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UVB light upregulates prostaglandin synthases and prostaglandin receptors in mouse keratinocytes

Adrienne T. Black¹, Joshua P. Gray¹, Michael P. Shakarjian², Vladimir Mishin¹, Debra L. Laskin¹, Diane E. Heck², and Jeffrey D. Laskin^{3,*}

¹Department of Pharmacology and Toxicology, UMDNJ-Robert Wood Johnson Medical School, 170 Frelinghuysen Road, Piscataway, NJ 08854

²Department of Pharmaceutics, Rutgers University, UMDNJ-Robert Wood Johnson Medical School, 170 Frelinghuysen Road, Piscataway, NJ 08854

³Department of Environmental and Occupational Medicine, UMDNJ-Robert Wood Johnson Medical School, 170 Frelinghuysen Road, Piscataway, NJ 08854

Abstract

Prostaglandins belong to a class of cyclic lipid-derived mediators synthesized from arachidonic acid via COX-1, COX-2 and various prostaglandin synthases. Members of this family include prostaglandins such as PGE₂, PGF_{2α}, PGD₂ and PGI₂ (prostacyclin) as well as thromboxane. In the present studies we analyzed the effects of UVB on prostaglandin production and prostaglandin synthase expression in primary cultures of undifferentiated and calcium-differentiated mouse keratinocytes. Both cell types were found to constitutively synthesize PGE₂, PGD₂ and the PGD₂ metabolite PGJ₂. Twenty-four hr after treatment with UVB (25 mJ/cm²), production of PGE₂ and PGJ₂ increased, while PGD₂ production decreased. This was associated with increased expression of COX-2 mRNA and protein. UVB (2.5 – 25 mJ/cm²) also caused marked increases in mRNA expression for the prostanoid synthases PGDS, mPGES-1, mPGES-2, PGFS and PGIS, as well as expression of receptors for PGE₂ (EP1 and EP2), PGD₂ (DP and CRTH2) and prostacyclin (IP). UVB was more effective in inducing COX-2 and DP in differentiated cells and EP1 and IP in undifferentiated cells. UVB readily activated keratinocyte PI-3-kinase (PI3K)/Akt, JNK and p38 MAP signaling pathways which are known to regulate COX-2 expression. While inhibition of PI3K suppressed UVB-induced mPGES-1 and CRTH2 expression, JNK inhibition suppressed mPGES-1, PGIS, EP2 and CRTH2, and p38 kinase inhibition only suppressed EP1 and EP2. These data indicate that UVB modulates expression of prostaglandin synthases and receptors by distinct mechanisms. Moreover, both the capacity of keratinocytes to generate prostaglandins and their ability to respond to these lipid mediators are stimulated by exposure to UVB.

Keywords

mPGES; PGDS; EP1; DP; cyclooxygenase; prostacyclin

*To whom correspondence should be addressed, Jeffrey D. Laskin, Ph.D., Department of Environmental and Occupational Medicine, UMDNJ-Robert Wood Johnson Medical School, 170 Frelinghuysen Road, Piscataway, NJ 08854, Email: jlaskin@eohsi.rutgers.edu.

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Introduction

Eicosanoids are derived from arachidonic acid released from cell membranes through the action of phospholipases. Three major classes of enzymes are involved in generating eicosanoids: cyclooxygenases (COX), which produce prostaglandins, lipoxygenases, which generate leukotrienes and hydroxyeicosatetraenoic acids, and cytochrome P450 enzymes which mediate the synthesis of 19- and 20-hydroxyeicosatetraenoic acids (HETEs), epoxyeicosatrienoic acids (EETs) and diHETEs (see Fig 1 for a summary of eicosanoid metabolism) (Funk, 2001). In the skin, COX-derived metabolites are known to control many aspects of inflammation, cell growth control, apoptosis and tumor development (Trifan and Hia, 2003). Two key COX enzymes have been extensively characterized, COX-1, a constitutive enzyme, and COX-2, an enzyme induced by many irritants and inflammatory agents (Fitzpatrick, 2004). These enzymes mediate the synthesis of the prostanoid precursor, prostaglandin H₂ (PGH₂) (Fitzpatrick, 2004). Metabolism of PGH₂ leads to the generation of PGD₂, PGE₂, PGF₂, PGI₂ and thromboxane A₂ which are synthesized by PGD₂ synthase, PGE₂ synthase, PGF₂ synthase, PGI₂ synthase and thromboxane A₂ synthase, respectively (Helliwell *et al.*, 2004). Exposure to UVB light, which is known to cause cutaneous inflammation and cancer (Hruza and Pentland, 1993), has been reported to upregulate COX-2 in human and mouse skin (Buckman *et al.*, 1998; Isoherranen *et al.*, 1999; Chen *et al.*, 2001; Bachelor *et al.*, 2005), a process linked to many of its biological effects (Fitzpatrick, 2004). Increased concentrations of PGE₂, PGF_{2α} and PGD₂ have been detected in UVB-exposed skin (Black *et al.*, 1980a) and, in cultured keratinocytes, PGE₂ is the predominant prostaglandin identified following UVB exposure (Miller *et al.*, 1994).

At present, little is known about the effects of UVB on expression of the prostanoid synthases in keratinocytes and this represents the focus of the present studies. We found that UVB was highly effective in inducing not only COX-2, but also mPGES-1, mPGES-2, PGDS, PGFS and PGIS in primary cultures of undifferentiated and calcium-differentiated mouse keratinocytes. UVB also induced expression of receptors for PGE₂, PGD₂ and PGI₂. These responses were regulated by Akt and MAP kinases, two signal transduction pathways known to be activated by UVB light. Coordinate upregulation of the prostaglandin biosynthetic enzymes and prostaglandin receptors in keratinocytes may be an important mechanism contributing to the biological actions of UVB in the skin.

