB) following UVB exposure as detailed in Materials and methods.
Effect of silibinin on UVB-induced ERK1/2 phosphorylation

First, we conducted studies to assess the effect of silibinin treatments in three different protocols on a single acute UVB irradiation-caused ERK1/2 phosphorylation with a view to study time-kinetics in this experiment. Exposure of mice to UVB resulted in a very moderate ERK1/2 phosphorylation without any change in total ERK1/2 protein levels at 1 and 4 h (data not shown); however, later time-points of 8, 12 and 24 h showed stronger effects with maximum response in ERK1/2 phosphorylation at 12 and 24 h after UVB exposure (Figure 1B). The inhibitory effect of silibinin on UVB-induced ERK1/2 phosphorylation was evident in all three treatment protocols at all time-points, without any measurable changes in total ERK1/2 protein; actin immunoblots showed equal protein loading in each case (Figure 1B). These results suggest that at least part of silibinin’s protective effect against UVB-induced tumorigenesis in SKH-1 hairless mouse skin could be via an inhibition in UVB-caused ERK1/2 activation.

Effect of silibinin on UVB-caused JNK phosphorylation

Next we assessed the effect of UVB irradiation on another member of the MAPK family, JNK, and its modulation by silibinin treatments. Similar to ERK1/2, a single UVB exposure of mice resulted in a moderate phosphorylation of JNK1/2 at 1 and 4 h (data not shown) but a stronger effect was evidenced after 8–24 h after irradiation (Figure 2). Pre- or post-treatment of silibinin topically or its dietary feeding strongly inhibited UVB-induced JNK1/2 phosphorylation at all time-points studied (Figure 2). In each case, immunoblotting for total levels of JNK1/2 showed that the observed changes in JNK1/2 phosphorylation were not due to measurable changes in total JNK1/2; actin immunoblots showed equal protein loading in each case (Figure 2).

Effect of silibinin on UVB-induced MAPK/p38 phosphorylation

Recent reports show that, in addition to ERK1/2 and JNK1/2, MAPK/p38 activation is crucial for various mitogen/promoter-induced cell transformation (34). Based on our findings showing that silibinin treatments inhibit UVB-induced ERK1/2 and JNK1/2 phosphorylation, we assessed the effect of UVB alone on MAPK/p38 phosphorylation in SKH-1 mouse skin epidermis and its modulation by silibinin treatments. A single acute UVB exposure of the animals resulted in MAPK/p38 phosphorylation in skin epidermis starting 8 h and sustained till 24 h after irradiation (Figure 3); and this was similar to our results for ERK1/2 and JNK1/2. Furthermore, like our other findings in the case of ERK1/2 and JNK1/2, silibinin treatments in three different protocols showed a strong protective effect against UVB-induced MAPK/p38 phosphorylation at all time-points studied without any change in total p38 levels; and actin immunoblots showed equal protein loading in each case (Figure 3).

Effect of silibinin on UVB-induced AKT phosphorylation

We then assessed the effect of silibinin treatments on UVB-induced AKT phosphorylation, which is a crucial cell survival molecule activated together with MAPKs in response to many
mitogens and survival stimuli including UV (35). A single acute exposure of mice to UVB irradiation also induced AKT phosphorylation at ser473 with almost comparable time-kinetics to MAPKs (Figure 4); however, topical application or dietary feeding of silibinin also showed strong inhibitory effects on UVB-caused AKT phosphorylation at all time-points (Figure 4), without similar changes in total AKT protein levels which were also confirmed using actin immunoblots for equal loading (Figure 4).

Silibinin inhibits phosphorylation of MAPKs and AKT in chronic UVB-exposed mouse skin epidermis

Based on our findings showing that silibinin treatment in three different protocols inhibits a single acute UVB-caused MAPK and AKT phosphorylation in mouse skin epidermis, we also extended our observations to assess silibinin effects on these UVB-caused molecular alterations under a chronic UVB irradiation protocol. Under identical study protocols including silibinin treatments, mice were either unexposed (control), exposed with UVB at 180 mJ/cm²/day dose for 5 days, topically applied with silibinin (9 mg/200 µl acetone/mouse) 30 min prior to or immediately after each UVB exposure, or fed with silibinin (1% w/w in diet) for 2 weeks prior to the start of UVB irradiation and continued on silibinin diet during the exposure. Animals were killed 24 h after the last UVB exposure and skin epidermal lysates were analyzed for MAPKs and AKT phosphorylation by western blotting. The chronic UVB exposure resulted in a strong phosphorylation of ERK1/2, and topical application of silibinin prior to or immediately after each UVB irradiation or its dietary feeding very strongly inhibited this ERK1/2 phosphorylation (Figure 5). UVB exposure of mice for five consecutive days also resulted in a strong phosphorylation of other MAPKs JNK1/2 and p38 as well as AKT, and similar treatments with silibinin showed strong inhibition in the UVB-caused phosphorylation of these molecules (Figure 5).

