



UVB light regulates expression of antioxidants and inflammatory mediators in human corneal epithelial cells

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ABSTRACT

The cornea is highly sensitive to ultraviolet B (UVB) light-induced oxidative stress, a process that results in the production of inflammatory mediators which have been implicated in tissue injury. In the present studies, we characterized the inflammatory response of human corneal epithelial cells to UVB (2.5–25 mJ/cm²). UVB caused a dose-dependent increase in the generation of reactive oxygen species in the cells. This was associated with increases in mRNA expression of the antioxidants Cu,Zn superoxide dismutase (SOD), Mn-SOD, catalase and heme oxygenase-1 (HO-1), as well as the glutathione S-transferases (GST), GSTA1-2, GSTA3, GSTA4, GSTM1, and mGST2. UVB also upregulated expression of the proinflammatory cytokines, IFN γ , IL-1 β , TGF β and TNF α , and enzymes important in prostaglandin (PG) biosynthesis including cyclooxygenase-2 (COX-2) and the PG synthases mPGES-2, PGDS, PGFS and thromboxane synthase, and in leukotriene biosynthesis including 5-lipoxygenase (5-LOX), 15-LOX-2, and the epidermal and platelet forms of 12-LOX. UVB was found to activate JNK and p38 MAP kinases in corneal epithelial cells; ERK1/2 MAP kinase was found to be constitutively active, and its activity increased following UVB treatment. Inhibition of p38 blocked UVB-induced expression of TNF α , COX-2, PGDS and 15-LOX-2, while JNK inhibition suppressed TNF α and HO-1. These data indicate that UVB modulates corneal epithelial cell expression of antioxidants and proinflammatory mediators by distinct mechanisms. Alterations in expression of these mediators are likely to be important in regulating inflammation and protecting the cornea from UVB-induced oxidative stress.

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Abbreviations: COX-1, cyclooxygenase-1; COX-2, cyclooxygenase-2; FLAP, 5-lipoxygenase activating protein; GST, glutathione S-transferase; mGST, microsomal GST; HO-1, heme oxygenase-1; LTA₄ hydrolase, leukotriene A₄ hydrolase; LTC₄ synthase, leukotriene C₄ synthase; LOX, lipoxygenase; 5-LOX, 5-lipoxygenase; 12-LOX-epi, 12-lipoxygenase-epidermal type; 12-LOX-plt, 12-lipoxygenase platelet-type; IFN, interferon; IL, interleukin; PGE₂, prostaglandin E₂; mPGES-1, microsomal PGE₂ synthase-1; mPGES-2, microsomal PGE₂ synthase-2; PGD₂, prostaglandin D₂; PGDS, PGD₂ synthase; PGF₂, prostaglandin F₂; PGFS, PGF₂ synthase; PGI₂, prostaglandin I₂; PGIS, PGI₂ synthase; PGH₂, prostaglandin H₂; SOD, superoxide dismutase; TXA₂, thromboxane A₂; TXAS, TXA₂ synthase; TGF β , transforming growth factor beta; TNF α , tumor necrosis factor alpha.

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1. Introduction

The higher energy wavelengths of ultraviolet light from sunlight including UVB (290–320 nm) are known to cause oxidative stress and ocular injury [1,2]. The corneal epithelium serves as a protective barrier, absorbing UVB light and minimizing damage to the interior eye including the lens and retina. However, in excessive amounts, UVB light can injure the cornea, a process that causes aberrant epithelial cell growth and differentiation, as well as cell death via necrosis and apoptosis [3,4]. This can result in corneal opacities or photokeratitis [5–7]. UVB-induced corneal injury is thought to be initiated by oxidative stress due to the localized generation of reactive oxygen species (ROS) by corneal epithelial cells [8,9]. ROS, including superoxide anion, hydrogen peroxide and hydroxyl radicals, can damage DNA, resulting in mutations [10]. ROS can also oxidize proteins and lipids, leading to the generation of highly toxic electrophilic species including malondialdehyde and 4-hydroxynonenal, which can initiate inappropriate or altered cellular signal transduction pathways and contribute to toxicity [10].

A critical step in limiting oxidative damage involves upregulation of antioxidants that remove or detoxify ROS [11]. UVB light has been shown to upregulate the antioxidant enzymes, glutathione peroxidase, heme oxygenase-1 (HO-1) and the glutathione S-transferases GST1A-2 and GSTM1 in mouse keratinocytes [12] and glutathione peroxidase, GSTA4 and GSTP1 in human and rat skin [13,14]. In a rabbit eye model, reduced corneal expression of antioxidants including catalase, glutathione peroxidase and superoxide dismutase (SOD) was observed following UVB light treatment, a process thought to accelerate oxidative stress and tissue injury [15]. UVB-induced oxidative stress in the skin is also associated with the initiation of an inflammatory response characterized by the generation of cytokines, growth factors and lipid-derived eicosanoids [16]. Previous studies in mouse and human keratinocytes and corneal epithelial cells showed that exposure to UVB light stimulated production of proinflammatory cytokines and growth factors including tumor necrosis factor- α (TNF α), interleukin (IL)-1 β , transforming growth factor β (TGF β) and interferon- γ (IFN γ) [7]. In skin keratinocytes, UVB light increased the release of PGE₂ and leukotriene B₄, a process associated with upregulation of cyclooxygenase-2 (COX-2) and prostaglandin and leukotriene synthases, enzymes mediating the generation of prostaglandins and leukotrienes [17–19].

At present, little is known about the mechanisms by which corneal epithelial cells respond to UVB light-induced oxidative stress and this represents the focus of the present studies. We found that human corneal epithelial cells were highly responsive to UVB light, which effectively upregulated mRNA expression of proinflammatory cytokines, growth factors and enzymes important in mediating prostaglandin and leukotriene biosynthesis, as well as antioxidants. Moreover, these responses were regulated by mitogen activated protein kinases. Coordinate regulation of the production of inflammatory mediators is likely important in mediating UVB light induced corneal inflammation and tissue injury. Alterations in antioxidants may be a key adaptive mechanism of controlling oxidative stress and protecting against ROS-induced cytotoxicity following exposure to UVB light.

2. Materials and methods

2.1. Chemicals and reagents

Rabbit polyclonal antibodies to p38, phospho-p38, JNK, phospho-JNK, ERK1/2, and phospho-ERK1/2 were purchased from Cell Signaling Technology (Beverly, MA), and horseradish peroxidase-labeled goat anti-rabbit secondary antibodies and detergent-compatible protein assay reagents from Bio-Rad Laboratories (Hercules, CA). M-MLV Reverse Transcriptase was from Promega (Madison, WI) and the Western Lightning enhanced chemiluminescence kit (ECL) from Perkin Elmer Life Sciences, Inc. (Boston, MA). SYBR Green Master Mix and other PCR reagents were from Applied Biosystems (Foster City, CA). SP600125 was purchased from Calbiochem (La Jolla, CA). Cell culture reagents, 2',7'-dichlorofluorescein-diacetate (DCFH-DA) and keratinocyte growth medium with supplement kits were purchased from Invitrogen Corp. (Carlsbad, CA). Tri reagent, SB203580, protease inhibitor cocktail and all other chemicals were Sigma (St. Louis, MO) unless otherwise indicated.

