Inhibition of p38 Mitogen-Activated Protein Kinase and Phosphatidylinositol 3-Kinase Decreases UVB-Induced Activator Protein-1 and Cyclooxygenase-2 in a SKH-1 Hairless Mouse Model

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Abstract

Activation of activator protein-1 (AP-1) and increased expression of cyclooxygenase-2 (COX-2) have been clearly shown to play a functional role in UVB-induced skin tumor promotion. In this study, we examined UVB-induced signal transduction pathways in SKH-1 mouse epidermis leading to increases in COX-2 expression and AP-1 activity. We observed rapid increases in p38 mitogen-activated protein kinase (MAPK) signaling through activation of p38 MAPK and its downstream target, MAPK activated protein kinase-2. UVB also increased phosphatidylinositol 3-kinase (PI3K) signaling as observed through increases in AKT and GSK-3β phosphorylation. Activation of the p38 MAPK and PI3K pathways results in the phosphorylation of cyclic AMP–responsive element binding protein, which was also observed in UVB-irradiated SKH-1 mice. Topical treatment with SB202190 (a specific inhibitor of p38 MAPK) or LY294002 (a specific inhibitor of PI3K) significantly decreased UVB-induced AP-1 activation by 84% and 68%, respectively, as well as COX-2 expression. Our data show that in mouse epidermis, UVB activation of the p38 MAPK and PI3K pathways leads to AP-1 and COX-2 expression.

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Introduction

Nonmelanoma skin cancers (NMSC) composed of squamous cell carcinoma (SCC) and basal cell carcinoma are the most frequently diagnosed cancers in the United States, with >1,000,000 cases reported annually (1). The major etiologic factor in the development of NMSC is solar radiation, as SCCs and basal cell carcinomas occur primarily on sun-exposed regions of the body (2). More specifically, the UVB (280–320 nm) spectrum of light, and to a lesser extent UVA (320–400 nm), has been implicated in the development of NMSC (3, 4). UVB acts as a complete carcinogen, capable of acting in the three phases of tumor development: initiation, promotion, and progression. Despite increased use of sunscreens and sunblocks, the incidence of NMSC has not decreased in the United States due to an aging population and increases in solar radiation reaching the Earth’s surface due to ozone depletion (5). These factors necessitate the development of new targeted approaches for the chemoprevention of NMSC.

In the studies done herein, we describe UVB-induced signaling events leading to activator protein-1 (AP-1) activation and cyclooxygenase-2 (COX-2) expression in vivo. AP-1 is composed of heterodimers of Fos and Jun family members and homodimers of Jun family members. The AP-1 family of transcription factors has been shown to play a role in a variety of cellular processes, including cell proliferation, differentiation, apoptosis, and tumorigenesis (6). Several lines of evidence suggest a role of AP-1 in skin tumor formation induced by UVB. Inhibition of AP-1 activation in malignant mouse SCC lines inhibits the ability of these malignant cells to form tumors on s.c. injection into athymic nude mice (7). Decreases in UVB-induced AP-1 by sodium salicylate or perillyl alcohol have also been correlated with inhibition of skin tumor formation (8, 9). More recently, work from our laboratory has shown that AP-1 plays a functional role in the development of UVB-induced SCC in SKH-1 mice. Epidermal expression of a dominant-negative c-jun (TAM67) mutant under the control of a human keratin K14 promoter showed a significant decrease in AP-1 activity in response to an acute UVB exposure. Expression of TAM67 also reduced the number of tumors per mouse by 58% and tumor sizes by 79% compared with nontransgenic littermates in UVB skin carcinogenesis studies (10).

