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Advances in Brief

Roles of Akt and Glycogen Synthase Kinase 3β in the Ultraviolet B Induction of Cyclooxygenase-2 Transcription in Human Keratinocytes[1]

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Abstract

Ultraviolet B (UVB)-induced cyclooxygenase-2 (COX-2) expression plays an important role in UVB tumor promotion. We examined whether Akt and glycogen synthase kinase 3β (GSK-3β), components of the phosphatidylinositol 3′-kinase pathway, are involved in UVB induction of COX-2 transcription. UVB caused Akt phosphorylation at both Thr-308 and Ser-473 that was inhibited by LY294002, a phosphatidylinositol 3′-kinase inhibitor. LY294002 also decreased the expression of endogenous COX-2 protein and a luciferase construct driven by COX-2 promoter. Similarly, UVB caused phosphorylation of GSK-3β (Ser-9) and presumably inactivation of GSK-3β. Inhibition of GSK-3β by lithium induced endogenous COX-2 protein expression and COX-2 promoter activity. Finally, overexpression of a dominant-negative Akt mutant or wild-type GSK-3β suppressed UVB-mediated induction of COX-2 promoter. These studies suggest that inactivation of GSK-3β through activation of Akt plays an important role in the UVB induction of COX-2 transcription.

Introduction

UVB[3] irradiation from sun exposure is thought to be the major cause of human nonmelanoma skin carcinoma (1). One possible mechanism for UVB-induced carcinogenesis involves its ability to induce COX-2 expression. UVB has been found to induce COX-2 expression in human skin (2), and oral administration of a selective COX-2 inhibitor, celecoxib, produces a decrease in the tumor number and multiplicity in UV-treated hairless mice (3). There is growing evidence suggesting a role of COX-2 in tumorigenesis. Studies in rodents indicate that nonsteroidal anti-inflammatory drugs, which inhibit both COX-1 and COX-2, reduce carcinogen-induced intestinal cancer (4). Epidemiological studies also suggest that aspirin reduces the risk of colorectal (5), skin (6), and breast (7) cancer. Studies with COX-2 knockout mice have further demonstrated the role of COX-2 in intestinal and skin cancers. Deficiency in COX-2 reduces the number of intestinal polyps in the Min mouse and decreases the number of papillomas in a mouse skin initiation/promotion model (8, 9). Consistent with this proposal, COX-2 was up-regulated in human and rodent intestinal tumors (10) and in rodent skin papillomas (8), and most tumor-promoting agents also induce COX-2 expression (2, 11).

UVB activates a variety of signaling pathways, particularly MAP kinases that include p38, JNK, ERK1/2 (12). All of them have been implicated in the induction of COX-2, depending on cell types and stimuli (13, 14). Our previous work with human keratinocytes indicated that UVB induced COX-2 expression at both mRNA and protein levels, and p38 MAP kinases played a major role in this process.[4] Recently, it was reported that inactivation of GSK-3β by activation of the Wnt pathway induces COX-2 expression (15). Because UVB has been reported to activate the PI 3-kinase pathway (16), which also leads to inactivation of GSK-3β, it is possible that this pathway plays a role in the COX-2 transcription. In addition, GSK-3β has been shown to negatively regulate transcriptional factors including c-Jun (17), NF-AT (18), and CREB (19), all of which have been implicated in the induction of COX-2 expression in different cell types in response to various stimuli (14, 20). Therefore, we examined whether UVB inactivates GSK-3β through activation of the PI 3-kinase pathway in a human keratinocyte cell line, HaCaT, and whether this pathway plays a role in UVB-induced COX-2 transcription.

Materials and Methods

Plasmids. The luciferase construct, PhPES2(−327/+59), containing the wild-type COX-2 promoter fragment, was prepared and cloned into the pGL2 basic vector as described (21). Dominant-negative Akt mutant Akt AAA and wild-type GSK-3β plasmids were gifts from Dr. Woodgett (Ontario Cancer Institute, Toronto, Ontario, Canada).

Cells and Transfections. HaCaT cells were cultured in DMEM supplemented with 10% fetal bovine serum and penicillin/streptomycin. Transient transfections were performed using Lipofectamine Plus reagent (Life Technologies, Inc., Rockville, MD) according to the manufacturer's protocol. Briefly, cells were plated in six-well plates the day before transfection and grown to ~90% confluence. One to 2 μg of plasmid DNA/well were transfected. Transfections were allowed to proceed for 4–6 h, and cells were washed twice and allowed to recover for ~18 h in DMEM before UVB or drug treatment. Transfection efficiency was corrected by cotransfection of *Renilla* luciferase plasmid (Promega Corp., Madison, WI). The ratio between the reporter plasmid and *Renilla* luciferase plasmid was 20:1 for COX-2 reporters.