2.2. Cells and UVB light treatments

The human corneal epithelial (HCE) cell line was kindly provided by Kaoru Araki-Sasaki, Kinki Central Hospital (Hyogo, Japan) and grown in keratinocyte serum-free medium (K-SFM), supplemented with fetal bovine serum (5%), gentamicin (0.05%), epidermal growth factor (0.05 μ g/ml) and bovine pituitary extract (0.05 mg/ml). The cells were originally isolated from donor corneas

and immortalized using a recombinant SV40-adenovirus [20]. HCE cells were exposed to UVB light using a bank of 2 Westinghouse FS40BL light bulbs, calibrated with an International Light IL-1700 UV-radiometer as previously described [12,21]. Briefly, cells (2×10^5) were seeded into 6 well culture dishes and allowed to grow 3–4 days until approximately 90% confluent. Cells were then exposed to UVB (2.5–25 mJ/cm²) in phosphate-buffered saline (PBS); PBS was then removed and the cells were refed with medium. The times of exposure to UVB light were 30 s, 1 min, 2 min and 5 min. In control experiments, cells were mock irradiated for 5 min. In some experiments, the p38 MAP kinase inhibitor, SB203580 (10 μ M), or the JNK kinase inhibitor, SP600125 (20 μ M), was added to the medium and the cells preincubated at 37 °C for 3 h prior to UVB treatment. Cell viability following UVB treatment was assessed by measuring cell growth as determined by protein content of the cultures. Low doses of UVB light (2.5–10 mJ/cm²) had no effects of cell growth. At 25 mJ/cm², we found a 15% inhibition of cell growth.

2.3. Western blotting

Cell lysates were prepared from control and UVB light treated cells using 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) as previously described [12]. Protein lysates (20 μ g) were separated on 10% SDS-polyacrylamide gels and then transferred to nitrocellulose. After incubation in blocking buffer (5% dry milk Tris-buffered saline with 0.1% Tween 20), the membranes were incubated overnight at 4 °C with primary antibodies followed by horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. Protein expression was visualized using ECL reagents.

2.4. Measurement of ROS production

Intracellular hydrogen peroxide production was measured using DCFH-DA and flow cytometry as previously described [22]. Briefly, the cells were incubated with DCFH-DA (5 μ M) in PBS for 15 min, washed with PBS, exposed to UVB light (2.5–25 mJ/cm²), and then immediately analyzed on a Beckman Coulter Cytomics FC 500 flow cytometer (Miami, FL). Control cells were mock irradiated. In preliminary experiments we found similar DCF fluorescence in cells mock irradiated for 30 s, 1 min, 2 min and 5 min. Therefore, we used a 5 min mock irradiation for control experiments. Data were analyzed using Beckman Counter CXP software.

2.5. Real-time PCR

RNA was isolated from the cells using Tri Reagent (Sigma) following the manufacturers protocol. RNA was converted to cDNA using M-MLV reverse transcriptase. The cDNA was diluted 1:10 in RNase-DNase-free water for PCR analysis. For each gene, a standard curve was generated from serial dilutions of cDNA. All values were normalized to β -actin ($n=3$). The control was assigned a value of one and changes in gene expression calculated relative to this control. Real-time PCR was performed on an ABI Prism 7900 Sequence Detection System (Life Technologies, Carlsbad, CA) using 96-well optical reaction plates. SYBR-Green was used for detection of the 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 (Life Technologies) and the oligonucleotides were synthesized by Integrated DNA Technologies, Inc. (Coralville, IA). The forward and reverse (5'–3') primer sequences used are listed in Table 1.

Table 1
Realtime PCR primer sequences.

Gene	Forward (5' → 3')	Reverse (5' → 3')
β-actin	AAAGACCTGTACGCCAACAC	GTCATACTCCTGCTTGCTGAT
catalase	CGGAGATTCAACTGCGCAA	GAATGCCCGCACTGAGTAA
COX-1	GTACCGCAAGAGGTTTGGCA	ACGAGCTCTCGGAAGGAGG
COX-2	GCCTGATGATTGCCCCGACT	GCTGGCCCTCGCTTATGATCT
cPLA2	GATTGCAATTCTGCACGTGATG	CCACCTGAACCAATATGGC
FLAP	AAGTGGAGCACGAAGCAGG	CGGTCTCTGGAAGCTCCTC
GSTA1-2	TTGATGTTCCAGCAAGTGCC	CACCAGCTTCATCCCATCAAT
GSTA3	TTCTGCCCTTATGTCGACCTG	TGATCAAGGCAATCTTGGCAT
GSTA4	GCTCCACTATCCCAACGGAA	AAAACCATCTCACGGACTCC
GSTM1	AGCAACGCCATCTTGTGTAC	CTCCCCACACAGGTTGTGC
GSTP1	CTATGGGAAGGACCAGCAGG	CCGTCATTACCATGTCCAC
HO-1	GCTCAAAAAGATTGCCAGAG	GCGGTAGAGCTGCTTGAAC
IL-1β	CACACAGCAAGGCTTCAGG	GTCATGGCCACAACAACCTG
IFNγ	GGCTTTTCAGCTCTGCATCG	TCCTGGCAGTAACAGCCAAGA
5-LOX	TCGAGTTCCCTGTACCG	TCAGGACAACCTCGACATCG
12-LOX-epi	ACCGACCTCTTGTGGGAAC	CCCCACAATGGTCAGTGAGAT
12-LOX-plt	AGATGGAGGCCAATGGGAAG	GAGGCTGAATCTGGATGACCA
15-LOX-1	GAGATCACTGAAATCGGGCTG	GACAGGAAACCTCGGTCCT
15-LOX-2	TGATATTACCTGCTCCGCC	CAAACCTGCCCTGCCTGACA
LTA4 hydrolase	TGAAGTTTACCCGGCCCTTA	GGATTTGTCAAAGGCAGCA
LTC4 synthase	AGTACTTCCCGCTGTCTCTCG	GAAAGAAGATGCCCGGCAC
mGST1	GCCCACCTGAATGACCTTGA	GAGGCCAATTCCAAGAAATGG
mGST2	GAACCTGATCCTGCTGGCTG	CTTTGCTGACAGGCCGAGA
mGST3	CCACCCGCGTATAGCTTCTG	ACTCGTCCAACAATCCAGGC
mPGES-1	CACCGGAACGACATGGAGAC	GACGAAGCCCAGGAAAAGG
mPGES-2	GATGTACGTGGTGGCCATCA	CTCTTCTCCGACGCTCAC
PGDS	CAAGGCTGGTGACTTTACGGA	AGTTAGCGACGGCAGGAATG
PGFS	CTCTGTACCACCTGGGAGGC	GGCCAATCTGCATCTTAC
PGIS	AGAGTATCCTTTGGCAAGCGG	GGAGAGTGGTCTGTCGAG
Cu,Zn-SOD	GTCGTAGTCTCTGCAGCGTC	CTGGTTCGAGGACTGCAA
Mn-SOD	TCTGGACAACCTCAGCCCT	GCAACTCCCTTTGGGTTCT
TGFβ	GAAGGGACAAATCGCTTAGC	TGTAGACTCTTCCCGGTTAG
TNFα	CCTGCCCCAATCCCTTATT	CCCTAAGCCCCCAATCTCT
TXAS	CATCCATAATGGTCCCACTGG	CGTCTCGGTTCTTATGGGC

2.6. Statistical analysis

Data were evaluated using Student's *t*-test and differences were considered statistically significant at $p < 0.05$.