Materials and Methods

Chemicals and reagents

Rabbit polyclonal antibodies to p38, phospho-p38, JNK, phospho-JNK, ERK 1/2, phospho-ERK 1/2, Akt and phospho-Akt were from Cell Signaling Technology (Beverly, MA), and rabbit anti-keratin-1, keratin-10 and filaggrin from Covance Research Products (Berkley, CA). Goat polyclonal anti-COX-2 and donkey anti-goat secondary antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Horseradish peroxidase-labeled goat anti-rabbit secondary antibodies, Silver Stain Plus and detergent-compatible protein assay reagents were obtained from Bio-Rad Laboratories (Hercules, CA). M-MLV Reverse Transcriptase was from Promega (Madison, WI) and collagen IV from BD Biosciences (San Diego, CA). The Western Lightning enhanced chemiluminescence kit (ECL) was from Perkin Elmer Life Sciences, Inc. (Boston, MA). HPLC prostaglandin standards were purchased from Cayman Chemical (Ann Arbor, MI). SYBR Green Master Mix and other PCR reagents were from Applied Biosystems (Foster City, CA). Cell culture reagents and 2-(2, 3-naphthalimino)ethyl-trifluoromethanesulphonate (NE-OTf) were purchased from Invitrogen Corp (Carlsbad, CA). SP600125 and Wortmannin were from Calbiochem (La Jolla, CA). SB203580, protease inhibitor cocktail, arachidonic acid and all other chemicals were from Sigma (St. Louis, MO).

Cells

Primary keratinocytes were isolated from the epidermis of C57BL neonatal mice and cultured as previously described (Hager *et al.*, 1999). Cells, grown in six-well collagen IV-coated plates, were placed in serum-free low calcium (0.05 mM) Keratinocyte Growth Medium (Cambrex, Walkersville, MD) to maintain their undifferentiated phenotype. Differentiation was induced by the addition of calcium (0.15 mM) to the medium (Yuspa *et al.*, 1988), and was confirmed by morphological changes (Figure 2) and expression of differentiation markers including keratin 1, keratin 10 and filaggrin (Black *et al.*, 2008), as analyzed by Western blotting (see below). For some experiments, primary keratinocytes were obtained from the Yale University Cell Culture Facility (New Haven, CT).

UVB light treatment

UVB was generated from a bank of 2 FS40BL bulbs calibrated using an International IL-1700 UV-radiometer. According to the manufacturer, the spectrum of the bulbs was approximately 85% UVB (290–320 nm), 1% UVA (320–400 nm) and less than 1% UVC (< 290 nm) with the remainder composed of visible light. Undifferentiated and differentiated cells were grown to 90% confluence and exposed to UVB (2.5–25 mJ/cm²) in phosphate-buffered saline (PBS) as previously described (Black *et al.*, 2008). Following UVB treatment, the PBS was removed and the cells refed with Keratinocyte Growth Medium containing 0.05 mM (low) or 0.15 mM (high) calcium. In some experiments, the p38 MAP kinase inhibitor SB203580 (10 μM), the JNK kinase inhibitor SP600125 (20 μM), the PI3-kinase inhibitor wortmannin (0.1 μM) or DMSO control was added to the medium, and the cells incubated at 37°C for 3 hr prior to UVB treatment as previously described (Black *et al.*, 2008). When the cells were refed after UVB exposure, the medium contained the same concentrations of inhibitors. The cytotoxicity of UVB treatment was assayed using trypan blue exclusion when the cells were collected for mRNA and protein. The viability (SEM, n = 4) was 97% ± 2% for control cells and 94% ± 3% for cells treated with the highest dose of UVB (25 mJ/cm²).

Western blotting

Western blotting was performed as previously described (Black *et al.*, 2008). Briefly, cell lysates were prepared using an SDS-lysis buffer (10 mM Tris-base and 1% SDS, pH 7.6 supplemented with a protease inhibitor cocktail consisting of 4-(2-aminoethyl)benzenesulfonyl fluoride, aprotinin, bestatin hydrochloride, N-(trans-epoxysuccinyl)-L-leucine 4-guanidinobutylamide, EDTA and leupeptin). Proteins (20 μg) from lysates were separated on 7.5% or 10% SDS-polyacrylamide gels and then transferred to nitrocellulose membranes. After incubating the membranes in blocking buffer (5% dry milk Tris-buffered saline containing 0.1% Tween 20) for either 1 hr at room temperature or overnight at 4°C, the membranes were then incubated either 1 to 2 hr at room temperature or overnight at 4°C with primary antibodies followed by horseradish peroxidase-conjugated secondary antibodies for 1 hr at room temperature. Protein expression was visualized using enhanced chemiluminescence (ECL) reagents. Equal protein loading was confirmed through silver staining of the gels.

Analysis of prostaglandins

Confluent monolayers of undifferentiated cells were exposed to UVB (25 mJ/cm²) and refed with 1 ml culture medium. Arachidonic acid (10 μM) was added to the medium 24 hr later. After an additional 20 min at 37°C, prostaglandin metabolites were extracted from the cells and medium by the addition of 1 ml of ice-cold methanol. Both cells and medium were collected, centrifuged (12,000 × g for 10 min at 4°C) and aliquots of the supernatants evaporated to dryness under helium. Prostaglandin metabolites were then derivatized as described previously (Yue *et al.*, 2004). Briefly, the dried extracts were solubilized in anhydrous acetonitrile, supplemented with *N,N*-diisopropylethylamine catalyst (dried with 5

Å molecular sieves) and NE-OTf (2 mg/ml in anhydrous acetonitrile). After incubation in a dessicator for 30 min at 4°C, the samples were evaporated to dryness under helium. The derivatized samples were then dissolved in methanol and analyzed using a Shimadzu HPLC 10A system (Kyoto, Japan) fitted with a Beckman Ultrasphere C18 column (4.6 mm × 250 mm) (Fullerton, CA) and a Shimadzu RF-551 fluorescence detector. The excitation and emission wavelengths of the fluorescence detector were set at 260 and 396 nm, respectively. Derivatized prostaglandins were separated at a flow rate of 1.0 ml/min using a step gradient consisting of 85% mobile phase A (acetonitrile:water:acetic acid, 25:75:0.1): 15% mobile phase B (100 % acetonitrile) for 25 min, 50% A: 50% B for 15 min; and 15% B for 10 min.