COX-2 is the rate-limiting enzyme in arachidonic acid metabolism leading to prostaglandin synthesis. Currently, two isoforms of the COX-2 enzyme have been identified (COX-1 and COX-2). COX-1 is a constitutively expressed enzyme in most tissues, whereas COX-2 is considered the inducible form of the enzyme stimulated by growth factors, cytokines, and tumor-promoting agents (11). UVB has been shown to increase COX-2 expression in human skin and in cultured keratinocytes (1). Increased expression of COX-2 has also been reported in...
SCC biopsies when compared with non-sun-exposed skin (1). Studies using selective COX-2 inhibitors have shown a significant decrease of UVB-induced skin tumors in hairless mice (12, 13). UV is capable of inducing phospholipase A2 activation, allowing release of arachidonic acid from membrane-bound phospholipids, and producing prostaglandin G2, which is then further reduced to prostaglandin H2. Increases in prostaglandins have a variety of effects, including increases in cell proliferation and angiogenesis, common events in tumorigenesis (14).

Previous work from our laboratory has established a role for p38 mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K) in UVB-induced AP-1 activation and COX-2 expression in the immortalized human keratinocyte cell line, HaCaT. p38 MAPK is one of the three serine/threonine kinases comprising the MAPK family in addition to extracellular-regulated signal kinases and c-Jun NH2-terminal kinases. There are five isoforms of p38 MAPK, including α, β1, β2, γ, and stress-activated protein kinase 4. MAPK signaling is stimulated by various stimuli, including growth factors, cytokines, and stressful stimuli, such as UVB (15, 16). These kinases play an important role in the signaling events leading to proto-oncogene activation (15, 16). PI3K is a heterodimeric protein composed of a catalytic subunit (p110) and a regulatory subunit (p85), the signaling of which is frequently deregulated in carcinogenesis (17, 18). On stimulation, the catalytic subunit phosphorylates phosphatidylinositol PtdIns(4)P or PtdIns(4,5)P2 to produce PtdIns(3)P, PtdIns(3,4)P2, and PtdIns(3,4,5)P3 (19-21). AKT is a serine/threonine kinase-activated downstream of PI3K with a well-established role in promoting cell survival (22).

Published work has established a role for p38 MAPK and PI3K signaling in UVB-induced AP-1 activation and COX-2 expression in cultured human keratinocytes. Additionally, both COX-2 and AP-1 seem to play a functional role in UVB-induced epidermal skin tumor formation. In this study, we determined in mouse epidermis the role of UVB-induced p38 MAPK and PI3K signaling in the induction of AP-1 activation and COX-2 expression.

**Results**

**UVB Induces p38 MAPK and AKT Signaling Pathways In vivo**

Mice were exposed to a single dose of UVB (6 kJ/m2) and dorsal epidermal protein was isolated at various time points following irradiation. Four mice were used for each time point. Increases in p38 phosphorylation were detected using an antibody recognizing the activating sites of phosphorylation of p38, Tyr180 and Thr182. Blots were stripped and reprobed with an antibody specific for total levels of p38 MAPK. Densitometric analysis was done and is shown as levels of phosphorylated p38 MAPK normalized to total p38 MAPK. The average of the relative densitometric units from Western blots probed with phosphospecific normalized to total p38 MAPK is shown in Fig. 1A. A statistically significant increase in normalized phosphorylated p38 MAPK levels was observed at 0.25, 0.5, and 1 hour postirradiation (Fig. 1A). Increases in p38 MAPK phosphorylation can be observed in mice (labeled individually as A-D) at 0.25 and 0.5 hour (Fig. 1B). UVB irradiation did not affect total levels of p38 MAPK. Afaq et al. showed that UVB induced p38 MAPK phosphorylation with multiple doses, however we are able to show that a single acute dose is sufficient for p38 activation (23). To confirm that increases in observed p38 phosphorylation induced by UVB also caused increases in p38 activity, we looked downstream at MAPK activated protein kinase-2 (MAPKAPK-2) phosphorylation. Western analysis was done for MAPKAPK-2 phosphorylation at Thr422 as well as total levels of MAPKAPK-2. Using densitometric analysis, a statistically significant increase in MAPKAPK-2 phosphorylation was detected at 0.25, 0.5, 2, and 12 hours (Fig. 2A). The pattern of MAPKAPK-2 phosphorylation follows UVB-induced activation of p38 MAPK, albeit prolonged. In probing for total levels of MAPKAPK-2, a shift in migration can be observed consistent with the pattern of MAPKAPK-2 phosphorylation. Our laboratory has shown previously that the retardation in migration in MAPKAPK-2 is due to increases in phosphorylation (16).

