Silibinin Inhibits Inflammatory and Angiogenic Attributes in Photocarcinogenesis in SKH-1 Hairless Mice

Mallikarjuna Gu, Rana P. Singh, Sivanandhan Dhanalakshmi, Chapla Agarwal, and Rajesh Agarwal

Abstract

Sunscreens partially filter UVB and, therefore, could partially prevent skin cancer; however, efficient approaches are desired to effectively prevent photocarcinogenesis. It is hypothesized that nontoxic pharmacologically active natural compounds can increase photoprotective effects. Our completed studies suggest that silibinin, a bioactive phytochemical, strongly prevents photocarcinogenesis; however, its mechanism is not fully understood. Herein, for the first time, we used a clinically relevant UVB dose (30 mJ/cm²/day) to examine the photoprotective effect and associated mechanisms of silibinin in SKH1 hairless mice. Topical or dietary silibinin treatment caused a strong protection against photocarcinogenesis in terms of delay in tumor appearance, multiplicity, and volume. Analyses of normal skin, uninvolved skin from tumor-bearing mice, and skin tumors showed a statistically significant decrease (P < 0.05–0.001) in inducible nitric oxide synthase (iNOS) and cyclooxygenase 2 (COX-2) levels by silibinin. Concomitantly, phospho–signal transducers and activators of transcription 3 (Tyr705) and phospho-p65(Ser536) were also decreased by silibinin, which are potential upstream regulators of iNOS and COX-2. Simultaneously, silibinin also decreased UVB-caused increase in cell proliferation and microvessel density. In tumors, hypoxia-inducible factor 1α (HIF-1α) and vascular endothelial growth factor protein levels were decreased by silibinin. Further analysis showed that silibinin inhibited UVB-caused phosphorylation and nuclear translocation of STAT3 and p65, as well as nuclear factor κB (NF-κB) DNA binding activity. Together, these results suggest that silibinin causes a strong protective effect against photocarcinogenesis via down-regulation of inflammatory and angiogenic responses, including skin inflammation and edema (25); however, its comprehensive mechanisms are not understood. Present study, for the first time, was designed to use a chronic UVB dose in physiologic range to induce photocarcinogenesis in mice. Animal exposure to this UVB dose (30 mJ/cm²/day) is in close range of human exposure to sunlight and, therefore, has more translational significance (26) compared with higher doses used in previous studies. We studied end points of photocarcinogenesis, including tumor incidence, multiplicity, and volume, and underlying molecular alterations.

Introduction

UVB radiation–induced skin damages can potentially cause precancerous and cancerous skin lesions, mostly nonmelanoma skin cancer, and also accelerate skin aging. It involves an imbalance of endogenous antioxidant system, leading to an increase in free radical levels and inflammation. Along with production of reactive oxygen species, UVB also causes direct DNA damage to exert its detrimental effect (1, 2). In photocarcinogenesis, oxidative stress and activation of various signaling cascades, including receptor and nonreceptor tyrosine and serine/threonine kinases, play major role in clonal expansion of UVB-initiated cells into visible skin tumors (3). Signal transducers and activators of transcription 3 (STAT3) and phospho-p65 subunit of nuclear factor κB (NF-κB) are key mediators for many cytokines/growth factors–receptors signaling (4). Both these transcription factors are constitutively active in many human tumors and contribute to oncogenesis, including skin tumorigenesis (5).

Recent studies reveal that persistently active STAT3 induces tumor angiogenesis by up-regulation of vascular endothelial growth factor (VEGF) expression and also modulates immune functions in favor of tumor immune evasion (6). Activated NF-κB often facilitates transcription of numerous genes, including inducible nitric oxide synthase (iNOS) and cyclooxygenase 2 (COX-2), involved in inflammation and tumorogenesis (7). Both iNOS and NF-κB are induced by UVB, and because NF-κB responsive elements are functionally present in iNOS, it indicates a possible role of UVB-induced NF-κB activation in iNOS expression (8). COX-2 is also involved in enhancing cell proliferation and up-regulation of angiogenesis (9, 10). UVB is shown to enhance COX-2 protein level in both human and mouse skin (10–15). Another molecule, hypoxia-inducible factor 1α (HIF-1α) is also known to increase VEGF expression (16). Studies with genistein, all-trans retinoic acid and carbazol have shown to antagonize UVB-caused inflammation and angiogenesis through different signal transduction pathways (17–19). These studies indicate potential role of iNOS and COX-2 in UVB-caused inflammation, angiogenesis, and skin tumorigenesis.