Real-time polymerase chain reaction

RNA was isolated from the cells using Tri Reagent following the protocol provided by the manufacturer. The RNA was converted to cDNA using M-MLV reverse transcriptase. For each gene to be tested, a standard curve composed of serial dilutions of a pool of the cDNA from the samples was used as a reference. All values were normalized to β -actin ($n = 3$, $\pm$ SEM). The undifferentiated control was assigned a value of 1, and the values of both the undifferentiated and differentiated samples were calculated relative to this control. Real-time PCR was performed on an ABI Prism 7900 Sequence Detection System using 96- well optical reaction plates. SYBR-Green was used for detection of fluorescent signal and the standard curve method was used for relative quantitative analysis. The primer sequences for the genes were generated using Primer Express software (Applied Biosystems) and the oligonucleotides were synthesized by Integrated DNA Technologies, Inc. (Coralville, IA). The forward and reverse sequences used are listed in Table 1.

Statistical analysis

Data are expressed as mean $\pm$ SEM. Statistical differences between the means were determined using two-way analysis of variance (ANOVA) and were considered statistically significant at $p < 0.05$.

Results

Effects of UVB on prostaglandin production in keratinocytes

In initial experiments we analyzed prostaglandin production by primary cultures of undifferentiated and differentiated murine keratinocytes. These cells were found to constitutively synthesize several prostaglandins important in regulating inflammation including PGE₂, PGD₂, PGJ₂ and PGA₂ (Fig 3 and not shown). PGA₂ and PGJ₂ are cyclopentenone prostaglandins formed through the spontaneous dehydration of PGE₂ and PGD₂, respectively (Straus and Glass, 2001). Treatment of the cells with UVB (25 mJ/cm²) markedly increased production of PGE₂ and PGJ₂, but decreased production of PGD₂. No alterations were evident in the production of PGA₂ and there were no major differences between undifferentiated and differentiated cells (not shown).

Effects of UVB on expression of arachidonic acid mobilizing enzymes

To analyze mechanisms mediating prostaglandin production in keratinocytes, we measured mRNA expression of enzymes controlling the release of arachidonic acid from cell membranes. These included cytosolic phospholipase A₂ (cPLA₂), which acts directly on phospholipid membranes to release arachidonic acid (Balsinde *et al.*, 2002), as well as phospholipase C β 1 (PLC β 1), which acts indirectly by generating diacylglycerol (DAG), a metabolite that is hydrolyzed by diacylglycerol lipase (DAGL) to monoacylglycerol (MAG) and then to arachidonic acid by monoacylglycerol lipase (MAGL) (Rebecchi and Pentyla, 2000). Real time PCR analysis revealed that keratinocytes express mRNA for cPLA₂, PLC β 1, DAGL and

MAGL (Fig. 4). While UVB had no major effects on cPLA₂ or MAGL expression, expression of PLCβ1 increased 8-fold and DAGL decreased 2–3 fold. Although the differentiation status of keratinocytes has been reported to be important in regulating biological responses to UVB light (Tron *et al.*, 1999), we found no major differences in expression of arachidonic acid mobilizing enzymes between undifferentiated and calcium-differentiated keratinocytes (Fig. 4).

Effects of UVB on expression of COXs, prostaglandin synthases, and prostaglandin receptors

Two isoforms of COX have been identified; COX-1 and COX-2, which catalyze the two-step oxidation of arachidonic acid to PGH₂, the precursor for PGE₂, PGF_{2α} and PGD₂ as well as prostacyclin and thromboxane (see Fig. 1). UVB was found to stimulate expression of COX-2 mRNA and protein in both undifferentiated and differentiated cells (Fig. 5). Differentiated cells appeared to be more sensitive to UVB when compared to undifferentiated cells. In contrast, no major changes in COX-1 expression were noted in either cell type. PGE₂ is the major prostaglandin generated in the skin and is produced by the action of the microsomal PGE₂ synthases, mPGES-1 and mPGES-2 (Murakami *et al.*, 2003). We found that mRNA expression of these enzymes increased 4–6-fold in both undifferentiated and differentiated keratinocytes following exposure to UVB (Fig. 6). Interestingly, UVB also upregulated expression of PGFS, PGDS and PGIS but not TXAS, the enzymes responsible for the formation of PGF_{2α}, PGD₂, PGI₂ and TXA₂, respectively (Fig. 6). In general, no major differences were noted between the undifferentiated and differentiated cell types. These data are consistent with our findings that PGE₂ and PGJ₂ production is increased in keratinocytes in response to UVB (Fig. 3). We also found that differentiated cells contained approximately two times more constitutive PGFS when compared to undifferentiated cells. Notably, PGFS expression in these cells was approximately two-fold higher across the range of UVB doses tested (2.5 – 25 mJ/cm²) (Fig. 6).