3. Results

3.1. UVB light-induced oxidative stress in HCE cells

In initial studies, we determined if UVB light causes oxidative stress in corneal epithelial cells by quantifying its ability to generate intracellular hydrogen peroxide. Using the hydroperoxy-

sensitive probe DCFH-DA in conjunction with flow cytometry, we found that HCE cells generated low basal levels of hydrogen peroxide (Fig. 1). UVB (2.5–25 mJ/cm²) treatment resulted in a dose-dependent increase in hydrogen peroxide production; a 20–30-fold increase in hydrogen peroxide production was evident after exposure to the maximal UVB dose tested.

3.2. UVB light alters mRNA expression of antioxidant enzymes

We next examined the effects of UVB light on expression of primary antioxidant enzymes. UVB light treatment of HCE cells resulted in increased mRNA expression of Cu,Zn-SOD, Mn-SOD, catalase and HO-1 (Fig. 2). For each of these genes, maximal effects were observed at 5–10 mJ/cm². At the highest dose of UVB (25 mJ/cm²), mRNA expression was reduced to near basal levels. The glutathione *S*-transferases (GST) consists of a class of secondary intracellular antioxidant enzymes that function to eliminate oxidized macromolecules and xenobiotics via glutathione conjugation and include both cytosolic (GSTA, GSTM and GSTP families) and microsomal (mGST1, mGST2 and mGST3) enzymes [23–26]. Significant increases in mRNA expression of the GSTA family including GSTA1–2, GSTA3 and GSTA4, as well as GSTM1 and mGST2 were noted, in HCE cells following UVB light treatment (Fig. 2). In contrast, a 2-fold decrease in mGST3 expression was evident after exposure of the cells to 25 mJ/cm² UVB light. No major changes were observed in mRNA expression for GSTP1 or mGST1 at any dose of UVB tested.

3.3. UVB light alters mRNA expression of enzymes generating eicosanoids

Arachidonic acid, released from the phospholipid membrane by phospholipase A₂, is oxidized by cyclooxygenase (COX) and

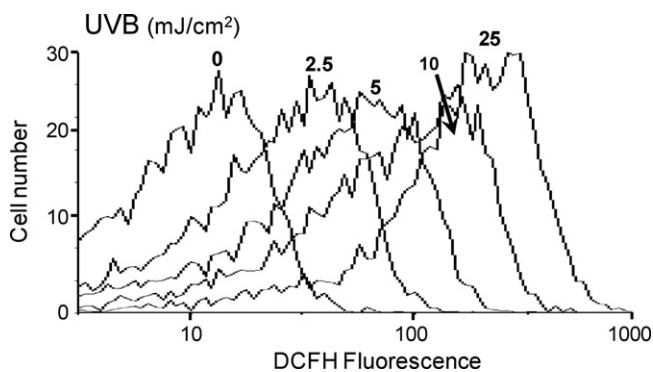


Fig. 1. UVB induces oxidative stress in human corneal epithelial cells. HCE cells were incubated with DCFH-DA (5 μM) for 15 min. Cells were then rinsed with PBS, exposed to 2.5, 5, 10 or 25 mJ/cm² UVB light and analyzed by flow cytometry for DCF fluorescence. Control cells were treated with DCFH-DA and mock irradiated. The panel shows that UVB light increased DCF fluorescence intensity which is directly proportional to hydrogen peroxide production. Increases in fluorescence intensity are presented on a log scale.

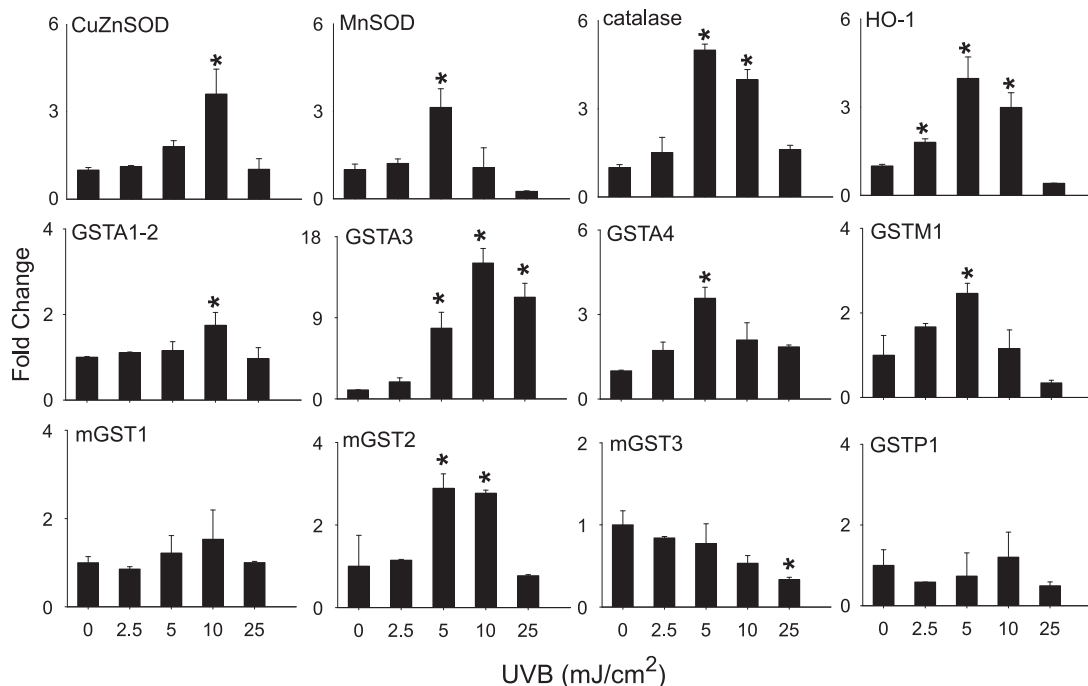


Fig. 2. Effects of UVB light on antioxidant enzyme gene expression. HCE cells were exposed to UVB (2.5–25 mJ/cm²) or control. After 24 h, RNA was extracted and analyzed by real-time PCR. Data are presented as fold change in gene expression relative to controls. Each bar is the mean \pm SE ($n = 3$). * Significantly different from control ($p < 0.05$).

lipoxygenase (LOX) enzymes to generate prostaglandins (PG) and leukotrienes, respectively [19]. UVB light treatment of HCE cells resulted in a dose-related increase in expression of mRNA for COX-2, microsomal PGE₂ synthase (mPGES-2), PGD₂ synthase (PGDS), PGF₂ synthase (PGFS), and thromboxane synthase (TXAS) (Fig. 3). Maximal increases in expression of these genes were observed at 10 mJ/cm² UVB. In contrast, at the highest dose of UVB tested, a 2-fold decrease in mPGES-1 and PGI₂ synthase (PGIS) expression was evident (Fig. 3). UVB light had no effect on COX-1 mRNA expression.