UVB Treatment. Cells were grown to 90% confluence and then serum starved for 24 h. Cells were then washed with PBS and exposed to UVB radiation. A bank of two SF20 UVB lamps (National Biological Corporation, Twinsburg, OH), providing a peak emission of 313 nm, was used. Control cells were mock irradiated. For drug treatment, drugs were added 1 h before UVB irradiation and continued after UVB irradiation until harvest.

Luciferase Assay. After experimental treatments, cells were washed two times with cold PBS, lysed in a passive lysis buffer provided in the dual luciferase kit (Promega), and assayed for luciferase activity according to the manufacturer's protocol. The data were presented as a ratio between firefly and *Renilla* luciferase activities. The luciferase activity of cells with stable transfection of COX-2 reporters was assayed using a regular luciferase kit (Promega).

Western Analysis. Cells were lysed in a buffer containing 20 mM Tris-Cl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium PP_i, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 10 mM NaF, 50 μg/ml aprotinin, 50 μg/ml leupeptin, and centrifuged at 14,000 rpm for 10 min. Protein concentration in the supernatant was determined by Bio-Rad D_C reagent (Bio-Rad Laboratories, Hercules, CA). For Western analysis, 30–40

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[3] The abbreviations used are: UVB, ultraviolet B; COX, cyclooxygenase; GSK-3β, glycogen synthase kinase 3β; PI 3-kinase, phosphatidylinositol 3′-kinase; CRE, cyclic AMP-responsive element; CREB, CRE binding protein; MAP, mitogen-activated protein; ERK, extracellular signal-regulated kinase.

[4] Chen *et al.*, submitted for publication.

μg of protein were resolved on a 10% of SDS-polyacrylamide gel. The protein was transferred to a polyvinylidene difluoride membrane by electroblotting. The membrane was then blocked in 5% nonfat dry milk TBST [10 mM Tris (pH 8.0), 150 mM NaCl, and 0.05% Tween 20] at room temperature for 1 h. Primary antibody was diluted at 1:1000 in 5% BSA/TBST for antibodies against phospho-Akts, Akt, and phospho-GSK-3β (New England Biolabs, Inc., Beverly, MA) or in 5% nonfat dry milk/TBST at 1:4000 for anti-COX-2 antibody and 1:2000 for anti-α-tubulin (Santa Cruz Biotechnology, Santa Cruz, CA). The membrane was incubated at room temperature for 2–3 h and washed three times for 5 min each with TBST. The membrane was then incubated with corresponding horseradish peroxidase-conjugated secondary antibodies in 5% nonfat dry milk/TBST at room temperature for 1 h and washed three times for 5 min each with TBST. Antigen-antibody complexes were detected with LumiGlo reagent (New England Biolabs, Inc.).

Results

UVB Induced the Phosphorylation of Akt at Ser-473 and Thr-308 That Was Dependent on the Activity of PI 3-Kinase. To study whether UVB activates Akt, we studied the effect of UVB treatment on Akt phosphorylation. Phosphorylation of Akt at Ser-473 and Thr-308 is required for full activation of Akt (22). UVB at a dose of 250 J/m^2 rapidly induced Akt phosphorylation at both Ser-473 and Thr-308 as early as 1 h, persisting for up 16 h after irradiation. The increase in phospho forms of Akt was not attributable to a change in the level of total Akt, which was not affected by UVB treatment (Fig. 1*A*). Although it is not certain which enzyme is responsible for Akt phosphorylation at Ser-473, Akt phosphorylation at Thr-308 is dependent on the activity of PDK, which in turn is activated by the product of PI 3-kinase phosphatidylinositol 3,4,5-trisphosphate (22). We found that UVB-induced Akt phosphorylation at Thr-308 was dependent on PI 3-kinase, because LY294002, an inhibitor of PI 3-kinase, decreased Akt phosphorylation at this site. Similarly, Akt phosphorylation at Ser-473 was also inhibited by LY294002 (Fig. 1*B*). These results indicated that UVB-induced Akt phosphorylation at both Thr-308 and Ser-473 sites depended on PI 3-kinase activity.