Prostaglandins initiate their biological activity by binding to specific membrane receptors (Narumiya *et al.*, 1999). To date, four PGE₂ receptors have been identified (EP1, EP2, EP3 and EP4). We found that expression of EP1 and EP2 was induced by UVB light (approximately 4- and 6-fold, respectively) in both undifferentiated and differentiated keratinocytes (Fig. 7). In contrast, no major changes were noted in expression of EP3 or EP4. Interestingly, at 10 and 15 mJ/cm² UVB, greater increases in EP1 and EP2 expression were observed in undifferentiated keratinocytes when compared to differentiated cells. Expression of the two known receptors for PGD₂, DP and CRTH2, were also increased following UVB exposure. Although differentiated cells were more responsive to UVB in terms of DP receptor expression, no major differences were observed in CRTH2 expression between the cell types. UVB also induced expression of the prostacyclin receptor, IP, and the maximal responses were greater in undifferentiated cells (16-fold) when compared to the differentiated cells (8-fold). No significant alterations were detected in the expression of the PGF_{2α} receptor FP or the thromboxane A₂ receptor TP.

Role of phosphoinositide-3-kinase (PI3K)/Akt and MAP kinase signaling in UVB-induced alterations in prostaglandin synthase and prostaglandin receptor expression

UVB was found to upregulate p38 and JNK MAP kinases in a dose-dependent manner in both undifferentiated and differentiated keratinocytes (Fig. 8) and the effects of UVB on JNK were more pronounced. In contrast, only small increases in the ERK1/2 MAP kinase were evident in undifferentiated cells. The undifferentiated cells also responded to UVB with activation of Akt. This appeared to be due to increased expression of total Akt protein. In differentiated cells, high constitutive levels of activated Akt were noted and this did not change with UVB exposure.

We next used inhibitors of p38, JNK and Akt to evaluate the role of these kinases in regulating expression of COX-2, mPGES-1 and PGIS, three genes that were highly responsive to UVB. SB203580, a p38 kinase inhibitor, and SP600125, a JNK kinase inhibitor, were found to markedly suppress UVB-induced COX-2 expression in both undifferentiated and differentiated keratinocytes (Fig 9 and not shown). In contrast, mPGES-1 expression decreased in response to the JNK inhibitor and the p38 inhibitor was effective in suppressing mPGES-1 only at the highest dose of UVB (25 mJ/cm²). Whereas UVB-induced expression of PGIS was unchanged following p38 inhibition, JNK inhibition effectively suppressed expression of this enzyme (Fig. 9). The role of PI3K/Akt in regulating expression of these genes was assessed using Wortmannin, an inhibitor of PI3K. As observed with the MAP kinases, expression of COX-2 and mPGES-1, but not PGIS, was markedly reduced by wortmannin. No major differences were noted in the effects of MAP kinase or Akt kinase inhibition on UVB-induced gene expression in undifferentiated and differentiated keratinocytes (data not shown).

We also examined the effects of p38, JNK and Akt inhibitors on keratinocyte expression of EP1, EP2, CRTH2, DP and IP, the prostanoid receptors upregulated in response to UVB. UVB-induced EP1 expression was significantly reduced by p38, but not JNK inhibition (Fig 10). In contrast, expression of EP2 was decreased by JNK, but only slightly with p38 inhibition. Kinase inhibition also differentially affected UVB-induced expression of the two PGD₂ receptors; CRTH2 was reduced by JNK inhibition, DP was unaffected. No major alterations were detected in IP expression with the kinase inhibitors. PI3K inhibition suppressed UVB-induced expression of CRTH2, while no changes were observed in expression of EP1, EP2, DP or IP.

Discussion

Previous work has demonstrated that exposure of mouse and human skin to ultraviolet light leads to alterations in the production of lipid-derived mediators including PGE₂, PGF_{2α} and PGD₂ (Black *et al.*, 1980a; Miller *et al.*, 1994). These eicosanoids are known to mediate many skin reactions and a number of drugs that inhibit their production effectively suppress skin inflammation (Fogh and Kragballe, 2000; Wilgus *et al.*, 2003). The present studies demonstrate that primary cultures of mouse keratinocytes constitutively produce these prostaglandins, as well as low levels of PGJ₂. Each of these prostaglandins or their metabolites is known to be important in regulating cell growth, and their constitutive production in keratinocytes is presumably important in skin homeostasis (Fogh and Kragballe, 2000). As previously observed in mouse and human skin (Black *et al.*, 1980b; Ruzicka *et al.*, 1983), UVB stimulated production of PGE₂ in undifferentiated and differentiated cultures of primary mouse keratinocytes. PGE₂ is a potent proinflammatory mediator and it has been shown to play a key role in the development of UVB-induced skin cancer (Furstenberger *et al.*, 1989). UVB was also found to cause marked increases in PGJ₂ in keratinocytes, but to reduce PGD₂ production. Decreased production of PGD₂ is consistent with an earlier report demonstrating that UVB suppressed production of this arachidonic acid metabolite in hairless mouse skin (Ruzicka *et al.*, 1983). PGD₂ is relatively unstable and degrades by spontaneous dehydration and isomerization reactions to PGJ₂ which is metabolized to a variety of biological active metabolites (Kikawa *et al.*, 1984). Increased levels of PGJ₂ in keratinocytes after UVB may be due to increased PGD₂ biosynthesis (see Figure 1). PGJ₂, and the PGE₂ dehydration product PGA₂, contain a reactive unsaturated carbonyl prostane which forms Michael adducts with cellular thiols including glutathione and cysteine residues in cellular proteins (Straus and Glass, 2001). A variety of signaling molecules important in mediating inflammation in response to UVB are modulated by these cyclopentenones including NF-κB (Rossi *et al.*, 2000) and PPARγ (Shibata *et al.*, 2002). Moreover, 15-deoxy-Δ^{12,14}-PGJ₂, a highly active anti-inflammatory metabolite of PGJ₂, is known to potentiate tumor formation in a mouse skin carcinogenesis model (Millan *et al.*, 2006). These data suggest that cyclopentenones including

PGJ₂ and its metabolites may be important in mediating, at least in part, the biological actions of UVB in the skin.