We next determined if UVB light altered expression of the LOX family of enzymes. Increases in mRNA expression for 15-LOX-2, LTC₄ synthase, 12-LOX, 5-LOX, FLAP and LTA₄ hydrolase were observed in HCE cells after UVB treatment (Fig. 3). While increases in expression of LTC₄ synthase, 5-LOX, FLAP and LTA₄ hydrolase were evident after exposure to 5–10 mJ/cm² UVB, 15-LOX-2 and epidermal type 12-LOX increased continuously over the dose range tested (2.5–25 mJ/cm²) (Fig. 3). Cytokine expression was also analyzed as a marker of UVB-induced inflammation. UVB caused a dose-dependent increase in IFN γ , TNF α , TGF β and IL-1 β mRNA expression with maximal effects at 10–25 mJ/cm² (Fig. 4).

3.4. Role of MAP kinase signaling in regulating expression of antioxidants and inflammatory proteins

In our next series of studies, we analyzed the role of MAP kinases in UVB-induced alterations in gene expression. UVB treatment (10–25 mJ/cm²) resulted in a dose-dependent activation of JNK and p38 MAP kinase expression in HCE cells, as measured by increases in the phosphorylated forms of the enzymes (Fig. 5). In contrast, relatively small increases in activated ERK1/2 were detected at doses of 2.5–15 mJ/cm² UVB (Fig. 5). HCE cells also constitutively expressed activated phosphorylated ERK1/2 kinases, but not JNK or p38 kinase. To determine if JNK and p38 MAP kinases mediated UVB-induced changes in mRNA expression in HCE cells, we used specific enzyme inhibitors. Treatment of the cells with the p38 MAP kinase inhibitor, SB203580, markedly suppressed UVB-induced expression of COX-2, 15-LOX-2 and

TNF α while expression of PGDS and IFN- γ was decreased. In contrast, inhibition of JNK kinase using SP600125 markedly suppressed expression of HO-1 and TNF α and decreased PGDS, 15-LOX-2 and IFN- γ (Fig. 6)

4. Discussion

ROS are key mediators of many of the cytotoxic effects of UVB light in epithelial cells [27,28]. ROS generated by UVB can trigger oxidative stress including lipid peroxidation, protein oxidation and DNA damage, as well as cytokine and growth factor signaling leading to changes in gene expression [29,30]. Consistent with previous studies in mouse and human keratinocytes [12,31], we found that UVB light readily stimulates the formation of intracellular ROS in human corneal epithelial cells, as measured by the production of hydrogen peroxide. These findings are in accord with reports that UVB alters expression of markers of oxidative stress in human and rabbit corneal epithelial cells, and in rabbit corneal tissue [32–34].

We also found that UVB upregulates expression of both primary and secondary antioxidant enzymes, which most likely reflects an adaptive cytoprotective response to oxidative stress [35,36]. Thus, UVB increased mRNA expression of Mn-SOD and Cu,Zn-SOD, HO-1 and catalase. Mn-SOD is a mitochondrial-associated enzyme, while Cu,Zn-SOD is cytosolic. Coordinate regulation of these antioxidants, as well as catalase, is presumably key in protecting different intracellular compartments in corneal epithelial cells from UVB-induced increases in formation of superoxide anion and hydrogen peroxide, respectively [37]. We have previously shown that mRNA expression for catalase and Cu,Zn-SOD are not affected by UVB in mouse keratinocytes, while Mn-SOD is suppressed [12]. Differences in expression of these enzymes in cornea and skin may be due to unique characteristics of epithelial cells in different tissues. Increased expression of these enzymes in the cornea may be required to protect against ROS-induced damage to the lens and/or retina at higher fluences of UVB [9,15]. HO-1, the rate-limiting enzyme in the catabolism of the prooxidant heme, functions as an antioxidant/antiinflammatory protein and is induced in many cells

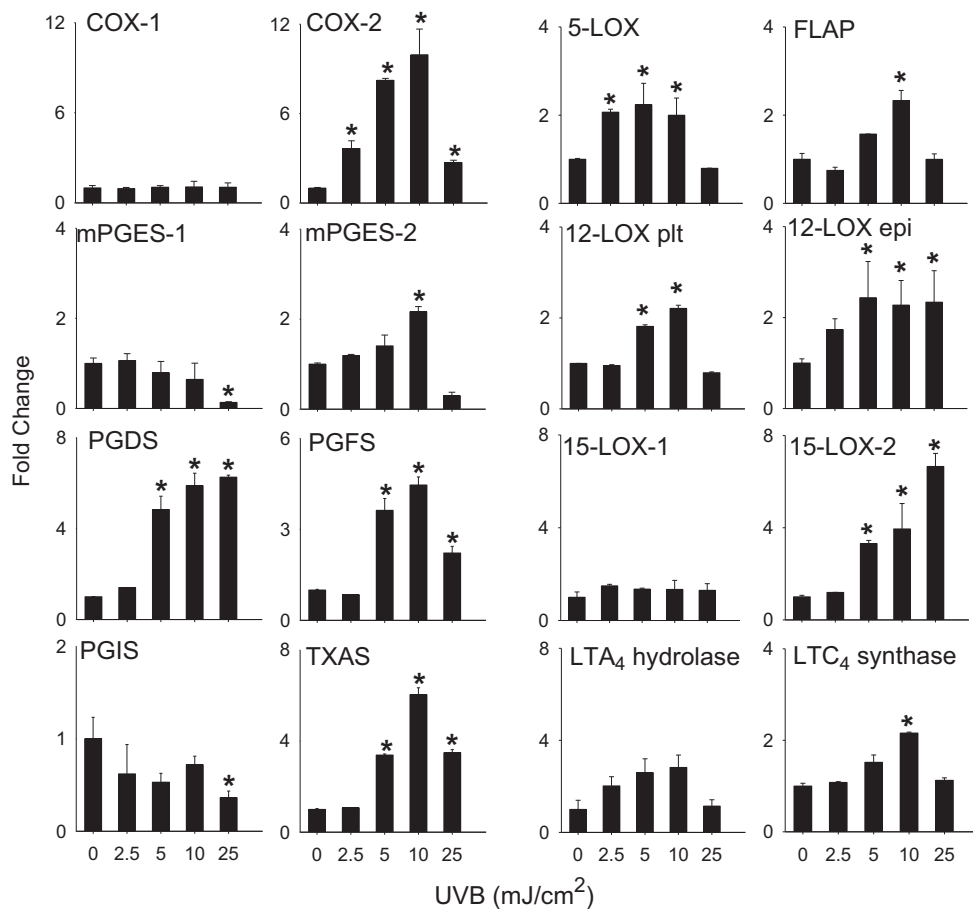


Fig. 3. Effects of UVB light on expression of the eicosanoid biosynthetic enzymes. HCE cells were exposed to UVB (2.5–25 mJ/cm²) or control. After 24 h, RNA was extracted and analyzed by real-time PCR. Data are presented as fold change in gene expression relative to controls. Each bar is the mean \pm SE ($n = 3$). * Significantly different from control ($p < 0.05$).