Our recent studies have shown that silibinin, a naturally occurring flavanolignan, causes strong protection against photocarcinogenesis (20–24) and inhibits UVB and chemical tumor promoters–induced skin inflammation and edema (25); however, its comprehensive mechanisms are not understood. Present study, for the first time, was designed to use a chronic UVB dose in physiologic range to induce photocarcinogenesis in mice. Animal exposure to this UVB dose (30 mJ/cm²/day) is in close range of human exposure to sunlight and, therefore, has more translational significance (26) compared with higher doses used in previous studies. We studied end points of photocarcinogenesis, including tumor incidence, multiplicity, and volume, and underlying molecular alterations.

Materials and Methods

Experimental design. Female SKH-1 hairless mice (5 weeks old) were from Charles River (Wilmington, MA). Silibinin (Sigma, St. Louis, MO) diet (1%, w/w) was prepared by Dyets (Bethlehem, PA). UVB light source was four FS-40-T-12-UVB sunlamps with UVB Spectra 305 Dosimeter (Daavlin, Bryan, OH), emitting 80% radiation within 280 to 340nm with 314-nm peak
as described earlier (21). Treatment groups, each with 25 mice, were (a) unirradiated and untreated (control); (b) 9 mg silibinin in 200 μL acetone per mouse topical (SBT); (c) 1% silibinin fed in diet (SBF); (d) 30 mJ/cm² UVB, 5 days/week for 25 weeks (UVB); (e) SBT 30 min before UVB (SB + UVB); (f) SBT immediately after UVB (UVB + SBT); and (g) SBF and UVB (SBF + UVB). Rationale for these protocols was based on completed studies showing silibinin efficacy against photocarcinogenesis and acute UVB-caused changes in mouse skin after identical treatments but with higher UVB dose (21, 23). Body weight and diet consumption were recorded throughout the experiment. Mice were sacrificed when all the mice had tumors in groups 4 to 7 (25 weeks post-UVB), and results were analyzed for tumor incidence, multiplicity, and volume. Parts of skin and tumors were collected and fixed in buffered formalin for immunohistochemical analyses, and remainder flash frozen in liquid nitrogen for molecular analyses.

**Immunohistochemical staining.** Tissue processing, serial section preparation, and other details are described recently (21–24). Sections were incubated with 1:100 dilution for iNOS (BD Biosciences, San Diego, CA), COX-2, platelet/endothelial cell adhesion molecule 1 (PECAM-1; M-20), proliferating cell nuclear antigen (PCNA; Dako, Glostrup, Denmark) and 1:50 dilution for phospho-STAT3(Tyr705;D3A7), phospho-p65(Ser276;Cell Signaling, Beverly, CA) in PBS for 1 h at room temperature followed by overnight at 4°C. Then sections were incubated with appropriate biotinylated secondary antibody (1:250 dilution) for 1 h at room temperature, followed by 45 min incubation with conjugated horseradish peroxidase (HRP) streptavidin, and with 3,3′-diaminobenzidine for 10 min at room temperature. Sections were counterstained with Harris hematoxylin. To rule out nonspecific staining, sections were incubated with N-Universal Negative Control mouse or rabbit antibody (Dako). Immunohistochemical analyses were done using Zeiss Axioscop 2 microscope (Carl Zeiss, Inc., Jena, Germany).

**Western immunoblotting.** Total tissue lysates and nuclear and cytosolic fractions were prepared as published earlier (21, 23, 27). Equal amounts of protein from desired samples were resolved on Tris-glycine gel, transferred to nitrocellulose membrane and blocked for 1 h with 5% nonfat dry milk. Membranes were incubated with primary antibody for phosphorylated and total STAT3 and p65 (Cell Signaling), iNOS (BD Biosciences), HIF-1α (Novus Biologicals, Littleton, CO), COX-2, VEGF (Santa Cruz, Santa Cruz, CA), and α-tubulin (Neomarkers, Fremont, CA) overnight at 4°C, and then with HRP-conjugated secondary antibody, followed by enhanced chemiluminescence. Purity of cytosolic and nuclear fractions was established by blotting membranes with anti–glutathione S-transferase (GST)-α (MBL, Nagoya, Japan) and histone H1 (Lab Vision, Fremont, CA) antibodies. Blots were scanned with Amsa Scan 600 with minimum background.