To investigate mechanisms mediating prostaglandin production in keratinocytes, we characterized the effects of UVB on expression of the enzymes regulating their biosynthesis. The initial step of prostanoid biosynthesis is mobilization of arachidonic acid from phospholipid membranes through the action of phospholipases (Funk, 2001). Previous studies reported that UVB stimulates arachidonic acid release from membrane phospholipids (DeLeo *et al.*, 1985; Punnonen *et al.*, 1987), as well as cPLA₂ synthesis and activity in skin (Kang-Rotondo *et al.*, 1993; Gresham *et al.*, 1996). In contrast, we found no major changes in cPLA₂ mRNA expression in undifferentiated or differentiated mouse keratinocytes following UVB light treatment. An alternative pathway for arachidonic acid release from cell membranes is via activation of PLC, forming 1,2-diacylglycerol and inositol-1,4,5-triphosphate (Rebecchi and Penttyala, 2000). Inositol-1,4,5-triphosphate causes calcium release from intracellular stores (Berridge and Irvine, 1984); both 1,2-diacylglycerol and calcium activate protein kinase C, a signaling enzyme that can cause phosphorylation and activation of cPLA₂ (Lin *et al.*, 1993). We found that PLCβ1 mRNA expression increased 24 hr after UVB. On the contrary, there were no changes or small decreases in mRNA expression in undifferentiated and differentiated cells for DAG lipase and MAG lipase, the two enzymes that sequentially hydrolyze diacylglycerol to form arachidonic acid (Rebecchi and Penttyala, 2000). This is consistent with a report of increased diacylglycerol levels in keratinocytes after UVB exposure (Punnonen and Yuspa, 1992). Thus, either increases in PLCβ1 or in the activities of DAG or MAG lipase may be sufficient to stimulate arachidonic acid production in mouse keratinocytes.

It is well recognized that UVB-induced prostaglandin production in the skin is mediated by COX-2 (Fitzpatrick, 2004). Increased expression of this enzyme has been directly associated with many UVB-induced skin inflammatory reactions including edema, erythema, keratinocyte proliferation and epidermal hyperplasia (Rodriguez-Burford *et al.*, 2005). Changes in COX-2 expression have also been linked to the accumulation of DNA damage and mutations, as well as the development of premalignant lesions and skin cancer (Muller-Decker *et al.*, 1998; Fischer *et al.*, 2007). We found that COX-2 mRNA and protein were increased in mouse keratinocytes following UVB exposure. These data are consistent with earlier reports showing that UVB effectively induces COX-2 in mouse and human keratinocytes (Isoherranen *et al.*, 1999; Black *et al.*, 2008). Our data also demonstrate that differentiated cells are more sensitive to UVB-induced upregulation of COX-2 mRNA and protein, a finding consistent with previous studies showing that UVB-induced COX-2 expression in mouse skin occurs primarily in the suprabasal, differentiated keratinocytes (Athar *et al.*, 2001). At the present time, the mechanisms underlying the increased sensitivity of differentiated cells to UVB are not known although it should be noted that abnormal differentiation occurs in mouse epidermis following overexpression of COX-2 (Neufang *et al.*, 2001). Differentiation related changes in mRNA expression for enzymes regulating arachidonic acid release and other prostanoid synthases and receptors were not observed following UVB treatment (see further below) indicating that this response was selective for COX-2.

The effects of UVB on expression of the various prostanoid synthases in keratinocytes have not been investigated previously. The synthesis of PGE₂ is catalyzed by the microsomal prostaglandin E synthases mPGES-1 and mPGES-2 (Murakami *et al.*, 2002), both of which were upregulated by UVB in undifferentiated and differentiated keratinocytes. mPGES-1 is a glutathione-dependent enzyme and member of the membrane associated proteins involved in eicosanoid and glutathione metabolism (MAPEG) superfamily. This enzyme is known to be upregulated in response to proinflammatory stimuli, a process thought to be critical for PGE₂ production during inflammatory responses (Claveau *et al.*, 2003). Our findings showing that mPGES-2 is also upregulated by UVB is in contrast to earlier studies indicating that this

is a constitutively expressed enzyme, and suggest that mPGES-2, like mPGES-1, is also important in regulating increases in PGE₂ production in the skin during inflammation. Coordinate increases in expression of COX-2 and mPGES-1 have been previously described (Murakami *et al.*, 2000; Murakami *et al.*, 2003). UVB-induced increases in COX-2, mPGES-1 and mPGES-2 in the skin are likely to be important for maximal stimulation of PGE₂ production. It should be noted that mPGES-1 also possesses glutathione transferase and peroxidase activity (Murakami *et al.*, 2002), and increases in this enzyme may also be key in reducing cytotoxic lipophilic hydroperoxides generated in the skin following exposure to UVB.

Also of interest is our findings that exposure to UVB resulted in increased expression of two (EP1 and EP2) of the four known keratinocyte PGE₂ receptors. This indicates that UVB not only upregulates the capacity of keratinocytes to generate PGE₂, but also their responsiveness to this prostaglandin. PGE₂ receptors belong to a family of G-protein coupled receptors that function via distinct signaling mechanisms (Narumiya *et al.*, 1999). All of the known receptors, including EP1 and EP2, are expressed in mouse skin and several have been directly linked to UVB- and phorbol ester-induced skin inflammation and carcinogenesis (Thompson *et al.*, 2001; Sung *et al.*, 2006; Brouxhon *et al.*, 2007; Chun *et al.*, 2007). Thus, UVB has previously been reported to stimulate expression of EP1, EP2 and EP4 in mouse skin, but to reduce expression of EP3 (Tober *et al.*, 2007). Moreover, modulating EP receptor expression or activity using genetic and pharmacological approaches has demonstrated that EP1, EP2 and/or EP4 are critical mediators of the actions of UV light in the skin (Tober *et al.*, 2006). Also of note was our observation that EP1 and EP2 relative mRNA levels increased at intermediate doses of UVB (10 and/or 15 mJ/cm²), suggesting that undifferentiated cells were more responsive to UVB than differentiated cells in this parameter. Recent studies using techniques in immunohistochemistry have shown that these receptors are localized in suprabasal keratinocytes in mouse skin (Tober *et al.*, 2007).