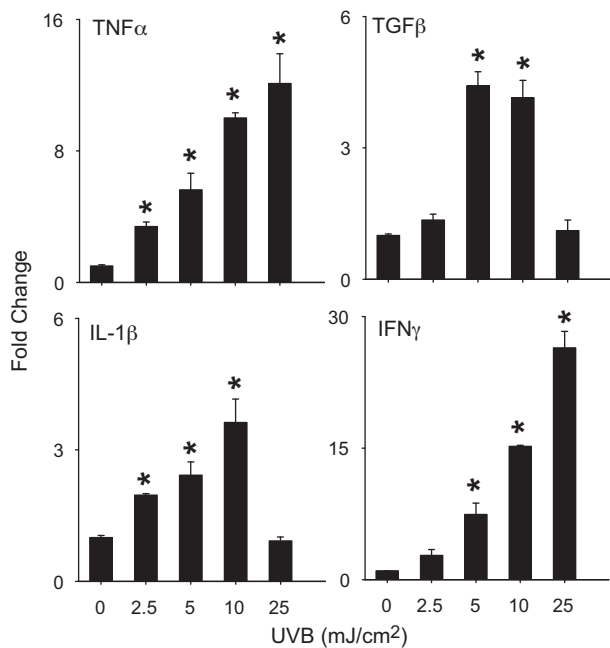


Fig. 4. Effects of UVB light on cytokine expression. HCE cells were exposed to UVB (2.5–25 mJ/cm²) or control. After 24 h RNA was extracted and analyzed by real-time PCR. Data are presented as fold change in gene expression relative to controls. Each bar is the mean \pm SE ($n = 3$). * Significantly different from control ($p < 0.05$).

by oxidative stress [38]. Our findings of increased mRNA expression for HO-1 in corneal epithelial cells is consistent with earlier studies showing that UVB induces HO-1 mRNA and protein in keratinocytes [12]. In the rabbit cornea, HO-1 has been shown to attenuate corneal inflammation and accelerate wound healing after injury. Increased expression of HO-1 in human corneal epithelial cells following UVB treatment may be important not only in limiting oxidative stress and photokeratitis, but also in mitigating UVB-induced tissue injury [39].

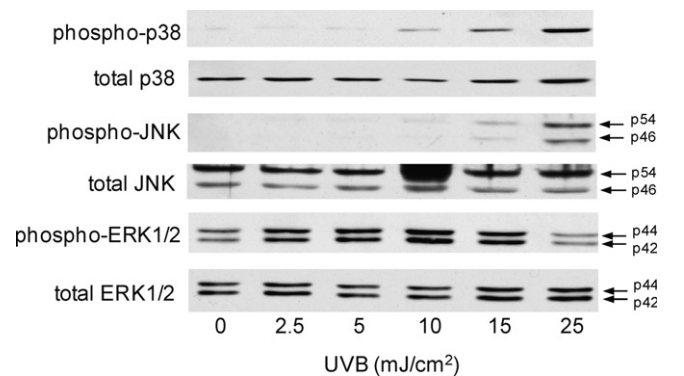


Fig. 5. Effects of UVB on MAP kinase expression in HCE cells. HCE cells were exposed to UVB (2.5–25 mJ/cm²) or control. After 15 min, cells were lysed and analyzed for total and phosphorylated p38, JNK, and ERK1/2 MAP kinases by Western blotting. One representative blot from three experiments is shown.

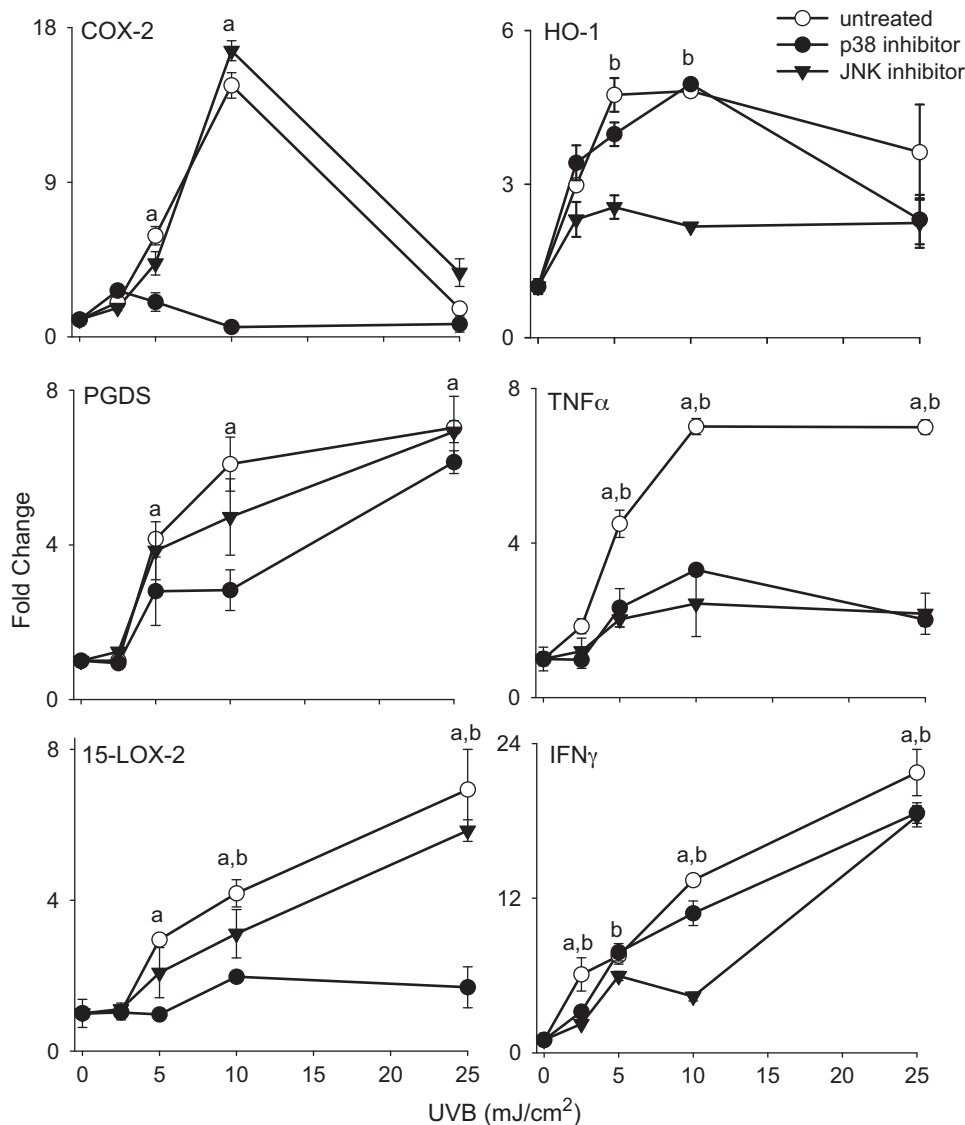


Fig. 6. Effects of p38 and JNK MAP kinase inhibition on UVB-induced gene expression. Cells were incubated with SB203580 (10 μ M), a p38 MAP kinase inhibitor, or SP600125 (20 μ M), a JNK MAP kinase inhibitor, for 3 h and then exposed to UVB (2.5–25 mJ/cm²) or control. After 24 h, RNA was extracted and analyzed by real-time PCR. Data are presented as fold change in gene expression relative to untreated controls. Differences were considered statistically significant at $p < 0.05$: ^a p38 kinase inhibitor treatment versus control, ^b JNK kinase inhibitor treatment versus control.