We observed a statistically significant increase in AKT phosphorylation following UVB (6 kJ/m2) at 0.5 and 1 hour postirradiation (Fig. 3A). Blots were stripped and reprobed with total AKT for densitometric analysis, with no changes in total AKT observed with UVB irradiation. Western blots showing increases in AKT phosphorylation are shown in Fig. 3B at 0.5 and 1 hour postirradiation. GSK-3β is phosphorylated at Ser9 by activated AKT, thereby inactivating its activity. This inhibition of activity prevents the phosphorylation of cyclic AMP–responsive element binding protein (CREB) at Ser133, an inactivating site of phosphorylation. We therefore sought to determine if AKT induced by UVB also activated GSK-3β in vivo. An increase in phosphorylation of GSK-3β at Ser9 was observed following UVB irradiation at 0.25, 0.5, and 1 hour postirradiation (Fig. 4A and B). p38 MAPK signaling and PI3K signaling work cooperatively to increase the activity of CREB. Increases in CREB activity were observed 0.25, 0.5, and 1 hour postirradiation at Ser133, an activating site of phosphorylation induced by p38 signaling (Fig. 5A and B).

**UVB Induces COX-2 and c-Fos Expression**

We analyzed the expression of c-Fos (Fig. 6A and B) and COX-2 (Fig. 7A and B) from 0 to 48 hours postirradiation following a single dose of UVB (6 kJ/m2). Basal levels of c-Fos were detected at 0 hour with marginally significant increases at 0.25 and 24 hours postirradiation (P = 0.10). As expected, low basal expression of COX-2 was detected at 0 hour, but significant increases were apparent at 12 hours postirradiation with maximal induction detected at 48 hours postirradiation. As observed previously in vitro (16, 24), UVB increased the expression of c-Fos and COX-2 in SKH-1 hairless mice.

**Topical Treatment with SB202190 Inhibits p38 Activity**

SB202190 [4-(4-fluorophenyl)-2-(4-hydroxyphenyl)-5-(4-pyridyl)1H-imidazole] is a reversible inhibitor of two isoforms of p38 (p38α and p38β1). SB202190 acts by binding to the ATP binding pocket of the kinase, preventing phosphorylation of p38 MAPK and PI3K Regulate AP-1 and COX-2 In vivo

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downstream targets. Following pretreatment with 5 μmol SB202190 or acetone for 1 hour, mice were UVB irradiated (6 kJ/m²). Immediately following irradiation, SB202190 or acetone was reapplied. We evaluated inhibition of p38 activity by looking at inhibition of its downstream target, MAPKAPK-2 (Fig. 8A). At 6 hours postirradiation, MAPKAPK-2 phosphorylation was again observed in all acetone-treated mice as seen in Fig. 2. A significant decrease in the phosphorylation of MAPKAPK-2 (Thr²²²) was observed in mice treated with SB202190, confirming that topical treatment was effective in inhibition of p38 activity. Blots were stripped and reprobed with total MAPKAPK-2 and β-actin to serve as loading controls. A shift in the migration of total MAPKAPK-2 was observed (as in Fig. 2), indicative of increased phosphorylation in UVB-irradiated mice as reported in previously published work (16).