An important finding from our work is that UVB also stimulated PGE₂ biosynthesis, as well as expression of mRNA for enzymes mediating production of PGD₂, PGF_{2α} and prostacyclin, and their respective receptors. These data are generally consistent with the results of our metabolism studies. PGD₂ and its receptors, DP and CRTH2, are known to be involved in chronic allergic skin inflammation (Angeli *et al.*, 2004; Satoh *et al.*, 2006). The marked increases in PGFS and PGIS that we noted in response to UVB also suggest that eicosanoids produced by these enzymes may be important mediators of cutaneous inflammation (Muller *et al.*, 2000; Takahashi *et al.*, 2002). Increases in expression of receptors for PGD_{2α} and prostacyclin provide further evidence that UVB stimulates both the capacity of keratinocytes to produce these prostaglandins and cellular responsiveness to these mediators. Additionally, UVB was more effective in inducing expression of IP in undifferentiated cells and DP in differentiated cells. These data suggest that the two cell types may respond distinctly to prostacyclin and PGD₂, respectively.

It is well recognized that UVB activates a number of signaling pathways leading to inflammatory gene expression, a process referred to as 'the UV response' (Heck *et al.*, 2004). Initially characterized by activation of the immediate early genes, c-fos and c-jun and various transcription factors including NFκB (Heck *et al.*, 2004), more recent studies have demonstrated that PI3K/Akt and MAP kinase signaling are also key to the response (Bode and Dong, 2003). We found that MAP kinase and Akt were readily activated by UVB in mouse keratinocytes; however, this response was differentiation-dependent. Thus, while the ERK1/2, JNK, p38 MAP kinases and Akt were activated by UVB in undifferentiated cells, in differentiated cells, only JNK and p38 kinase were activated. Differentiated cells were also found to contain high constitutive levels of activated Akt. Differential activation of Akt in differentiated cells was most likely due to the higher intracellular concentrations of calcium which have been shown to activate the PI3K signaling pathway leading to Akt phosphorylation

(Yuspa *et al.*, 1988). These later findings are also consistent with studies showing that the PI3K/Akt pathway is activated during mouse keratinocyte differentiation both in culture and in the intact epidermis (Calautti *et al.*, 2005). It is possible that activation of Akt is important in the process of keratinocyte differentiation. Differences in UVB-induced PI3K and MAP kinase signaling in undifferentiated and differentiated keratinocytes may contribute, at least in part, to their distinct responses following UVB treatment, with respect to expression of COX-2, EP1, EP2, IP and DP.

Previous studies have demonstrated that inhibition of p38 MAP kinase effectively suppresses UVB-induced inflammation in mouse skin including expression of proinflammatory cytokines and COX-2 production (Hildesheim *et al.*, 2004; Kim *et al.*, 2005). Similarly, we found that UVB-induced COX-2 expression is regulated via p38 kinase, as well as JNK and Akt kinase. The role of these kinases in controlling expression of enzymes downstream of COX-2, however, is not known. We found that UVB-induced expression of mPGES-1 and PGIS were suppressed by an inhibitor of JNK. Interestingly, mPGES-1, but not PGIS, was also suppressed by PI3K/Akt inhibition indicating that the two prostanoid synthases are controlled by distinct mechanisms. Our data indicate that JNK is also important in controlling CRTH2 and EP2 expression, while p38 kinase regulates EP1 and EP2. Moreover, PI3K/Akt is selectively involved in UVB-induced CRTH2 expression. Thus, each of the prostaglandin receptors also appears to be regulated distinctly. At the present time, signaling mechanisms regulating UVB-induced expression of PGIS, DP and IP in keratinocytes remain to be determined.

Work on the role of eicosanoids in UVB-induced skin inflammation and carcinogenesis has largely been limited to the study of COX-2 expression and PGE₂ signaling. Several studies have shown a link between prostaglandin production and the initiation and progression of skin carcinogenesis (Fischer *et al.*, 1999; Wilgus *et al.*, 2003; Chun *et al.*, 2007). Our studies demonstrate that UVB also stimulates a number of additional synthases important in prostaglandin production, as well as prostaglandin receptors and each may be critical for mediating the biological effects of UVB in mouse skin (see Figure 11 for summary of prostaglandin synthase and prostaglandin receptor genes upregulated by UVB and those regulated by MAP kinases and/or PI3K). Whether or not the genes induced by UVB contribute to its biological actions will depend on levels of expression of functional proteins for the prostaglandin synthases and receptors as well as amounts of prostaglandins produced in keratinocytes. Further studies are needed to more clearly define the prostaglandins produced by keratinocytes and their roles in modulating the responses of the skin to UVB.