The GST enzymes are secondary antioxidants that conjugate reduced glutathione to electrophilic compounds, decreasing their potential for inducing cytotoxicity [23]. Preferred substrates have been identified for different classes of GSTs. For example, GSTP1 and the GSTA family of enzymes are known to detoxify lipid peroxidation products, a reaction important in breaking radical-forming chain reactions, while GSTM1 protects against protein oxidation [23–25]. Total GST activity has been reported to increase in the skin following UVB treatment [23,40,41]. UVB also increases mRNA expression of GSTA1–2 and GSTA4 in primary cultures of undifferentiated and differentiated mouse keratinocytes [12]. Similarly, the present studies demonstrate UVB upregulated mRNA's for these GSTs, as well as for GSTA3 and GSTM1 in corneal epithelial cells. Microsomal GSTs consist of a class of enzymes that function to protect against microsomal lipid peroxidation. UVB exposure resulted in increased expression of mGST2 which may play a role in mitigating UVB-induced oxidative stress in the microsomal compartment of the corneal cells. Consistent with earlier findings in mouse keratinocytes [12], we found that mGST3 decreased following UVB treatment of corneal

epithelial cells. mGST3 has been reported to mediate glutathione conjugation to leukotriene A₄, a reaction important in generating proinflammatory leukotrienes (see further below) [24]. In both corneal epithelial cells and keratinocytes, decreases in mGST3 may limit production of proinflammatory lipid mediators and contribute to the resolution of inflammation following UVB treatment.

As highly active arachidonic acid-derived mediators, prostaglandins are known to regulate inflammation and evidence suggests that they may contribute to UVB-induced tissue injury [42]. For example, in human skin and rabbit eyes, toxic doses of UVB light stimulate production of PGE₂, PGF_{2α} and PGD₂ [18,43]. Moreover, in both of these tissues, non-specific inhibitors of COX-2 reduce prostaglandin biosynthesis and prevent tissue injury. Hence, treatment of human skin with diclofenac, which inhibits UVB light-induced erythema, also reduces dermal levels of PGE₂, while treatment of rabbits with indomethacin, which reduces UVB light-induced corneal injury, suppresses ocular levels of PGE₂ and PGF_{2α} [6,44]. Our studies demonstrate that UVB light induces mRNA for enzymes that generate prostaglandin biosynthesis in corneal epithelial cells including COX-2, mPGES-2, PGDS and PGFS

and are consistent with earlier studies on the effects of UVB on rabbit ocular tissue [18,43]. Previous studies have shown that both rat and rabbit corneas have the capacity to synthesize thromboxanes [45,46]. Our data show that UVB light can upregulate mRNA expression of thromboxane synthase. Taken together, these data suggest that arachidonic acid-derived thromboxanes as well as prostaglandins may be important mediators of UVB light toxicity in the cornea.

Leukotrienes and their metabolites are pleiotropic autotoxins that regulate inflammation and wound healing in the cornea [47,48]. We found that UVB light treatment of corneal epithelial cells increased expression of mRNA for 5-LOX and its activator protein FLAP, an important pathway for generating 5-hydroperoxyeicosatetraenoic acid and its dehydration product LTA₄ from arachidonic acid. LTC₄ synthase is key for the generation of the cysteinyl leukotrienes (LTC₄, LTD₄ and LTE₄) while LTA₄ hydrolase mediates the conversion of LTA₄ to LTB₄. We found that UVB light also upregulates LTC₄ synthase, an enzyme important in mediating LTC₄ biosynthesis. Earlier work has shown that corneal inflammation is associated with increased production of LTB₄ [49]. Both LTB₄ and the cysteinyl leukotrienes are proinflammatory and function by binding to specific G protein coupled receptors on target cells. The actions of the leukotrienes in inflammation are multifactorial. For example, both LTB₄ and the cysteinyl leukotrienes stimulate chemokine production and endothelial cell-leukocyte adhesion, while LTB₄ is a potent neutrophil chemoattractant [48]. Active neutrophil infiltration into the cornea is known to be associated with infection and injury and increased production of LTB₄ may be an important mechanism mediating this process.

UVB light was also found to increase cornea epithelial mRNA expression for the platelet and epithelial forms of 12-LOX, as well as 15-LOX-2, enzymes mediating the generation of anti-inflammatory and protective lipid mediators, including the lipoxins and the docosahexaenoic acid-derived protectins and resolvins [50,51]. Recent studies have shown that lipid mediators such as lipoxin A₄ and the neuroprotectin D1 are generated in the cornea where they inhibit chemokine formation and promote corneal wound healing [52,53]. The importance of these enzymes in corneal epithelial function is also evident by the findings that mice deficient in 12/15-LOX synthesize reduced amounts of lipoxin A₄, and exhibit defects in corneal reepithelialization and neutrophil recruitment following wounding [52]. The precise mechanisms by which lipoxin A₄ and neuroprotectin D1 promote wound healing in the cornea are not known and may involve enhanced epithelial cell proliferation and/or stimulation of pro-survival repair pathways which lead to inhibition of apoptosis [52,54].

It is well recognized that MAP kinase signaling cascades regulate the action of many growth factors and inflammatory mediators [55]. Earlier studies demonstrated that UVB is a potent activator of MAP kinase signaling, a process thought to be due to direct or indirect activation of growth factor and/or cytokine receptors and subsequent down-stream signaling, referred to as “the UV response” [56,57]. Our laboratory and others have shown that UVB is a potent inducer of MAP kinase signaling in keratinocytes [12,21]. These enzymes control expression of genes important in regulating cell growth and differentiation as well as oxidative stress and inflammation [56]. The present studies show that corneal epithelial cells are also highly responsive to UVB-induced MAP kinase signaling, specifically JNK and p38 MAP kinases. These enzymes regulate UVB-induced mRNA for enzymes that generate both eicosanoids and inflammatory mediators, although their expression is regulated by distinct mechanisms. Thus, while both COX-2 and 15-LOX-2 expression are controlled by p38 MAP kinase, JNK regulates HO-1 and IFN γ and both kinases regulate TNF α . Generally similar results have been reported in UVB treated mouse and human skin and/or keratinocytes [21,58–62].