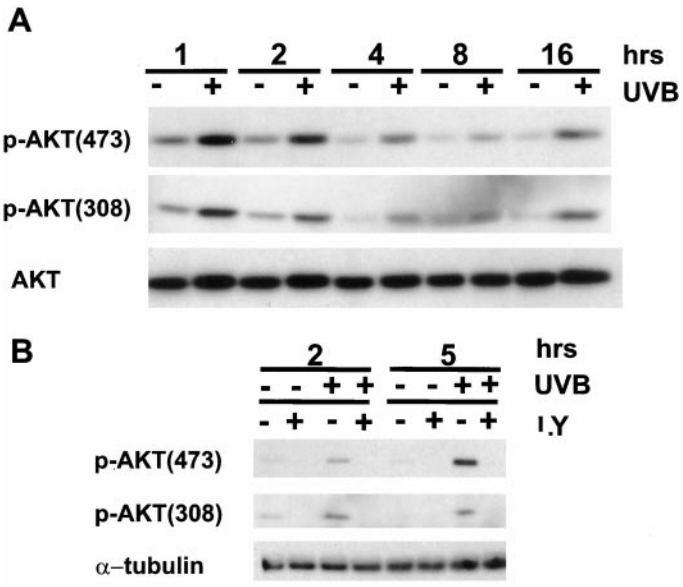

Fig. 1. UVB induces Akt phosphorylation that is dependent on PI 3-kinase. *A*, time course for UVB-induced Akt phosphorylation at Thr-308 or Ser-473. Forty μg of whole cell lysate were resolved on 10% polyacrylamide gel and probed with an antibody recognizing phospho-Akt [Ser-473; *p-Akt(473)*] and phospho-Akt [Thr-308; *p-Akt(308)*]. The same blot was then reprobed with an antibody for total Akt. *B*, effect of PI 3-kinase inhibitor LY294002 (*LY*) on Akt phosphorylation. Cells were pretreated with 40 μM LY294002 in serum-free medium for 0.5 h and then irradiated with 250 J/m^2 UVB or mock treated. After UVB treatment, cells were again cultured in serum-free medium containing inhibitors and harvested at the indicated time points.

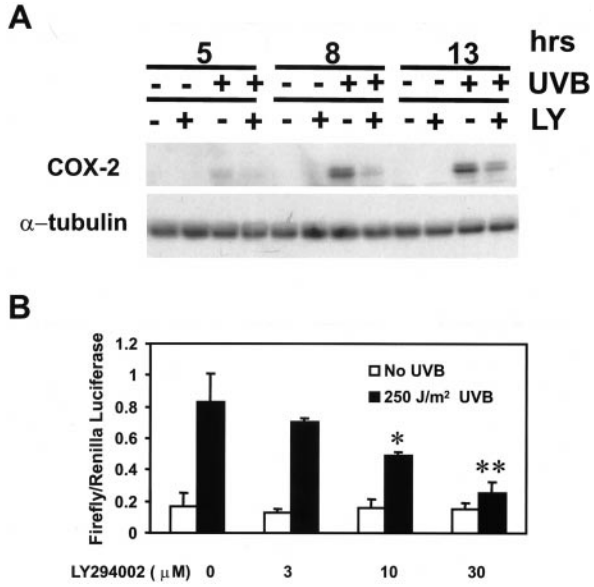

Fig. 2. PI 3-kinase inhibitor LY294002 inhibits UVB induction of COX-2 protein and COX-2 promoter activity. *A*, effect of LY294002 (*LY*) on UVB induction of COX-2 protein. Cells were treated with LY294002 and processed for Western blotting as described in Fig. 1. Proteins were detected with an antibody against COX-2 or α-tubulin. *B*, effect of LY294002 on the UVB induction of COX-2 promoter activity. HaCaT cells were transfected with 0.8 μg of phPES2(−327/+59) and 40 ng of pRL-TK and allowed to recover for 18 h. Cells were pretreated with 40 μM LY294002 in serum-free medium for 0.5 h and then irradiated with 250 J/m^2 UVB or mock treated. After UVB treatment, cells were again cultured in serum-free medium containing inhibitors and harvested 12 h after UVB or mock treatment. Shown are representative results of three experiments. *Columns*, means; *bars*, SD; $n = 3$. *, $P < 0.05$ and **, $P < 0.01$, compared with the corresponding no-drug group, respectively.