**Electrophoretic mobility shift assay.** Consensus sequences of double-stranded NF-κB oligonucleotide (5′-AGT TGA GGC GAC TTT CCC AGG C-3′ and 3′-TCA ACT CCC CTT AAA GGA TCC G-5′ from Santa Cruz) were end-labeled with γ-32P-ATP (3000 Ci/mmol at 10 mCi/mL) as per manufacturer’s protocol (Promega, Madison, WI). Labeled probe was separated from free γ-32P-ATP using G-25 Sephadex column. Nuclear extract (8 μg) was incubated with 5× gel shift binding buffer (Promega) and then with 32P-labeled NF-κB probe for 20 min at 37°C. In super shift and competition assays, nuclear extract was incubated with anti-p65 or p50 antibody or unlabeled-oligo before adding labeled NF-κB oligo. Unlabeled mutant NF-κB oligonucleotide (Santa Cruz) was also used to further establish the specificity of DNA-protein complex. DNA retardation gel (6%) was used to resolve DNA-protein or DNA-protein-antibody complexes followed by gel drying and autoradiography.

**Statistical analysis.** Data were analyzed using SigmaStat 2.03 software, and statistical significance of difference between UVB-alone versus all other groups was determined by ANOVA, followed by Bonferroni t test for multiple comparisons. Between groups, comparison was done by t test. P < 0.05 was considered statistically significant.

**Results**

Silibinin inhibits physiologically relevant dose of UVB-caused tumorigenesis in mouse skin. Exposure of mice to 30 mJ/cm² UVB dose resulted in development of skin tumors in all mice by 18 weeks; however, it took 21 weeks to get tumors in all mice in three different silibinin-treated plus UVB-exposed groups (data not shown). First tumor appearance in UVB-alone animals occurred at 14th week, which was delayed by 2 weeks in silibinin-treated groups (data not shown). Silibinin treatments in three different protocols showed lower tumor numbers throughout the experiment, and at the end of study at 25 weeks, 26% to 45% (P < 0.001) decrease in tumor number per mouse was observed compared with UVB group (Fig. 1A). Tumor volume per mouse also decreased from 96.2 ± 15.6 mm³ in UVB group to 25.2 ± 4.7, 30.1 ± 2.8, and 21.4 ± 1.5 mm³ in presilibinin or postsilibinin topical or dietary group, respectively, accounting for 69% to 78% (P < 0.001) decrease (Fig. 1B). Overall, three different modes of silibinin treatments (pre-UVB or post-UVB topical or dietary) showed almost similar effects on tumor number and volume. None of the silibinin treatments caused any decrease in diet consumption (Fig. 1C) or body weight (Fig. 1D) compared with controls. Together, these results convincingly indicate that silibinin, as opposed to sunscreens, acts at molecular level to provide protective effect against photocarcinogenesis without any toxicity. In further studies, potential molecular targets of silibinin in its photoprotective effects were examined.

**Silibinin treatments decrease iNOS expression in UVB-exposed mouse skin and skin tumors.** Induction in iNOS expression is a potential mechanism to induce many physiologic, as well as pathophysiologic, processes, including inflammation and angiogenesis (7). Thus far, the effect of physiologic dose of UVB has not been investigated on iNOS. Here, we observed that chronic exposure of physiologic dose of UVB strongly increases the immunoreactivity of iNOS throughout the skin epidermis compared with sham-irradiated and only-silibinin (topical or diet) treated groups of mice (data not shown). Quantification of immunostaining showed 22 ± 2% iNOS-positive cells in UVB-alone group; however, pre-UVB or post-UVB topical silibinin or its feeding showed 14 ± 1%, 12 ± 1%, and 12 ± 1% iNOS-positive cells, respectively, accounting for 36% to 46% (P ≤ 0.001) decrease (Fig. 2A, left). Similarly, strong immunostaining for iNOS was observed in UVB-irradiated skin tumors, which was also decreased in the tumors from silibinin-treated groups (data not shown). Quantification for iNOS-positive cells in skin tumors showed 34 ± 4% cells in UVB-alone group, which was decreased by 29% to 34% to 24 ± 2% (P ≤ 0.05), 25 ± 2% (P ≤ 0.01), and 23 ± 2% (P ≤ 0.01) iNOS-positive cells in pre-UVB or post-UVB topical or dietary silibinin group, respectively (Fig. 2A, right). These results were further confirmed by immunoblot analysis. Consistent with immunostaining data, physiologic dose of chronic UVB exposure strongly increased iNOS levels in both epidermis (Fig. 2C, left) and skin tumors (Fig. 2C, right) compared with normal skin, which were profoundly decreased in silibinin-treated epidermis (Fig. 2C, left) and tumor (Fig. 2C, right) samples. Overall, these results suggest iNOS as a potential molecular target in photoprotective effects of silibinin.