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List of Abbreviations

CRTH2, Chemoattractant receptor-homologous molecule expressed on T helper type 2 cells
 COX-1, cyclooxygenase-1
 COX-2, cyclooxygenase-2
 cPLA₂, cytosolic phospholipase A₂
 DAGL, diacylglycerol lipase
 EETs, epoxyeicosatrienoic acids
 HETEs, 19- and 20- hydroxyeicosatetraenoic acids
 mPGES-1, microsomal PGE₂ synthase-1

mPGES-2, microsomal PGE₂ synthase-2
 MAGL, monoacylglycerol lipase
 PI3K, phosphoinositide-3-kinase
 PLC, phospholipase C
 PGE₂, prostaglandin E₂
 PGD₂, prostaglandin D₂
 DP, PGD₂ receptor
 PGDS, PGD₂ synthase
 PGF₂, prostaglandin F₂
 FP, PGF₂ receptor
 PGFS, PGF₂ synthase
 PGH₂, prostaglandin H₂
 PGI₂, prostaglandin I₂
 IP, PGI₂ receptor
 PGIS, PGI₂ synthase
 TXA₂, thromboxane A₂
 TP, TXA₂ receptor
 TXAS, TXA₂ synthase

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Figure 1. The cyclooxygenase branch of arachidonic acid metabolism

Arachidonic acid is mobilized directly from membrane phospholipids by phospholipase A₂ (cPLA₂) and indirectly through the sequential actions of phospholipase C-β1 (PLCβ1), diacylglycerol lipase (DAGL) and monoacylglycerol lipase (MAGL). Arachidonic acid can then be oxidized to form various eicosanoids by cyclooxygenases (COX), lipoxygenases (LOX) or cytochrome P450 monooxygenases. For prostaglandin metabolism, arachidonic acid is metabolized by cyclooxygenases (COX-1 and COX-2) to produce an intermediate prostaglandin, PGH₂. Further metabolism by the prostanoid synthases, microsomal prostaglandin E₂ synthase (mPGES), prostaglandin F₂ synthase (PGFS), prostaglandin D₂ synthase (PGDS), prostaglandin I₂ synthase (PGIS) or thromboxane A₂ synthase (TXAS), forms prostaglandins (PGE₂, PGF_{2α} and PGD₂), prostacyclin (PGI₂) or thromboxane (TXA₂), respectively. PGD₂ can be further metabolized to PGJ₂ and Δ^{12,14}-PGJ₂ and 15-deoxy Δ^{12,14}-PGJ₂, while PGE₂ is spontaneously converted to PGA₂.

Figure 2. Morphology of undifferentiated and calcium-differentiated primary mouse keratinocytes
The micrograph shows the surface morphology of proliferating undifferentiated cells (upper panel) and differentiated cells (lower panel). The differentiation process was initiated by the addition of calcium (0.15 mM) to the medium as described in the Methods section.

Figure 3. Effects of UVB on prostaglandin production by keratinocytes

Cells were exposed to UVB (25 mJ/cm²) or control. After 24 hr, cells were pulsed with 10 μ M arachidonic acid and prostaglandin metabolites in the cultures were extracted, derivatized and analyzed by HPLC with fluorescence detection as described in the Materials and Methods. The peaks were quantified using the Shimadzu software and are given in arbitrary units. For the control cells, the amounts of prostaglandins produced were 4.5 (PGE₂), 14 (PGD₂), 65 (PGA₂) and 5 (PGJ₂). For the UVB-treated cells, the amounts were 22 (PGE₂), 1.5 (PGD₂), 64 (PGA₂) and 96 (PGJ₂).

Figure 4. Effects of UVB light on expression of enzymes that release arachidonic acid from cell membranes

Undifferentiated (closed circles) and differentiated keratinocytes (open circles) were exposed to UVB light at the indicated doses. After 24 hr, mRNA was isolated, reverse transcribed and resulting cDNA's analyzed by real-time PCR ($n = 3$, $\pm$ SE) for expression of cPLA₂, PLCβ1, DAGL and MAGL). ^aSignificant ($p < 0.05$) increase in UVB-treated undifferentiated cells relative to undifferentiated control; ^bSignificant ($p < 0.05$) increase in UVB-treated differentiated cells relative to undifferentiated control.

Figure 5. Effects of UVB light on expression of COX-1 and COX-2 in keratinocytes

Undifferentiated (closed circles) and differentiated (open circles) keratinocytes were exposed to UVB light at the indicated doses. After 24 hr, cells were analyzed for COX-1 and COX-2 mRNA expression by real-time PCR ($n = 3$, $\pm$ SE) (upper panels) or COX-2 protein expression by Western blotting (lower panel). ^aSignificant ($p < 0.05$) increase in UVB-treated undifferentiated cells relative to undifferentiated control; ^bSignificant ($p < 0.05$) increase in UVB-treated differentiated cells relative to undifferentiated control, ^cSignificant ($p < 0.05$) difference between UVB-treated undifferentiated and differentiated cells.

Figure 6. Effects of UVB light on mRNA expression of the prostanoid synthases

Undifferentiated (closed circles) and differentiated (open circles) keratinocytes were exposed to UVB light at the indicated doses. After 24 hr, mRNA expression of mPGES-1, mPGES-2, PGDS, PGFS, PGIS and TXAS was determined using real-time PCR ($n = 3$, $\pm$ SE). ^aSignificant ($p < 0.05$) increase in UVB-treated undifferentiated cells relative to undifferentiated control; ^bSignificant ($p < 0.05$) increase in UVB-treated differentiated cells relative to undifferentiated control; ^cSignificant ($p < 0.05$) difference between UVB-treated undifferentiated and differentiated cells.

Figure 7. Effects of UVB light on expression of the eicosanoid receptors

Undifferentiated (closed circles) and differentiated (open circles) keratinocytes were exposed to UVB light at the indicated doses. After 24 hr, cells were analyzed for mRNA expression of the prostanoid receptors by real-time PCR ($n = 3$, $27 \pm \text{SE}$). Panel A: Expression of PGE₂ receptors, EP1, EP2, EP3 and EP4, and PGD₂ receptors, DP and CRTH2. Panel B: Expression of PGI₂ receptor, IP, PGF_{2α} receptor, FP, and TXA₂ receptor, TP. For clarity, the prostaglandin for each receptor is shown in parenthesis in each figure. ^aSignificant ($p < 0.05$) increase in UVB-treated undifferentiated cells relative to undifferentiated control; ^bSignificant ($p < 0.05$) increase in UVB-treated differentiated cells relative to undifferentiated control, ^cSignificant ($p < 0.05$) difference between UVB-treated undifferentiated and differentiated cells.