In summary, our data demonstrate that corneal epithelial cells are highly sensitive to UVB light-induced oxidative stress. This is associated with increased mRNA expression for antioxidants, as well as proinflammatory mediators including cytokines and enzymes that generate prostaglandins and leukotrienes. UVB light also upregulates expression of enzymes that can generate mediators that downregulate inflammation and promote wound healing including lipoxin A₄ and neuroprotectin D1. Coordinate regulation of these mediators in the cornea is likely important in initiating, as well as controlling the resolution of inflammation and the repair of damaged cells and tissues. Whether or not the genes induced by UVB in the cornea contribute to its biological actions will depend on levels of expression of functional proteins and on production of both pro- and anti-inflammatory mediators. Further studies are needed to more clearly define the mediators produced by the corneal epithelial cells and their roles in modulating the responses of the cornea to UVB light.

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References

- [1] Kolozsvari L, Nogradi A, Hopp B, Bor Z. UV absorbance of the human cornea in the 240–400-nm range. *Invest Ophthalmol Vis Sci* 2002;43:2165–8.
- [2] Podskochy A. Protective role of corneal epithelium against ultraviolet radiation damage. *Acta Ophthalmol Scand* 2004;82:714–7.
- [3] Rogers CS, Chan LM, Sims YS, Byrd KD, Hinton DL, Twining SS. The effects of sub-solar levels of UV-A and UV-B on rabbit corneal and lens epithelial cells. *Exp Eye Res* 2004;78:1007–14.
- [4] Estil S, Olsen WM, Huitfeldt HS, Haaskjold E. UVB-induced formation of (6–4) photoproducts in the rat corneal epithelium. *Acta Ophthalmol Scand* 1997;75:120–3.
- [5] Cullen AP. Photokeratitis and other phototoxic effects on the cornea and conjunctiva. *Int J Toxicol* 2002;21:455–64.
- [6] Andley UP, Fritz C, Morrison AR, Becker B. The role of prostaglandins E₂ and F_{2 α} in ultraviolet radiation-induced cortical cataracts in vivo. *Invest Ophthalmol Vis Sci* 1996;37:1539–48.
- [7] Kennedy M, Kim KH, Harten B, Brown J, Planck S, Meshul C, et al. Ultraviolet irradiation induces the production of multiple cytokines by human corneal cells. *Invest Ophthalmol Vis Sci* 1997;38:2483–91.
- [8] Cejkova J, Stipek S, Crkovska J, Ardan T, Midelfart A. Reactive oxygen species (ROS)-generating oxidases in the normal rabbit cornea and their involvement in the corneal damage evoked by UVB rays. *Histol Histopathol* 2001;16:523–33.
- [9] Cejkova J, Stipek S, Crkovska J, Ardan T, Platenik J, Cejka C, et al. UV rays, the prooxidant/antioxidant imbalance in the cornea and oxidative eye damage. *Physiol Rev* 2004;53:1–10.
- [10] Halliwell B, Gutteridge JMC. Free radicals in biology and medicine. Oxford: Oxford University Press; 2007.
- [11] Droge W. Free radicals in the physiological control of cell function. *Physiol Rev* 2002;82:47–95.
- [12] Black AT, Gray JP, Shakarjian MP, Laskin DL, Heck DE, Laskin JD. Distinct effects of ultraviolet B light on antioxidant expression in undifferentiated and differentiated mouse keratinocytes. *Carcinogenesis* 2008;29:219–25.
- [13] Desmots F, Rissel M, Loyer P, Turlin B, Guilloze A. Immunohistochemical analysis of glutathione transferase A₄ distribution in several human tissues using a specific polyclonal antibody. *J Histochem Cytochem* 2001;49:1573–80.
- [14] Hiratsuka A, Saito H, Hirose K, Watabe T. Marked expression of glutathione S-transferase A4-4 detoxifying 4-hydroxy-2(E)-nonenal in the skin of rats irradiated by ultraviolet B-band light (UVB). *Biochem Biophys Res Commun* 1999;260:740–6.
- [15] Cejkova J, Stipek S, Crkovska J, Ardan T. Changes of superoxide dismutase, catalase and glutathione peroxidase in the corneal epithelium after UVB rays. Histochemical and biochemical study. *Histol Histopathol* 2000;15:1043–50.
- [16] Hruza LL, Pentland AP. Mechanisms of UV-induced inflammation. *J Invest Dermatol* 1993;100:355–415.