**Silibinin treatments decrease COX-2 expression in UVB-exposed mouse skin and skin tumors.** In addition to iNOS, COX-2 is also implicated in UVB-induced skin inflammation and photocarcinogenesis (12). To assess whether silibinin efficacy against UVB-induced skin inflammation and photocarcinogenesis also involves alteration in COX-2, tissue samples were analyzed for COX-2 levels. UVB-alone group showed increased immunoreactivity for COX-2 in both epidermis and skin tumors (data not shown). In control mice, 6 ± 1% COX-2–positive cells were observed in epidermis compared with ~3 ± 0.5% in topical or dietary silibinin
Silibinin Inhibits Inflammatory and Angiogenic Events

Effect of topical applications/dietary feeding of silibinin on physiologically relevant UVB-induced skin tumorigenesis in SKH-1 hairless mice. The treatment groups were unirradiated and untreated control (Control), 9 mg silibinin in 200 μL acetone per mouse topical (SBT), 1% silibinin fed in diet (SFB), 30 mJ/cm² UVB (UVB), 9 mg silibinin topical in 200 μL acetone per mouse 30 min before UVB exposure (SB + UVB), 9 mg silibinin topical immediately after UVB exposure (UVB + SBT), and 1% silibinin fed in diet and exposure with the same dose of UVB (SFB + UVB) as detailed in Materials and Methods. Tumor number per mouse (A) and tumor volume per mouse (mm³; B) were recorded at the end of 25 wks of experiment. Columns, mean of 20 mice in each group; bars, SE. Average diet consumption per mouse (g, C) and mean body weight per mouse (g, D) were also recorded throughout the experiment as the function of week from 20 mice in each group. The statistical significance of difference between UVB-treated and all other groups was determined by one-way ANOVA followed by Bonferroni t test for multiple comparisons. *, P < 0.001 versus UVB alone.

Silibinin inhibits cell proliferation in photocarcinogenesis. Persistent inflammation causes hyperproliferation in epidermis, and enhanced proliferation rate is hallmark of tumor cells (28). We therefore examined the effect of physiologic dose of UVB exposure without or with silibinin treatments on proliferation status of epidermis and skin tumors by measuring PCNA level. Quantitative analysis revealed 2 ± 0.6% PCNA–positive cells in unirradiated mouse epidermis, which were comparable with silibinin alone treatments (Fig. 3A, left). Chronic UVB exposure for 25 weeks showed 29 ± 3% PCNA–positive cells, which were decreased to 17 ± 2% (P < 0.001), 17 ± 2% (P < 0.001), and 20 ± 2% (P < 0.005) by pre-UVB or post-UVB topical or dietary silibinin, respectively (Fig. 3A, left). Similarly, in skin tumors, UVB-alone group showed 33 ± 1% PCNA–positive cells which were decreased to 20 ± 2% (P < 0.001), 18 ± 2% (P < 0.001), and 21 ± 2% (P < 0.001) by silibinin treatments, respectively (Fig. 3A, right). These results indicate the antiproliferative mechanism of silibinin in its photoprotective effect against physiologically relevant dose of UVB–caused skin tumorigenesis.