Inhibition of Akt Phosphorylation with a PI 3-Kinase Inhibitor Suppressed UVB-induced COX-2 Protein Expression and COX-2 Promoter Activity. After showing that Akt was activated through phosphorylation in HaCaT cells by UVB, we looked at whether this pathway plays a role in the induction of COX-2 expression by UVB. COX-2 protein expression was undetectable in mock treated control cells, and UVB at a dose of 250 J/m^2 induced COX-2 expression at 8 and 13 h after UVB treatment. Treatment with 40 μM LY294002 significantly, but not completely, decreased the UVB induction of COX-2 protein expression (Fig. 2*A*). Next, we studied whether inhibition of this pathway also suppresses the COX-2 promoter activity. phPES2(−327/+59), a construct that contains a COX-2 promoter sequence, was induced by UVB at a dose of 250 J/m^2. This induction was decreased by LY294002 in a dose-dependent manner, with nearly complete inhibition at 30 μM (Fig. 2*B*). Treatment with 40 μM LY294002 completely abolished induction of COX-2 promoter by UVB (data not shown). These data suggest that PI 3-kinase-dependent Akt activation may play a role in the UVB induction of COX-2 through transcriptional activation.

UVB Induced GSK3β Phosphorylation at Ser-9 and Inhibition of GSK-3β by Lithium-induced COX-2 Protein Expression and Promoter Activity. One of the major targets of Akt is GSK3β, which is inactivated upon phosphorylation at Ser-9 by Akt. In HaCaT cells, some GSK-3β exists in the phosphorylated form, which was increased upon UVB treatment. The increase was evident at 2 h and peaked around 8 h. Phosphorylation of GSK-3β was also partially dependent on PI 3-kinase activity because it was inhibited by LY294002, especially at later time points (Fig. 3*A*).

To further establish a causal relationship between the activation of the PI 3-kinase pathway and induction of the COX-2 expression, we investigated the effect of lithium, an inhibitor of GSK-3β, on COX-2 protein expression and COX-2 promoter activity. Treatment with 30

mM LiCl for 12 h alone produced an increase in COX-2 protein expression, although the induction appeared to be much smaller than that induced by UVB at a dose of 250 J/m^2. In contrast, treatment with the same concentrations of sodium did not induce COX-2 protein expression (Fig. 3*B*). Similarly, 30 mM lithium also induced luciferase expression driven by a COX-2 promoter sequence (Fig. 3*C*). The induction by lithium was comparable with that induced by UVB. Thus, inactivation of GSK-3β alone can induce the expression of COX-2, probably through transcriptional activation.

Overexpression of a Dominant-Negative Akt Mutant or Wild-Type GSK-3β Decreased the UVB Induction of the COX-2 Promoter Activity. To further confirm the role of Akt and GSK-3β in COX-2 induction by UVB, we made use of a dominant-negative Akt mutant (Akt AAA) and wild-type GSK-3β to examine the effect of inhibiting this pathway on the COX-2 promoter activity. PhPES2(−327/+59) was cotransfected into HaCaT cells with various amounts of Akt AAA, or wild-type GSK-3β, or empty vector. Both Akt AAA and GSK-3β dose-dependently decreased UVB transcriptional activation of the COX-2 promoter, with Akt AAA appearing to be more efficient in blocking the UVB induction of COX-2 transcription (Fig. 4). Therefore, the signaling pathway that involves activation of Akt and subsequent inactivation of GSK-3β appears to be essential for the transcriptional activation of the *COX-2* gene.

Discussion

Our previous work with the human keratinocyte cell line, HaCaT, indicates that UVB induces expression of COX-2 at both protein and mRNA levels.[4] By analyzing the regulation of the activity of the COX-2 promoter by UVB in HaCaT cells, we have also shown that