**FIGURE 1.** Activation of p38 MAPK by UVB in SKH-1 mouse epidermis. Mice were UVB irradiated (6 kJ/m²) and epidermal protein was harvested at various time points postirradiation. Four mice were treated per time point. Protein was resolved by SDS-PAGE and Western analysis was done. Blots were probed with phosphospecific antibodies for p38 MAPK and total p38 MAPK. Densitometric analysis was done normalizing phosphorylated p38 MAPK to total levels of p38 MAPK. Average of densitometric analysis for four mice per time point ± SE (A). *, P < 0.05, Student’s t test. UVB-induced activation of p38 MAPK in four individual mice (A-D) along with total levels of p38 MAPK (B).

**FIGURE 2.** UVB-induced MAPKAPK-2 phosphorylation in SKH-1 mouse epidermis. Four mice per group were UVB irradiated (6 kJ/m²) and collected at various time points postirradiation. Proteins were resolved by SDS-PAGE and Western analysis was done for phosphorylated MAPKAPK-2 (Thr²²²). Blots were stripped and reprobed for total MAPKAPK-2. Densitometric analysis was done for each group normalizing phosphorylated MAPKAPK-2 levels to total MAPKAPK-2. Average ± SE (A). Statistically significant increases in MAPKAPK-2 phosphorylation were observed at 0.25, 0.5 (B), 2, and 12 hours postirradiation. *, P < 0.05, Student’s t test.

**Topical Treatment with LY294002 Inhibits AKT Activation**

LY294002 [2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one] is a reversible inhibitor of PI3K that binds to the ATP binding pocket, preventing activation of downstream kinases. As with SB202190, mice were pretreated for 1 hour before irradiation with LY294002 (5 μmol) or acetone (vehicle control). LY294002 or acetone was reapplied immediately following UVB irradiation. Epidermal protein was harvested and evaluated for AKT phosphorylation, a downstream target of PI3K. UVB induced activation of AKT (Ser⁴⁷³) at 1 hour postirradiation in mice treated with vehicle as seen in Fig. 2A. All three mice treated with LY294002 had significant decreases in AKT phosphorylation compared with vehicle controls (Fig. 8B). UVB irradiation with LY294002 or acetone did not affect total levels of AKT.
SB202190 and LY294002 Inhibit AP-1 Activation and COX-2 Expression

AP-1 luciferase SKH-1 transgenic reporter mice were used to assess the effects of p38 MAPK inhibition and PI3K inhibition in UVB-irradiated mice. Mice were pretreated with SB202190 (5 μmol), LY294002 (5 μmol), or acetone for 1 hour before irradiation. Mice were also treated with drug or acetone following UVB irradiation. A total of 10 AP-1 luciferase reporter mice were used for each treatment group. Samples were collected before irradiation to serve as controls for individual mice. At 48 hours postirradiation, samples were collected and assayed for luciferase activity. Treatment with SB202190 showed an 84% reduction in luciferase activity compared with AP-1 luciferase activity in UVB-irradiated mice treated with acetone (\(P < 0.0001\); Fig. 9A). LY294002 also showed a significant reduction in UVB-induced AP-1 activation (\(P < 0.003\), 68% less than UVB-irradiated mice (Fig. 9B). AP-1 basal activity was not significantly altered at 0 and 48 hours postirradiation.

We decided to assess the effects of SB202190 and LY294002 on COX-2 expression at 48 hours postirradiation, as this was determined to be the point at which a significant increase was observed previously (Fig. 7). Mice were treated as described above with SB202190, LY294002, or acetone 1 hour before irradiation and immediately following UVB. Basal COX-2 expression was not detected in nonirradiated mice with or without drug treatment. Blots were stripped and reprobed with β-actin as a loading control. Inhibitory effects of SB202190 and LY294002 on UVB-induced COX-2 expression were also observed at 48 hours postirradiation (Fig. 9C).
Discussion

UVB (280-320 nm) is clearly established as a major etiologic factor in the development of NMSC (25). The development of UVB-induced skin tumors occurs as a result of changes at both genetic and epigenetic levels. UVB-induced DNA damage is a critical event in skin tumor initiation (26, 27), whereas skin tumor promotion and progression are dependent on alterations in gene expression due to epigenetic events (8, 28).