- [17] Nakaguma H, Takahashi H. Remarkable elevation of leukotriene B₄ in rat skin after induction of UV photodermatitis. *Inflammation* 1990;14:195–203.
- [18] Miller CC, Hale P, Pentland AP. Ultraviolet B injury increases prostaglandin synthesis through a tyrosine kinase-dependent pathway. Evidence for UVB-induced epidermal growth factor receptor activation. *J Biol Chem* 1994;269:3529–33.
- [19] Funk CD. Prostaglandins and leukotrienes: advances in eicosanoid biology. *Science* 2001;294:1871–5.
- [20] Araki-Sasaki K, Ohasi Y, Sasabe T, Hayashi K, Watanabe H, Tano Y, et al. An SV40-immortalized human corneal epithelial cell line and its characterization. *Invest Ophthalmol Vis Sci* 1995;36:614–21.
- [21] Black AT, Gray JP, Shakarjian MP, Mishin V, Laskin DL, Heck DE, et al. UVB light upregulates prostaglandin synthases and prostaglandin receptors in mouse keratinocytes. *Toxicol Appl Pharmacol* 2008;232:14–24.
- [22] Heck DE, Laskin DL, Gardner CR, Laskin JD. Epidermal growth factor suppresses nitric oxide and hydrogen peroxide production by keratinocytes. Potential role for nitric oxide in the regulation of wound healing. *J Biol Chem* 1992;267:21277–80.
- [23] Hayes JD, Flanagan JU, Jowsey IR. Glutathione transferases. *Annu Rev Pharmacol Toxicol* 2005;45:51–88.
- [24] Hayes JD, McLellan LI. Glutathione and glutathione-dependent enzymes represent a co-ordinately regulated defense against oxidative stress. *Free Radic Res* 1999;31:273–300.
- [25] Hayes JD, Pulford DJ. The glutathione S-transferase supergene family: regulation of GST and the contribution of the isoenzymes to cancer chemoprotection and drug resistance. *Crit Rev Biochem Mol Biol* 1995;30:445–600.
- [26] Mosialou E, Oiemonte F, Andersson C, Vos RM, van Bladeren PJ, Morgenstern R. Microsomal glutathione transferase: lipid-derived substrates and lipid dependence. *Arch Biochem Biophys* 1995;320:210–6.
- [27] Black HS. Potential involvement of free radical reactions in ultraviolet light-mediated cutaneous damage. *Photochem Photobiol* 1987;46:213–21.
- [28] Tyrrell RM. Ultraviolet radiation and free radical damage to skin. *Biochem Soc Symp* 1995;61:47–53.
- [29] Bender K, Blattner C, Knebel A, Iordanov M, Herrlich P, Rahmsdorf HJ. UV-induced signal transduction. *J Photochem Photobiol* 1997;37:1–17.
- [30] Halliday GM. Inflammation, gene mutation and photoimmunosuppression in response to UVR-induced oxidative damage contributes to photocarcinogenesis. *Mutat Res* 2005;571:107–20.
- [31] Sander CS, Chang H, Hamm F, Elsner P, Thiele JJ. Role of oxidative stress and the antioxidant network in cutaneous carcinogenesis. *Int J Dermatol* 2004;43:326–35.
- [32] Pauloin T, Dutot M, Joly F, Warnet JM, Rat P. High molecular weight hyaluronan decreases UVB-induced apoptosis and inflammation in human epithelial corneal cells. *Mol Vis* 2009;15:577–83.
- [33] Larrosa M, Lodovici M, Morbidelli L, Dolara P. Hydrocaffeic and p-coumaric acids, natural phenolic compounds, inhibit UV-B damage in WKD human conjunctival cells in vitro and rabbit eye in vivo. *Free Radic Res* 2008;42:903–10.
- [34] Lodovici M, Raimondi L, Guglielmi F, Gemignani S, Dolara P. Protection against ultraviolet B-induced oxidative DNA damage in rabbit corneal-derived cells (SIRC) by 4-coumaric acid. *Toxicology* 2003;184:141–7.
- [35] Franco AA, Odom RS, Rando TA. Regulation of antioxidant enzyme gene expression in response to oxidative stress and during differentiation of mouse skeletal muscle. *Free Radic Biol Med* 1999;27:1122–32.
- [36] Scandalios JG. Oxidative stress: molecular perception and transduction of signals triggering antioxidant defenses. *Braz J Med Biol Res* 2005;38:995–1014.
- [37] Zelko IN, Mariani TJ, Folz RJ. Superoxide dismutase multigene family: a comparison of the CuZn-SOD (SOD1), Mn-SOD (SOD2), and EC-SOD (SOD3) gene structures, evolution, and expression. *Free Radic Biol Med* 2002;33:337–49.
- [38] Maines MD, Panahian N. The heme oxygenase system and cellular defense mechanisms. Do HO-1 and HO-2 have different functions? *Adv Exp Med Biol* 2001;502:249–72.
- [39] Patil K, Bellner L, Cullaro G, Gotlinger KH, Dunn MW, Schwartzman ML. Heme oxygenase-1 induction attenuates corneal inflammation and accelerates wound healing after epithelial injury. *Invest Ophthalmol Vis Sci* 2008;49:3379–86.
- [40] Das M, Bickers DR, Santella RM, Mukhtar H. Altered patterns of cutaneous xenobiotic metabolism in UVB-induced squamous cell carcinoma in SKH-1 hairless mice. *J Biol Chem* 1985;84:532–6.
- [41] Sultana S, Alam A, Khan N, Sharma S. Inhibition of benzoyl peroxide and ultraviolet-B radiation induced oxidative stress and tumor promotion markers by cycloartenol in murine skin. *Redox Rep* 2003;8:105–12.
- [42] Wang D, Dubois RN. Eicosanoids and cancer. *Nat Rev Cancer* 2010;10:181–93.
- [43] Andley UP, Becker B, Hebert JS, Reddan JR, Morrison AR, Pentland AP. Enhanced prostaglandin synthesis after ultraviolet-B exposure modulates DNA synthesis of lens epithelial cells and lowers intraocular pressure in vivo. *Invest Ophthalmol Vis Sci* 1996;37:142–53.
- [44] Zhan H, Zheng H. The role of topical cyclo-oxygenase-2 inhibitors in skin cancer: treatment and prevention. *Am J Clin Dermatol* 2007;8:195–200.
- [45] Leahy KM, Ornberg RL, Wang Y, Zweifel BS, Koki AT, Masferrer JL. Cyclooxygenase-2 inhibition by celecoxib reduces proliferation and induces apoptosis in angiogenic endothelial cells in vivo. *Cancer Res* 2002;62:625–31.
- [46] Bazan HE, Birkle DL, Beuerman R, Bazan NG. Cryogenic lesion alters the metabolism of arachidonic acid in rabbit cornea layers. *Invest Ophthalmol Vis Sci* 1985;26:474–80.
- [47] Bazan HE. Cellular and molecular events in corneal wound healing: significance of lipid signalling. *Exp Eye Res* 2005;80:453–63.
- [48] Kenchegowda S, Bazan HE. Significance of lipid mediators in corneal injury and repair. *J Lipid Res* 2009;51:879–91.
- [49] Tjebbes GW, van Delft JL, van Haeringen NJ. Production of lipid mediators in experimental keratitis of rabbit eye. *J Lipid Mediat* 1993;8:87–93.
- [50] Serhan CN, Yacoubian S, Yang R. Anti-inflammatory and proresolving lipid mediators. *Annu Rev Pathol* 2008;3:279–312.
- [51] Liclican EL, Gronert K. Molecular circuits of resolution in the eye. *Scientific-WorldJournal* 2010;10:1029–47.
- [52] Gronert K, Maheshwari N, Khan N, Hassan IR, Dunn M, Laniado Schwartzman M. A role for the mouse 12/15-lipoxygenase pathway in promoting epithelial wound healing and host defense. *J Biol Chem* 2005;280:15267–78.
- [53] Biteman B, Hassan IR, Walker E, Leedom AJ, Dunn M, Seta F, et al. Interdependence of lipoxin A₄ and heme-oxygenase in counter-regulating inflammation during corneal wound healing. *FASEB J* 2007;21:2257–66.
- [54] Bazan NG, Calandria JM, Serhan CN. Rescue and repair during photoreceptor cell renewal mediated by docosahexaenoic acid-derived neuroprotectin D1. *J Lipid Res* 2010;51:2018–31.
- [55] Kyriakis JM, Avruch J. Mammalian mitogen-activated protein kinase signal transduction pathways activated by stress and inflammation. *Physiol Rev* 2001;81:807–69.
- [56] Bode AM, Dong Z. Mitogen-activated protein kinase activation in UV-induced signal transduction. *Sci STKE* 2003;2003:RE2.
- [57] Rosette C, Karin M. Ultraviolet light and osmotic stress: activation of the JNK cascade through multiple growth factor and cytokine receptors. *Science* 1996;274:1194–7.
- [58] Chen W, Tang Q, Gonzales MS, Bowden GT. Role of p38 MAP kinases and ERK in mediating ultraviolet-B induced cyclooxygenase-2 gene expression in human keratinocytes. *Oncogene* 2001;20:3921–6.
- [59] Einspahr JG, Bowden GT, Alberts DS, McKenzie N, Saboda K, Warneke J, et al. Cross-validation of murine UV signal transduction pathways in human skin. *Photochem Photobiol* 2008;84:463–76.
- [60] Bashir MM, Sharma MR, Werth VP. TNF-alpha production in the skin. *Arch Dermatol Res* 2009;301:87–91.
- [61] Matsuura K, Otsuka F, Fujisawa H. Effects of interferons on tumour necrosis factor alpha production from human keratinocytes. *Cytokine* 1998;10:500–5.
- [62] Yoshizumi M, Nakamura T, Kato M, Ishioka T, Kozawa K, Wakamatsu K, et al. Release of cytokines/chemokines and cell death in UVB-irradiated human keratinocytes. *CalCaT. Cell Biol Int* 2008;32:1405–11.