Silibinin inhibits angiogenesis in photocarcinogenesis. Apart from inflammation, iNOS and COX-2 are also implicated in angiogenesis; therefore, we next examined effect of physiologic dose of UVB exposure without or with silibinin treatments on angiogenic variables. Quantitative analyses of PECAM-1, an endothelial cell marker, immunostaining in control skin showed 2 ± 0.2% positive cells which did not alter after silibinin treatments (Fig. 3B, left). PECAM-1–positive cells increased to 8 ± 0.9% in UVB group which were decreased by silibinin treatments to 6 ± 0.6% (P < 0.01), 6 ± 0.8% (P < 0.01), and 6 ± 0.8% (P < 0.05) in pre-UVB or post-UVB topical or dietary group, respectively (Fig. 3B, left). Similarly, in skin tumors, there were 12 ± 1% PECAM-1–positive cells in UVB group (higher than UVB–treated skin) which were significantly reduced to 6 ± 0.6% (P < 0.001), 7 ± 0.9% (P < 0.001), and 8 ± 0.7% (P < 0.005) in silibinin treatment groups, respectively (Fig. 3B, right). These results suggest potential antiangiogenic activity of silibinin in its photoprotective effects, which we further investigated by immunoblotting for VEGF, a potent mitogen for endothelial cells. Compared with normal skin, chronic physiologic dose of UVB alone showed high level of VEGF expression in both epidermis (Fig. 3C, left) and skin tumors (Fig. 3C, right); however, silibinin treatments in three different protocols showed strongly reduced VEGF levels in both epidermis and skin tumor (Fig. 3C). Because HIF-1α is a potential transcription factor for VEGF expression, we next examined its...
Silibinin inhibits STAT3(Tyr705) phosphorylation and its nuclear translocation in photocarcinogenesis. Because we observed that iNOS, COX-2, and VEGF are up-regulated by UVB in both skin and tumors and were subsequently decreased by silibinin in its efficacy against photocarcinogenesis, we examined STAT3 status, which is known to transcriptionally activate these inflammatory and angiogenic molecules. Immunohistochemical analysis of skin for STAT3(Tyr705) phosphorylation, an activated form of STAT3, showed 3 ± 0.4% phospho-STAT3(Tyr705)-positive cells in unirradiated mice without or with silibinin treatments (Fig. 4A). When mice were exposed to UVB, an elevated level of phospho-STAT3 immunostaining was observed in epidermis (data not shown), which accounted for 28 ± 2% phospho-STAT3(Tyr705)-positive cells which were decreased by 29% to 46% to 15% (P < 0.001), 19% ± 2% (P < 0.001), and 20% ± 2% (P < 0.001) after three different silibinin treatments, respectively (Fig. 4A). Similarly, skin tumors from UVB group showed 32 ± 3% phospho-STAT3(Tyr705)-positive cells which were decreased by 25% to 44% to 22 ± 2% (P < 0.01), 24 ± 2% (P < 0.05), and 18 ± 2% (P < 0.001) in pre-UVB or post-UVB topical or dietary silibinin groups, respectively (Fig. 4B).

Skin samples were further analyzed by immunoblotting for phosphorylated and total STAT3 levels in cytosolic and nuclear fractions; tumor tissues could not be analyzed due to the limited amount. Consistent with immunostaining data, UVB-alone samples showed an increased level of phospho-STAT3(Tyr705) as well as total STAT3 in cytosolic extract from epidermis; however, this increase was more prominent in nuclear fraction compared with that of normal skin (Fig. 4C). Regarding silibinin-treated samples in three different protocols plus UVB, there was either no effect or only a slight decrease in both phosphorylated and total STAT3 levels in cytosolic fraction; however, both these levels decreased very strongly to almost normal skin level in nuclear fraction. Purity of cytosolic and nuclear fractions was established by also blotting membranes with anti–GST-p and histone H1 antibodies, which clearly showed immunoreactivity only for cytosolic and nuclear specific proteins (Fig. 4C), respectively. These results suggest that silibinin strongly inhibits UVB-induced STAT3(Tyr705) phosphorylation and its nuclear translocation, which is necessary for STAT3 transcriptional activity. These results also suggest that physiologic dose of UVB activates STAT3 signaling in photocarcinogenesis, which could effectively be targeted by silibinin in its photoprotective effects.