Increases in AP-1 and COX-2 have been clearly associated with skin tumor development primarily with chemical carcinogenesis protocols but more recently with studies involving UVB irradiation. Young et al. showed previously that epidermal expression of a dominant-negative c-jun (TAM67) decreased papilloma induction in a two-stage chemical carcinogenesis protocol using 7,12-dimethylbenz(a)anthracene as an initiator and 12-O-tetradecanoylphorbol-13-acetate as the promoting agent (29). Significant inhibition of okadaic acid–promoted tumor formation was also observed using TAM67 transgenic mice (30). Our laboratory has extended these observations in a model more relevant for UVB-induced human skin cancer by showing that decreasing AP-1 activation in TAM67 transgenic

![Graph A](image1.png)  
**FIGURE 5.** UVB-induced phosphorylation of CREB in SKH-1 mouse epidermis. Four SKH-1 were UVB irradiated (6 kJ/m²) and epidermal protein was harvested from 0 to 48 hours. Proteins were resolved by SDS-PAGE. Western analysis was done using phosphospecific antibodies for CREB (Ser133) and total CREB. Densitometric analysis of phosphorylated CREB normalized to total CREB revealed a statistically significant increase at 0.25 and 0.5 hours post-UVB irradiation. *, P < 0.05, Student’s t test.

![Graph B](image2.png)  
**FIGURE 6.** UVB-induced expression of c-Fos in SKH-1 mouse epidermis. SKH-1 cells were UVB irradiated and epidermal protein was resolved by SDS-PAGE. Blots were probed with c-Fos, and four mice were UVB irradiated per time point. Blots were stripped and reprobed for β-actin to serve as a loading control. Densitometric analysis of c-Fos normalized to β-actin revealed a marginally significant increase at 0.25 and 24 hours post-UVB irradiation. *, P = 0.10, Student’s t test.
mice also significantly inhibits UVB-induced SCC development (10). Two-stage chemical carcinogenesis studies using COX knockout mice have also implicated COX-2 metabolism in skin tumorigenesis. Deficiency of either COX-1 or COX-2 reduced skin tumor formation in these mice by 75% (31). Additionally, the use of celecoxib (a selective COX-2 inhibitor) or indomethacin (a nonspecific nonsteroidal anti-inflammatory drug) blocked UVB-induced skin tumor development in SKH-1 mice. We also confirmed previous findings that a single acute dose of UVB significantly induces COX-2 expression in mouse skin (12). More importantly, we provide evidence that p38 MAPK and PI3K play an important role in increasing both AP-1 and COX-2.

FIGURE 7. UVB-induced expression of COX-2 in SKH-1 mouse epidermis. SKH-1 cells were UVB irradiated and epidermal protein was resolved by SDS-PAGE. Blots were probed with COX-2. Four mice were UVB irradiated per time point. Blots were stripped and reprobed with β-actin to serve as a loading control. Densitometric analysis of COX-2 normalized to β-actin revealed a statistically significant increase at 6, 12, 24, and 48 hours post-UVB irradiation. *, P < 0.05, Student’s t test.

FIGURE 8. Effects of SB202190 and LY294002 on AKT and MAPKAPK-2 phosphorylation, respectively. A. Mice were pretreated for 1 hour with SB202190 (5 μmol) and UVB irradiated (6 kJ/m²). SB202190 was reapplied immediately following UVB exposure. Inhibition of p38 activity with topical treatment of SB202190 was observed by Western analysis of MAPKAPK-2 phosphorylation at Thr222. Epidermal protein was isolated at 6 hours postirradiation and Western analysis was done. Blots were stripped and reprobed with total MAPKAPK-2 and β-actin to serve as a loading control. B. Mice were treated for 1 hour before irradiation with LY294002 (5 μmol). Mice were UVB irradiated (6 kJ/m²), LY294002 was reapplied, and epidermal protein was harvested at 1 hour. To ensure that topical treatment with LY294002 inhibited PI3K activity, AKT phosphorylation was examined. Epidermal protein isolated at 1 hour postirradiation (maximal AKT induction) was resolved by SDS-PAGE and Western analysis was done with a phosphospecific antibody for AKT (Ser473). Blots were stripped and reprobed with total AKT to ensure that UVB had no effect on total levels of AKT and as a loading control.
and COX-2 promoter activity