Effect of silibinin on UVB-induced p53-positive cells

Tumor suppressor p53 plays a critical role in the regulation of cell cycle including DNA repair and programmed cell death (36) and UVB-caused DNA damage is known to induce p53 leading to cell cycle arrest for DNA repair or apoptosis depending on the extent of the damage (37). Several recent studies have also identified the causal role of UVB-induced MAPKs and AKT activation in p53 induction employing various mouse and human epidermal cells (8,36–39), suggesting that the agents influencing UVB-caused activation of MAPKs and AKT should also affect p53 levels and associated biological outcomes in terms of cellular proliferation and apoptosis. Consistent with this suggestion and based on our findings showing that silibinin inhibits both acute and chronic UVB-caused MAPKs and AKT phosphorylation, we analyzed the expression of p53 protein in the skin sections collected following different treatments in the chronic UVB irradiation protocol. Compared with sham irradiated controls showing almost no reactivity for p53 immunostaining (Figure 6A), repeated exposure of mice to UVB resulted in a strong immunoreactivity for the p53-positive cells (Figure 6B); however, silibinin treatment in three different protocols resulted in a further strong increase in p53-positive cells (representative
Silibinin inhibits UVB-induced signaling pathways

sunburn cells by H&E staining. Compared with sham-irradiated (Figure 6D) and only silibinin (topical or in diet) treated (data not shown) groups of mice showing weak and very few PCNA-positive cells, a very strong nuclear immunostaining for PCNA positive cells was observed throughout the epidermis of chronic UVB-irradiated mice (Figure 6E); however, topical application of silibinin prior to each UVB exposure resulted in a strong decrease in UVB-caused PCNA-positive cells (Figure 6F). Similar results were also observed when silibinin was applied topically immediately after each UVB irradiation or was fed through diet (immunohistochemistry staining data not shown). Quantification of immunohistochemistry results showed 31.06 ± 2.30% PCNA-positive cells in UVB alone group as compared with 4.77 ± 1.60% in control (Figure 7B). However, topical silibinin pre- or post-treatment or its dietary feeding decreased PCNA-positive cells to 13.91 ± 0.90, 17.78 ± 2.74 and 17.60 ± 1.31%, which were significantly (P < 0.001) lower than that of the UVB alone group (Figure 7B).

In the study analyzing the effect of chronic UVB exposure on apoptosis induction in mouse skin epidermis and its modulation by silibinin treatment, employing TUNEL staining, multiple UVB exposure showed a large number of TUNEL-positive cells (Figure 6H) as compared with sham-irradiated control (Figure 6G), which were also reduced strongly by different silibinin treatments (Figure 6I, data shown only for topical silibinin pre-treatment). Quantification of these results showed 31.44 ± 3.56% TUNEL-positive cells in UVB alone group as compared with 0.46 ± 0.18% in control (Figure 7C). However, topical silibinin pre- or post-treatment, or its dietary feeding decreased TUNEL-positive cells to 14.31 ± 1.47, 15.58 ± 1.16 and 23.22 ± 1.52%, which were significantly (P < 0.01-0.001) lower than that of the UVB alone group (Figure 7C). We also performed H&E staining for the determination of apoptotic cells that also showed similar response in terms of a strong increase in chronic UVB exposed skin (Figure 6K) samples compared with controls (Figure 6J) and a significant inhibition by silibinin treatments (H&E staining data shown only for topical silibinin pre-treatment followed by UVB, Figure 6L). In quantitative analyses, topical silibinin treatment prior to or immediately after each UVB irradiation, or its dietary feeding decreased UVB-induced apoptotic cells from 23.01 ± 1.87% to 10.28 ± 0.99, 13.46 ± 1.11 and 13.24 ± 0.80% (P < 0.001); as compared with 1.59 ± 0.39% in control (Figure 7D).

Discussion

Earlier studies have shown the photoprotective efficacy of silymarin against skin carcinogenesis (6,40). Silymarin, a crude form of silibinin, protects from UVB-caused tumor initiation, promotion and complete carcinogenesis (6). We recently showed that silibinin protects HaCaT cells from UVB-caused damages by protecting from, or enhancing apoptosis depending on the doses of UVB (41). We also showed that silibinin protects SKH-1 mice skin from UVB-induced thymine dimer formation and apoptosis (7). More recently, we observed that silibinin (both topical and oral) strongly inhibited UVB-induced skin tumorigenesis in long-term study (8); however, its modulatory effect on UVB-caused acute mitogenic signaling associated with tumor promotion has not been studied in mouse skin. The present study is an effort to investigate signaling events associated with...
the preventive efficacy of silibinin against UVB-induced cell proliferation, DNA damage, and apoptotic (sunburn) cells in mouse skin.