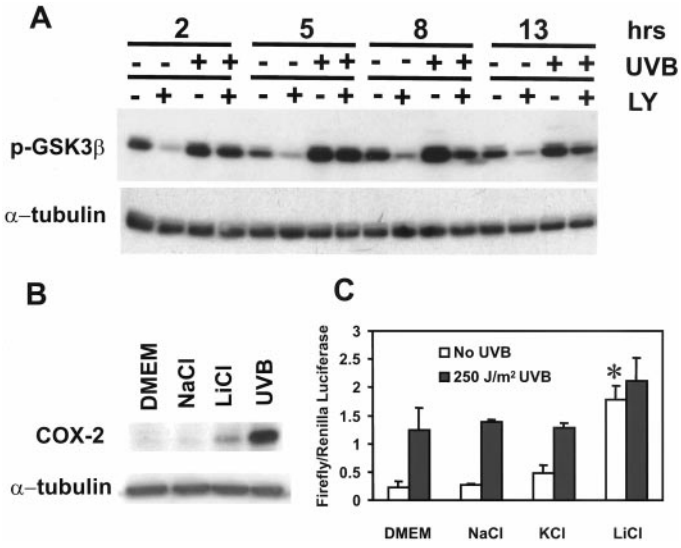

Fig. 3. UVB induces GSK-3β phosphorylation, and inhibition of GSK-3β by lithium induces COX-2 transcription. *A*, effects of UVB treatment and PI 3-kinase inhibitor LY294002 on phosphorylation of GSK-3β. Cells were treated with LY294002 and processed for Western blotting as described in Fig. 1. Proteins were detected with an antibody against phospho-GSK3β at Ser-9 (*p-GSK3*β) or α-tubulin. *B*, effect of lithium (*LiCl*) on endogenous COX-2 protein level. Cells were grown to near confluence and starved in serum-free medium for 24 h and then incubated with DMEM or 30 mM NaCl, LiCl, and harvested 12 h later. Forty μg of whole cell lysate were resolved on 10% polyacrylamide gel and probed with an antibody recognizing COX-2; the same blot was then probed with an antibody against α-tubulin. *C*, effect of lithium (*LiCl*) and UVB on the COX-2 promoter activity. HaCaT cells were transfected with 0.8 μg of phPES2(−327/+59) and 40 ng of pRL-TK and allowed to recover for 18 h. Cells were then irradiated with 250 J/m^2 UVB or mock treated. After UVB treatment, cells were cultured in serum-free medium (*DMEM*) or serum-free medium containing 30 mM *NaCl*, *KCl*, or *LiCl*, and harvested 12 h after UVB or mock treatment. Shown are representative results of three experiments. *Columns*, means; *bars*, SD; $n = 3$. $*$, $P < 0.01$, compared with the no UVB-treated DMEM group.

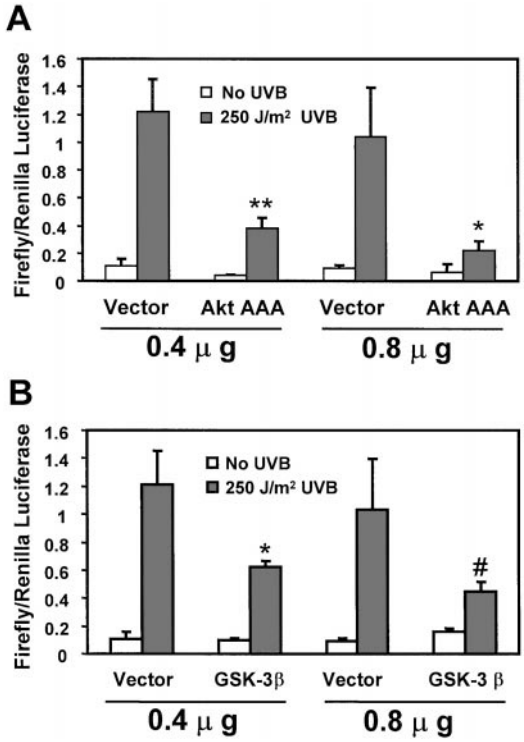

Fig. 4. Cotransfection of a dominant-negative Akt mutant (*A*) or wild-type GSK-3β (*B*) inhibits COX-2 promoter activity. HaCaT cells were transfected with 0.8 μg of COX-2 (−327/+59), 40 ng of pRL-TK, and a different amount of a dominant-negative Akt mutant (*Akt AAA*) plasmid, a wild-type GSK-3β (*GSK-3*β), or the corresponding vector. Cells were allowed to recover for 18 h and then irradiated with 250 J/m^2 of UVB or mock treated. Cells were harvested 12 h after UVB or mock treatment. Shown are representative results of three experiments. *Columns*, means; *bars*, SD; $n = 3$. $*$, $P < 0.05$, $**$, $P < 0.01$, and #, $P = 0.05$, compared with the group cotransfected with the corresponding amount of empty vector and irradiated with UVB, respectively.