UVB-induced AP-1 activation and COX-2 expression. To determine the effects of SB202190 and LY294002 on AP-1 activation, AP-1 luciferase reporter mice were pretreated with SB202190 (A), LY294002 (B), or acetone before and immediately following UVB irradiation. Ears were pre-punched to serve as a control for each group. Mice were UVB irradiated (6 kJ/m²) and ear punch biopsies were taken at 48 hours postirradiation. Luciferase activity was determined and normalized to total protein. Significant inhibition of UVB-induced AP-1 activation was observed with SB202190 (A; \( P < 0.0001 \)) and LY294002 (B; \( P < 0.003 \)) treatment. C. SKH-1 mice were pretreated for 1 hour before UVB irradiation (6 kJ/m²) with SB202190, LY294002, or acetone (vehicle control). Drug or vehicle was immediately reapplied following UVB exposure. Epidermal protein was isolated and pooled as described in Materials and Methods and resolved by SDS-PAGE. COX-2 expression was analyzed by Western analysis. Blots were stripped and reprobed with \( \beta \)-actin as a loading control.

SB202190 and LY294002 were used in these studies to implicate p38 MAPK and PI3K in UVB-induced AP-1 activation and COX-2 expression in vitro. Our laboratory has reported previously that UVB mediates increases in AP-1 activation and COX-2 through p38 MAPK and PI3K in the human keratinocyte cell line, HaCaT (16, 32-34). Therefore, we wanted to extend these studies into an in vivo system to specifically validate p38 MAPK and PI3K as potential molecular targets for the chemoprevention of NMSC. Both p38 MAPK and PI3K signaling events are involved in UVB-induced c-fos expression (16, 33). The use of a dominant-negative AKT or wild-type GSK-3\( \beta \) inhibited UVB-induced c-fos and COX-2 promoter activity in vitro (34, 35). Further analysis revealed that UVB induced the binding of CREB to the cyclic AMP response element and c-fos AP-1 site cis elements within the c-fos promoter (35). Similar in vitro studies with the COX-2 promoter revealed that CREB and activating transcription factor-1 were present on the COX-2 promoter in UVB-irradiated HaCaT cells (36). SB202190 decreased UVB-induced CREB/activating transcription factor-1 phosphorylation and COX-2 promoter activity in vitro. LY294002 also decreased UVB-induced COX-2 promoter activity.

p38 MAPK signaling results in the phosphorylation of CREB at Ser\(^{133} \), whereas activation of the PI3K pathway results in dephosphorylation at Ser\(^{129} \). Phosphorylation at Ser\(^{133} \) is an activating site of phosphorylation enhancing its ability to bind to cis promoter elements, whereas phosphorylation at Ser\(^{129} \) is inhibitory. Activation of the PI3K pathway serves to reduce phosphorylation at this inhibitory site of phosphorylation, allowing it to interact with CREB binding protein, a coactivator that regulates transcription through its interaction with basal transcription machinery (37). PI3K activation results in the phosphorylation of AKT and subsequently GSK-3\( \beta \) (Ser\(^{9} \)), inactivating its ability to phosphorylate CREB at Ser\(^{129} \) (34). We observed both phosphorylation of AKT (Ser\(^{177} \)) and GSK-3\( \beta \) (Ser\(^{9} \)) in UVB-irradiated SKH-1 mice, again confirming previously generated in vitro observations. Phosphorylated CREB binds to the c-fos AP-1 site and cyclic AMP response element of c-fos and the cyclic AMP response element of COX-2 in UVB-irradiated HaCaT cells (and not in mock-irradiated controls). In this study, we are able to show that UVB increases the activity of p38 MAPK and PI3K as observed through increases in their downstream targets, MAPKAPK-2 and AKT, respectively. UVB-induced activation of these pathways results in an increase in CREB phosphorylation at Ser\(^{133} \). Based on our findings in vitro, this increase in CREB may play a role in the observed increase in AP-1 activation and COX-2 expression.