Silibinin inhibits p65 phosphorylation and NF-κB activation in photocarcinogenesis. NF-κB transcription factor is activated by UVB that mediates many processes involved in inflammation and angiogenesis (7). In this regard, because iNOS, COX-2, as well as VEGF could also be transcriptionally activated by NF-κB (6, 7), we next assessed NF-κB activation in UVB-exposed skin and skin tumors. First, we did immunohistochemical analysis for...
phosphorylated p65(Ser276), an activated subunit form of NF-κB. In control mice receiving normal diet, 4 ± 0.6% phosphorylated p65(Ser276)–positive cells were observed in epidermis, which increased slightly (nonsignificant) by topical or dietary silibinin (Fig. 5A). UVB-alone exposure showed 7.5-fold increase (30 ± 2%) in phospho-p65(Ser276)–positive cells which were strongly decreased (by 57–63%) to 11 ± 1% (P < 0.001), 13 ± 1% (P < 0.001), and 12 ± 1% (P < 0.001) after pre-UVB or post-UVB topical or dietary silibinin treatment, respectively (Fig. 5A). Similarly, skin tumors from UVB-alone treatment had 41 ± 3% phospho-p65(Ser276)–positive cells, which were lowered by 27% to 34% to 30% to 3% (P < 0.001) by three silibinin treatments, respectively (Fig. 5B).

Once again, due to tumor tissue limitation, further studies were done only in skin samples analyzing phospho-p65(Ser536) and total p65 levels in both cytosolic and nuclear fractions, as well as NF-κB activation. It is important to emphasize here is that other than Ser276, phosphorylation of p65 at Ser536 increases its nuclear translocation (29, 30). UVB-alone exposure strongly increased phospho-p65(Ser536) level in both cytosolic and nuclear fractions compared with normal skin (Fig. 5C). All the three modes of silibinin treatments showed a very strong decrease in phospho-p65(Ser536) level in both cytosol and nucleus (Fig. 5C). Silibinin treatments did not show any considerable effect on total level of UVB-induced p65 in cytosol but strongly decreased it in the nucleus (Fig. 5C). Once again, purity of cytosolic and nuclear fractions was established by using anti–GST-π and histone H1 antibodies, which showed immunoreactivity only for cytosolic and nuclear specific proteins (Fig. 5C), respectively.

Consistent with increase in both Ser276 and Ser536 phosphorylation of p65, UVB treatment strongly increased NF-κB DNA binding activity, which was very strongly inhibited by silibinin treatments (Fig. 5D). Super shift assay indicated presence of both p65 and p50 subunits of NF-κB in DNA-protein complex (Fig. 5D). Competition assays with unlabeled NF-κB consensus oligo showing disappearance of band, and a lack of such effect when unlabeled mutant NF-κB oligo was used in electrophoretic mobility shift assay (EMSA) further established the specificity of DNA-protein complex (Fig. 5D). Overall, these results indicate that silibinin strongly inhibits UVB-induced NF-κB signaling in photocarcinogenesis to mediate its photoprotective effects.

Discussion

There are many novel findings in the present study related to physiologic dose of UVB-induced skin carcinogenesis. Specifically, physiologically relevant dose (30 mJ/cm²/day) of UVB (a) enhances iNOS and COX-2 expression, (b) promotes angiogenic response, (c) increases HIF-1α expression, (d) activates STAT3 and NF-κB signaling in both skin and tumors, and more importantly (e) these events were strongly suppressed by topical or dietary silibinin, leading to highly significant photoprotection (up to 78% decrease in tumor burden). Collectively, present study supports the hypothesis that chronic exposure to physiologically relevant UVB
dose alters molecular balance toward photocarcinogenesis. Additionally, these molecular changes can be effectively inhibited by nontoxic chemopreventive agents, like silibinin, to provide significant protection against photocarcinogenesis. Particularly, present findings suggest potential clinical benefits of silibinin in minimizing or preventing photodamage in human skin. Furthermore, almost similar effects of silibinin in topical post-UVB and dietary treatments versus topical pre-UVB protocol suggest that it is stable even after UVB irradiation (in pre-UVB application) and that silibinin primarily works at molecular levels through systemic distribution rather than as a sunscreen.

This study is different from previous work, in which six times higher UVB dose (180 mJ/cm²/day) was used, and still we observed photoprotective effect of silibinin (21). In this regard, the present study is one-step ahead in identifying molecular alterations in skin and tumors exposed to a chronic UVB dose which is in the range of exposure to humans (26). Therefore, silibinin efficacy observed in the present study in terms of a decrease in tumor number and a shrinkage of tumor size would have potential clinical significance. Further, consistent with earlier studies, silibinin did not show any adverse health effect, which further supports its possible clinical application.