one signaling pathway by which UVB induces COX-2 transcription involves the activation of p38 MAP kinases, phosphorylation of CREB/activating transcription factor-1, and activation of the CRE site in the COX-2 promoter.[5] However, inhibition of p38 MAP kinases does not completely inhibit the COX-2 promoter activity, suggesting that additional signaling pathways might also play a role in the UVB induction of COX-2 transcription. In the present study, we present evidence that two components of the PI 3-kinase pathway, Akt and GSK-3β, also play a role in the UVB-mediated induction of COX-2 transcription in the human keratinocytes. UVB treatment leads to Akt phosphorylation at both Thr-308 and Ser-473 sites, which is abolished by a PI 3-kinase inhibitor, LY294002. It has been shown that UVB activates PI 3-kinases in the human skin fibroblast (16). Therefore, the UVB-induced Akt phosphorylation may reflect the activation of PI 3-kinase in these cells. Alternatively, Akt phosphorylation is mediated through other signaling pathways, but a basal level of PI 3-kinase activity is required for Akt activation. In this regard, Normura *et al.*[6] have shown that suppression of p38 or ERK1/2 inhibits UVB induced Akt phosphorylation in JB6 mouse epidermal cells. We too have observed that UVB activates p38 MAP kinase (23), and SB202190, a p38 MAP kinase inhibitor, inhibits UVB-induced Akt phosphorylation at both Thr-308 and Ser-473 (data not shown). Therefore, in HaCaT cells, the activity of both PI 3-kinase and p38 kinases is required for UVB-induced Akt activation.

Akt activation plays an essential role in the COX-2 transcription induced by UVB. This conclusion is based on several observations:

[5] Tang *et al.*, submitted for publication.
[6] Nomura *et al.*, submitted for publication.

(*a*) inhibition of Akt phosphorylation using LY294002 completely suppresses the COX-2 promoter activity; (*b*) introduction of a dominant-negative Akt mutant also suppresses UVB-induced COX-2 promoter activity; and (*c*) LY294002 partially decreases the protein level of endogenous COX-2. Because UVB also actives p38 MAP kinases which have been shown to stabilize the COX-2 mRNA in a variety of types of cells (24, 25), we expected that inhibition of the *COX-2* gene transcription alone by LY294002 would not be able to totally abolish the UVB induction of COX-2 protein.

GSK-3β was the first Akt substrate shown to be inhibited upon phosphorylation by Akt (26). We find that GSK-3β is phosphorylated upon UVB irradiation that is also inhibited by PI 3-kinase inhibitor LY294002. It appears that there is an inverse correlation between the GSK-3β activity and COX-2 transcription. Inhibiting GSK-3β activity by lithium leads to activation of COX-2 promoter and induction of endogenous COX-2 protein, whereas overexpression of GSK-3β suppresses UVB-induced COX-2 transcription. Therefore, GSK-3β appears to be the major effector of Akt in regulating COX-2 transcription. A role of GSK-3β has also been demonstrated in the regulation of COX-2 expression through the Wnt pathway in mouse mammary epithelial cells (15). These authors have shown that inhibition of GSK-3β by lithium produces an increase in the COX-2 protein level.

Although we have not examined any downstream effectors of GSK-3β that may be involved in the transcriptional induction of COX-2 by UVB, several effectors could be suggested. GSK-3β has been shown to be able to negatively regulate the activity of several transcriptional factors. GSK-3β phosphorylates c-Jun in its DNA binding domain and decreases the affinity of c-Jun in binding to activator protein-1 sites (17). Phosphorylation of NF-AT by GSK-3β promotes its transport from nuclei, thus limiting its availability to activate its target gene (18). GSK-3β also phosphorylates CREB, but only when CREB is first phosphorylated at Ser-133, and phosphorylation of CREB by GSK-3 decreases its binding to the somatostatin gene *CRE* (19). We have demonstrated previously that, upon UVB treatment, CREB/activating transcription factor-1 are phosphorylated at Ser-133 through activation of p38 MAP kinases, and their activation contributes to the UVB induction of COX-2 transcription.[5] From this study, we speculate that UVB also prevents inactivation of CREB through a pathway that involves activation of Akt and inactivation of GSK-3β. Maximal activation of CREB may require its phosphorylation at Ser-133 through the p38 MAP kinase pathway and blocking of its inactivation by GSK-3β. Finally, the fact that the PI-3 kinase pathway plays an important role in the regulation of cell growth, proliferation, and survival (22) and is activated by UVB suggests that members of this pathway are potential targets for prevention and treatment of skin cancers.

Acknowledgments

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