MAPK family of proteins ERK1/2, p38 kinase and JNK are known to be involved in the activation of transcription factors NF-κB and AP-1 (19,20) implicated in tumor promotion. In promotion-sensitive mouse epidermal JB6 cells, ERK1/2 activation has been shown to be indispensable for transformation (42). Proven antioxidants N-acetyl cysteine and genistein prevent UV-induced skin damages through inhibition of one or more of mitogenic signaling molecules (43). Further, UV-induced MAPK signaling is also involved in the activation of matrix metalloproteinases causing degradation of skin tissues and photaging (43). Recent reports indicate that in tumor tissues, where p53 is constitutively activated, ERK1/2 was physically associated with mutant p53 and that activity of ERK1/2 was higher in tumor cells than in normal cells (44).

In the present study, UVB exposure resulted in the sustained activation of ERK1/2, which was inhibited by silibinin treatments in three different protocols. Similar to ERK1/2, MAPK/p38 kinase is activated in response to various cellular stresses, including UV (45). MAPK/p38 activity is consistently high in non-small cell lung cancer as compared with normal tissues (46). In JB6 cells, MAPK/p38 is also involved with AP-1 transactivation (47). These findings are suggestive of the crucial role of MAPK/p38 in UV-induced tumor promotion. Green tea polyphenols and glycolic acid inhibit UV-induced MAPK/p38 activation in SKH-1 mouse skin (48,49). In our study, UVB induced MAPK/p38 activation from as early as 4 h which remained sustained till 24 h after irradiation; however, silibinin treatment of mice by topical application (pre- or post-UVB exposure) or dietary feeding resulted in strong inhibition in UVB-induced MAPK/p38 activation. With regard to JNK, there are several reports showing the role of SAPK/JNK in cell survival, cell transformation and tumor promotion (50,51). One of the mechanisms by which antioxidants or chemopreventive agents suppress neoplastic transformation and skin tumor formation is through inhibition of JNK (48,52).

Also, JNK2-deficient mice have been shown to be less prone to TPA-induced skin tumorigenesis (51). Tumor promoters and mitogens increase cell proliferation through activation of transcription factor AP-1, which is effected through JNK-dependent activation of c-Jun and c-Fos, components of AP-1 (53). In the present study, silibinin treatments inhibited UVB-induced JNK1/2 activation, suggesting that inhibition of all three MAPKs activation by UVB, namely ERK1/2, MAPK/p38 and JNK1/2, possibly plays an important role in preventing the efficacy of silibinin against photocarcinogenesis.
AKT signaling is widely known to play a significant role in several processes associated with tumorigenesis, where in some cases AKT activation alone is reported to induce cancer development (54). Therefore, identification of inhibitors of the AKT pathway is suggested as a novel therapeutic strategy to deal with neoplastic development requiring AKT activation. AKT activation is known to regulate cyclin D stability causing cell cycle progression and cell proliferation (55). Consistent with these reports, we observed an activation of AKT (phospho-AKT) pathway in UVB-irradiated mouse skin as compared with unirradiated controls. Silibinin inhibited UVB-induced activation of AKT, suggesting that in addition to MAPKs, it could be another mechanism of its efficacy against photocarcinogenesis.

In the present study, consistent with our recent report showing silibinin inhibits UVB-induced cell proliferation, DNA damage/apoptosis and sunburn cells after 1–12 h of a single dose of UVB irradiation (7), silibinin inhibited these biological events in mouse skin even after five doses of UVB irradiation (chronic exposure) immunohistochemically analyzed 24 h after the last dose of UVB exposure. P53 accumulation is shown to be correlated with DNA damage and cell cycle arrest (56). Consistent with our earlier report showing silibinin enhancing UVB-induced p53 accumulation after 1–12 h of a single dose of UVB irradiation (7), silibinin treatment resulted in a further increase in UVB-induced (180 mJ/cm²/day for 5 days) p53 accumulation in mouse skin, suggesting a possible role in cell cycle arrest for DNA repair and inhibition of cell proliferation. Taken together, silibinin inhibits UVB-induced mitogenic signaling involving ERK1/2, JNK1/2 and MAPK/p38 kinases as well as AKT in SKH-1 mouse skin and associated biological responses in terms of proliferation and apoptosis, and these events could, in part, be responsible for its antitumor promoting effects against photocarcinogenesis.

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References


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