iNOS produces biological nitric oxide that plays a pivotal role in UV-induced inflammation and is implicated in the pathogenesis of various inflammatory diseases, including sunburn and pigmentation, as well as in different stages of tumorigenesis (7, 31). In an in vivo study, an acute and higher dose of UVB (200 mJ/cm²) is shown to induce iNOS expression in mouse skin (32). Also, UV is capable of inducing iNOS expression in keratinocytes (33). Interestingly, an in vitro study with lower doses of UVB (2 and 5 mJ/cm²) in murine keratinocytes has reported a moderate suppression in iNOS expression (33). However, in our in vivo study, chronic exposure of physiologic dose of UVB strongly induced iNOS expression in mouse skin as well as skin tumors, which is clinically relevant, as in humans, photosensitivity is shown to be positively associated with the level of iNOS expression (34, 35). Further, silibinin treatments down-regulated UVB-induced iNOS expression in both skin and tumors which were possibly associated with its photoprotective effects.

COX-2 is a key enzyme required for prostaglandins syntheses that mediate inflammatory responses (10). UVB induces COX-2 expression in both mouse and human skin (11–15) and potentially leads to photocarcinogenesis. However, UVB doses used in previous studies are considerably higher than that of human exposure. Recent studies also indicate a link between iNOS and COX-2 expression (36). A study using a single exposure of physiologic dose of UVB to cultured human keratinocytes has shown up-regulation of COX-2 expression (11). Consistent with these reports, our study with the same UVB dose showed increased COX-2 levels in both skin and skin tumor in hairless mice. Silibinin treatments substantially lowered COX-2 protein levels in both chronically exposed skin and skin tumors. Together, these findings suggest that chronic physiologic dose of UVB induces iNOS and COX-2 to mediate inflammatory and related processes in vivo and that topical or dietary silibinin treatment could effectively suppress them.

Because chronic inflammation is linked to enhanced cell proliferation and angiogenesis, our next focus was to assess the molecular markers associated with these biological processes. For proliferation status, the results showing higher number of PCNA-stained cells in both skin and skin tumors by UVB, and their lower number with silibinin treatments, further substantiate our above assumption. A similar result was obtained for cyclin D1 expression, another marker of cell proliferation (data not shown). Consistent with proliferation data, PECAM-1 analysis, an endothelial cell marker, showed a similar increasing trend in angiogenic response by physiologic dose of UVB which was decreased by silibinin treatments. VEGF is a potent mitogen for endothelial cells and is also secreted by chronically UVB-exposed skin epithelial or tumor cells for neoangiogenesis (6, 37). Our results for VEGF were in accord with PECAM-1 data which further support angiogenic role of chronic exposure of UVB in both skin and skin tumors. For the
first time, we also observed *in vivo* effect of UVB on HIF-1α induction in both tissues. HIF-1α transcriptionally activates VEGF and plays a crucial role in pathophysiologic angiogenesis (38). It is well established that overexpression of HIF-1α occurs in many human cancers (39, 40), and that targeting HIF-1α in tumor cells inhibits tumor growth (41, 42). Consistent with this, a possible HIF-1α-mediated up-regulation of VEGF by UVB was observed in our experimental conditions, which was targeted by silibinin. This *in vivo* observation is supported by a recent report in which UVB is shown to induce the expression of HIF-1α and VEGF in human keratinocytes in cell culture (43).

A critical role of STAT3 in promoting tumor cell survival, proliferation, angiogenesis, metastasis, and immune evasion has been shown in various pathophysiologic conditions, including carcinogenesis (44). However, to our knowledge, there is only one report with UVB in skin wherein authors have shown that a single-exposure of 180 mJ/cm² UVB dose increases Tyr705 phosphorylation and total protein level of STAT3 in hairless mouse skin (45). In our study, chronic physiologic dose of UVB showed similar effect on Tyr705 phosphorylation and total level of STAT3 in both skin and skin tumors; additionally, we also observed an increased nuclear translocation of STAT3 indicative of its potentially enhanced transcriptional activity. More importantly, UVB-induced activation of STAT3 was almost completely blocked by silibinin treatments. These findings imply that indeed STAT3 is a novel potential target for the prevention of photodamage as well as photocarcinogenesis.

NF-κB is a transcription factor with diverse activities linked to growth promoting potential and antiapoptotic responses of cells to a broad spectrum of stimuli that may result in malignant transformation of cells acquiring drug resistance and metastatic potential (46). Regarding photocarcinogenesis, there are some studies, mostly *in vitro* in keratinocytes, showing activation of NF-κB by UVB; however, there is no such animal study using the physiologically relevant dose of UVB to examine its effect on NF-κB. Therefore, our finding showing activation of NF-κB by UVB suggests that it could be a clinically relevant target to protect from photodamage and photocarcinogenesis. Further, this hypothesis is supported by the inhibitory effect of silibinin on NF-κB signaling in the same (preclinical) experimental design, which was associated with its protective effects against UVB.