We observed increases in CREB phosphorylation at 0.25, 0.5, and 1 hour post-UVB irradiation. Although UVB-induced increases in COX-2 protein were observed as early as 6 hours postirradiation, maximal UVB-induced expression of COX-2 and c-Fos was not observed until 48 hours postirradiation. CREB binding protein/p300 acts as a transcriptional adaptor, bridging phosphorylated CREB and basal transcriptional machinery, such as transcription factor IID, transcription factor IIB, and the RNA polymerase II holoenzyme (38, 39). These interactions are critical events, allowing efficient transcription to occur. As p300 contains a histone acetyltransferase domain, chromatin remodeling may be another component that enhances UVB-induced COX-2 and c-Fos expression (38, 39).

Although treatment with SB202190 and LY294002 inhibits UVB-induced AP-1 activation and COX-2 expression, long-term UVB carcinogenesis studies need to be conducted to address their efficacy in decreasing tumor incidence. In addition, the effects of inhibiting p38 MAPK and PI3K in the epidermis require further study. In a recent report by Hildesheim et al., C57BL/6J mice treated with SB202190 exhibited reduced apoptosis, inflammation, and hyperproliferation in response to UVR (40). As p38 MAPK may mediate...
effects on p53 activation and subsequent activation of DNA damage response genes, such as GADD45, inhibiting p38 MAPK activity may hinder the removal of damaged keratinocytes from the epidermis through increases in apoptosis (41). Effects on the G2-M checkpoint must also be given consideration, as inhibition of p38 MAPK has been shown to decrease Ser\textsuperscript{109} phosphorylation of Cdc25B, reducing its interaction with 14-3-3 proteins and decreasing progression through the checkpoint (42). Long-term effects of PI3K inhibitors in vivo on these downstream events in UVB-irradiated epidermis also require further study. PI3K is however well established to play a role in cell survival in response to various stimuli through phosphorylation of Bad, forkhead homologue in rhabdomyosarcoma, mammalian target of rapamycin, and nuclear factor-κ-B (43). Thus, the use of PI3K inhibitors may prove beneficial by inhibiting the protection from apoptosis normally conferred by activation of this pathway. Several studies have suggested that inhibition of UVB-induced PI3K increases apoptosis in cultured cells and in mouse epidermis (44–46). The studies presented herein examine the effects of SB202190 and LY294002 on UVB-treated SKH-1 mice using a single acute dose. Using a UVB carcinogenesis protocol, the long-term effects of these inhibitors on apoptosis, inflammation, and cell cycle will be addressed at a later time.

In the study presented here, a one-time acute UVB dose was used to validate p38 MAPK and PI3K signaling and downstream AP-1 activation and COX-2 expression. Previously, several groups have shown that multiple doses of UVB also result in increased AP-1 activation as well as COX-2 expression. Our laboratory has reported recently that AP-1 and COX-2 expression, upstream signaling events are thought to lead to downstream signaling and the activation of p38 and PI3K activation in UVB-induced AP-1 and COX-2 expression will be pursued in future studies.

In conclusion, topical application of SB202190 and LY294002 resulted in a significant decrease in p38 MAPK and PI3K activity and subsequent AP-1 activation and COX-2 expression. Because AP-1 and COX-2 have been clearly established to play a role in UVB-induced skin tumorigenesis, these findings are particularly significant, as pharmacologic inhibitors of p38 and PI3K may be effective in preventing the development of NMSC.