Figure 8. Effects of UVB on MAP and Akt kinases in keratinocytes

Undifferentiated and differentiated keratinocytes were exposed to UVB light at the indicated doses. After 15 min, cells were lysed and analyzed for the total and phosphorylated forms of p38, JNK, ERK1/2 MAP kinases, and Akt kinase by Western blotting as described in the Materials and Methods.

Figure 9. Effects of MAP kinase and Akt kinase inhibitors on UVB-induced expression of COX-2, mPGES-1 and PGIS

Undifferentiated keratinocytes were incubated with SB203580 (10 μ M), a p38 MAP kinase inhibitor, SP600125 (20 μ M), a JNK MAP kinase inhibitor, or wortmannin (0.1 μ M), a PI3 kinase inhibitor, for 3 hr and then exposed to UVB light at the indicated doses. After 24 hr, cells were analyzed for mRNA expression for COX-2 (upper panels), mPGES-1 (center panels) or PGIS (lower panels) by real-time PCR ($n = 3$, $\pm$ SE). Left panels, Effects of inhibitors of p38 (closed circles) and JNK MAP kinase (closed inverted triangles) on enzyme expression; Right panels, Effects of PI3K/Akt inhibitor (closed circles) on enzyme expression. Open circles are control cells in the absence of inhibitors. ^aSignificantly different from untreated control (p38 inhibitor); ^bSignificantly different ($p < 0.05$) from untreated control (JNK inhibitor); ^cSignificantly different ($p < 0.05$) from untreated control (PI3K inhibitor).

Figure 10. Effects of MAP kinase and Akt kinase inhibitors on UVB-induced expression of keratinocyte prostanoid receptors

Undifferentiated keratinocytes were incubated with inhibitors and exposed to UVB as described in the Legend to Figure 8. After 24 hr, cells were analyzed for mRNA expression of the prostanoid receptors by real-time PCR ($n = 3$, $\pm$ SE). Left panels: Effects of inhibitors of p38 (closed circles) and JNK MAP kinase (closed inverted triangles) on receptor expression. Right panels: Effects of PI3K/Akt inhibitor (closed circles) on receptor expression. Open circles are control cells in the absence of inhibitors. ^aSignificantly different from untreated control (p38 inhibitor); ^bSignificantly different ($p < 0.05$) from untreated control (JNK inhibitor); ^cSignificantly different ($p < 0.05$) from untreated control (PI3K inhibitor).

Figure 11. Summary of the effects of UVB on the cyclooxygenase biosynthetic pathway
 UVB light stimulates the release of arachidonic acid from keratinocyte cell membranes and increases COX-2 and various prostaglandin synthases and prostaglandin receptors (indicated by the arrows pointing upward) that contribute to its biological effects in the skin. Regulation of UVB-induced expression of the prostaglandin synthases and prostaglandin receptors by the MAP kinases (p38 and JNK) and/or PI3K are shown in parenthesis.

Table 1

Real-time PCR primer sequences (5'→3').

Gene	Forward (5'→3')	Reverse (5'→3')	Gene Bank ID
β-actin	TCACCCACACTGTGCCCATCTACGA	GGATGCCACAGGATTCCATACCC	NM_007393
COX-1	GTCCCAGAACCAGGGTGTCT	CACTGGTAGTTGTCGAGGCCA	NM_008969
COX-2	CATTCTTTGCCCAGCACTTCAC	GACCAGGCACCAGACCAAAGAC	NM_011198
CRTH2	GTCACACTGAAGCCGCTCTG	TGGAAGCTGGACCATCTCCT	NM_009962
DAGL	TTCGCCGAGTTCATTGACAG	TCTCAGGCACCATCATGCA	NM_144915
DP	TAAAGGAACTGCTGCCTGCC	AGGCGAACGTTTCGCATAAC	NM_008962
EP1	CTGGGCTTAACCAAGAGTGC	CCGGGAACCTACGCAGTGAAC	NM_013641
EP2	GTTTCACGTGCTGGTAACGGA	CAGGTTCCCAGCAGGTCAGT	NM_008964
EP3	ATGGGAAAGGAGAAGGAGTGC	AGCCAGGCGAACTGCAATTA	NM_011196
EP4	TTTGCGAATTCCGAGACCT	CTCTCAGAGTCCTGGCCCTG	NM_008962
FP	TCCAAGCAGCCAGTGTCTCC	GCAGGTTGTGTTTGCCATGA	NM_008966
IP	TGGGTCTTCATCCTTTCCG	CCAGAACTTGAGGCGTTGGA	NM_008967
MAGL	TGCTTTGGCATAACAGCTGCT	CATTGCTCGCTCCACTCTTG	NM_011844
cPLA ₂	AGCCATCCAGGCATCAAAGA	TGCCCTATCCCATCTTTGCA	NM_008869
PLCβ1	CTCCAAGCGAAACCAGGACA	CGGTGTGATTACCCCCATTC	NM_019677
PGDS	CTGCTCTGAGCAAATGGCTG	AGGACCAAACCCATCCACAG	NM_008963
mPGES-1	GGCCTTTCTGCTCTGCAGC	GCCACCGCGTACATCTTGAT	NM_022415
mPGES-2	AGCCCCTGGAAGAGGTCATC	CATTCATGGCCTTCATGGGT	NM_133783
PGFS	GAGGAAGTAGGGCTGGCCAT	CCTCACAGTGCCATCAGCAA	NM_027582
PGIS	ATCTGCTGCTCCCCAACTG	CTTTATCCCCGCTGACAAG	NM_008968
TP	TACAGTGTGCAGTACCCCGG	CTGTGTCCCGAGTGTGAGGA	NM_009325
TXAS	CCCCAAGCCTTCTCCTTTTG	AAACCCTGGCGGAAAAACAT	NM_011539