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**Figure 5.** Effect of physiologic dose of UVB and silibinin on NF-κB signaling in photocarcinogenesis. From the study detailed in Fig. 1, (A) skin and (B) skin tumor were analyzed for phospho-p65(Ser²⁷⁶)–positive cells by immunohistochemical staining. Percentage of phospho-p65(Ser²⁷⁶)–positive cells in normal skin from respective control groups, uninvolved skin from tumor-bearing mice, and skin tumors were recorded by counting the positively stained cells over total cells in four randomly selected fields (>400) from each of five randomly selected skin or tumor samples in each group. Treatments were as described in Fig. 2. Columns, mean of 20 fields per group; bars, SE. C, cytosolic and nuclear extracts from skin samples were analyzed for phospho-p65(Ser²⁷⁶) and total p65 by SDS-PAGE and immunoblotting using specific antibodies as mentioned in Materials and Methods. The purity of the cytosolic and nuclear protein fractions was established by also blotting the membranes with anti-GST-κ and histone H1 antibodies. D, nuclear extract prepared from skin was analyzed for NF-κB DNA binding by EMSA as described in Materials and Methods. Nuclear extract from UVB-treated skin sample was used for competition with cold NF-κB consensus oligo, super shift, and cold mutant NF-κB consensus oligo in the EMSA as described in Materials and Methods. *, P ≤ 0.001; £, P ≤ 0.01; ¥, P ≤ 0.05 versus UVB alone.
The inhibitory effects of silibinin on intermediates of angiogenic and proinflammatory signaling pathways, including HIF-1α, STAT3, and NF-κB transcription factors suggest that silibinin possibly targets cytokine and/or oxidative stress-induced activation of receptor/nonreceptor signaling cascade after UVB irradiation of mice or cells in culture. In this regard, we have shown that silibinin strongly inhibits UVB-induced and chemical tumor promoters–induced oxidative stress, activation of both mitogen-activated protein kinases and Akt signaling, and skin inflammation in mouse skin models (25). In addition, our cell culture studies suggest that silibinin inhibits UVB-induced NF-κB activation in mouse keratinocyte JB6 cells (47), tumor necrosis factor α (TNF-α)–induced NF-κB activation in prostate cancer cells (48) and cytokines mixture (IL-1β + IFNγ + TNF-α)–induced proinflammatory events in lung carcinoma A549 cells (49). Therefore, it is likely that as an upstream target, silibinin interferes with UVB-caused activation of receptor/nonreceptor signaling cascade to subsequently inhibit the activation of proinflammatory and angiogenic intermediate signaling molecules. More studies are needed in the future to further substantiate this assumption and to identify upstream targets of silibinin in photocarcinogenesis.

In summary, we have identified iNOS, COX-2, and VEGF as potentially important molecules in the regulation of inflammation and angiogenesis at physiologically relevant doses of UVB causing photocarcinogenesis. In this clinically relevant condition, we also identified three transcription factors, HIF-1α, STAT3, and NF-κB, which could potentially up-regulate iNOS, COX-2, and VEGF in response to UVB. More importantly, silibinin was found to inhibit these UVB-induced molecular alterations together with a strong photoprotective effect. Whereas these results establish correlations between inhibitory effects of silibinin on inflammatory and angiogenic attributes as they associate to its protective effect against photocarcinogenesis, future studies are needed to define specific role of each identified transcription factor in photoprotective efficacy of silibinin involving down-regulation of inflammatory and angiogenic molecules. Lastly, it is important to emphasize here that silibinin is physiologically achievable up to 165 μmol/L in mouse plasma (50) and is already in clinical trial for prostate cancer (48), and therefore, based on the findings of the present study; its clinical trial for photoprotective effects in humans is also warranted.

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References
35. Tsukazaki N, Watanabe M, Shimizu K, Hamasaki Y, Katayama I. Photoprovocation test and immunohistochemical analysis of inducible nitric oxide synthase

Cancer Research 2007; 67: (4). April 1, 2007 3490 www.aacrjournals.org

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