Materials and Methods

Breeding of AP-1 Luciferase Reporter Mice

AP-1 luciferase mice were obtained from Dr. Nancy Colburn (National Cancer Institute, Frederick, MD) on a DBA/2 background. These mice were subsequently bred onto a SKH-1 genetic background with mice obtained from Charles River Laboratories (Kingston, NY). Mice carrying the AP-1 luciferase transgene (after generation N5) were identified by PCR analysis of tail DNA. Luciferase primers used for identification were 5’-GCGGAATACTTCCGAAAATGTCC-3’ and 5’-CCTTAGG-TAACCCAGTAGTCC-3’ (29).

Analysis of AP-1 Luciferase Activity

Three skin punches (1.5 mm) were obtained from the right ear before irradiation for each mouse to serve as a control. These samples were snap frozen in liquid nitrogen and stored at −80°C until the luciferase assay was done. Three skin punches (1.5 mm) were obtained from the left ear at 48 hours postirradiation and stored as described previously. In drug-treated mice, the right ear was treated with SB202190, LY294002, or acetone for 1 hour before irradiation and immediately following irradiation to assess the effects of drug or vehicle on UVB-induced AP-1 luciferase activity. A BCA protein assay (Pierce, Rockford, IL) was done for protein quantification and luciferase activity was determined using the TD-20/20 luminometer (Turner Designs, Sunnyvale, CA).

UVB Irradiation and Treatment with Pharmacologic Inhibitors

Westinghouse FS-40 UVB lamps were purchased from National Biological Corporation (Twinsburg, OH) and used for
irradiation of mice. Fluence was determined using a UVX radiometer (Ultraviolet Products, San Gabriel, CA). SB202190 and LY294002 (LC Laboratories, Woburn, MA) were dissolved in acetonitrile and used at 5 μmol in these experiments. The inhibitors were reconstituted immediately before use and applied in a volume of 200 μL to the dorsal skin for 1 hour before irradiation. Control mice were treated with acetonitrile to serve as a vehicle control. Mice were treated with corresponding drugs postirradiation and samples were collected at various times.

Preparation of Epidermal Protein and Western Analysis

At various time points following UVB irradiation, mice were sacrificed. The dorsal skin was removed and snap frozen in liquid nitrogen and stored at −80°C. The epidermis was removed by scraping on dry ice. Histologic analysis determined that only epidermal layers were removed by this method. The samples were immediately placed in lysis buffer [1% Triton X-100, 10% glycerol, 137 mmol/L NaCl, 20 mmol/L Tris-HCl (pH 7.5), 1 μg/mL aprotinin, 1 μg/mL leupeptin, 1 mmol/L phenylmethylsulfonyl fluoride, 20 mmol/L NaF, 1 mmol/L disodium pyrophosphate, and 1 mmol/L Na3VO4] as described previously. Protein was determined using Bio-Rad DC Protein Assay (Hercules, CA) and resolved on 12.5% SDS-PAGE gels. Following transfer to polyvinylidene difluoride membranes, blots were blocked in 5% milk in TBS-Tween 20 for 1 hour. Antibodies for phosphospecific proteins were incubated overnight at 4°C and the antibodies were used at room temperature for 2 hours. Antibodies for phospho-p38, total p38, phospho-MAPKAPK-2, total MAPKAPK-2, phospho-AKT, total AKT, phospho-GSK 3β, total GSK-3β, phospho-CREB, total CREB (Cell Signaling, Beverly, MA), COX-2 (Cayman Chemical, Ann Arbor, MI), and c-Fos (Santa Cruz Biotechnology, Santa Cruz, CA) were used at 1:1,000 and β-actin (Sigma, St. Louis, MO) was used at 1:5,000. The appropriate horseradish peroxidase–conjugated secondary was incubated for 1 hour following washing with 3 × 10-minute wash with TBS-Tween 20. The membrane was again washed with TBS-Tween 20 following incubation with secondary and exposed to enhanced chemiluminescence (Amersham, Piscataway, NJ).

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