UVB Activates ERK1/2 and p38 Signaling Pathways via Reactive Oxygen Species in Cultured Keratinocytes

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We have previously shown that hydrogen peroxide is an important mediator of ultraviolet B induced phosphorylation of the epidermal growth factor receptor in human keratinocytes. Here we demonstrate that physiologic doses of ultraviolet B and hydrogen peroxide stimulate activation of two related but distinct mitogen-activated protein kinase pathways: extracellular regulated kinase 1 and 2 (ERK1/2), as well as p38, the mammalian homolog of HOG1 in yeast which is a major kinase for a recently identified stress-induced signaling pathway. The time-dependent activation of ERK1/2 and p38 are distinct, and ultraviolet B-induced ERK1/2 activation is downregulated more rapidly than p38. Using dihydrorhodamine or Amplex as specific fluorescent dye probes, we show that ultraviolet B-induced peroxides can be inhibited by ascorbic acid. Ascorbic acid strongly blocks ERK1/2 and p38 activation by ultraviolet B and hydrogen peroxide whereas pyrrolidine dithiocarbamate and butyl hydroxyanisole are less effective. Pyrrolidine dithiocarbamate was unable to inhibit ultraviolet B-induced p38 activation. Cell death was increased after ultraviolet B when ERK1/2 activation was attenuated by the specific inhibitor PD098059. The distinct time courses and extents of activation and inhibition of ERK1/2 and p38 indicate that these pathways are separate and regulated independently in keratinocytes. Specific types of reactive oxygen species induced by ultraviolet B as well as selective activation or inhibition of specific phosphatases may mediate these responses in keratinocytes. These findings demonstrate that reactive oxygen species are important multifunctional mediators of ultraviolet B-induced ERK1/2 and p38 signaling transduction pathways and suggest that ERK1/2 may play an important part in protecting keratinocytes from cell death following oxidative stress. Key words: extracellular regulated kinase/hydrogen peroxide/keratinocytes/mitogen-activated protein kinase/p38/reactive oxygen species/ultraviolet B. J Invest Dermatol 112:751–756, 1999

Ultraviolet radiation (UVR) is the primary cause for the vast majority of cutaneous malignancies each year in the U.S.A. (Miller and Weinstock, 1994). UVR is therefore the most efficient environmental carcinogen known and acts as both a tumor initiator as well as a tumor promotor (Romerdahl et al, 1989; Matsui and DeLeo, 1991). Skin exposure to UVB wavelength radiation, a minor but highly carcinogenic constituent of sunlight (Tornaletti and DeLeo, 1991), induces a range of biologic responses such as erythema, inflammation, hyperpigmentation, hyperplasia, skin cancer (Norris et al, 1993; Young, 1993; Gilchrist et al, 1996), DNA damage and mutations, induction of early and late gene responses (Sachsenmaier et al, 1994; Herrlich et al, 1994), systemic immune suppression (Kripke, 1994), and activation of dormant viruses (Herrlich et al, 1992).

UVR is a potent inducer of reactive oxygen species (ROS) including superoxide radical (·O2–), hydrogen peroxide (H2O2) and hydroxyl radical (·OH), which have been implicated in cutaneous aging as well as cancer and various inflammatory disorders (Cerutti, 1985; Darr and Fridovich, 1994). Among ROS, H2O2 as well as UVR have been reported to upregulate expression of genes such as c-fos and c-jun (Devary et al, 1992; Bender et al, 1997). Transcription of many early genes is mediated by the sequential activation of cytoplasmic protein kinases, and the mitogen-activated protein kinases (MAPK) play a major part in triggering and coordinating these gene responses (Karin, 1995).

Three structurally related but biochemically and functionally distinct MAPK signal transduction pathways have been identified and include the extracellular receptor kinases (ERK), c-jun N-terminal kinases (JNK) and p38 (also known as CSBP, RK, Mkp2), which is the mammalian homolog of yeast HOG1 (Su and Karin, 1996). ERK are primarily activated in response to growth factors and phorbol esters, whereas stress or inflammatory cytokines poorly activate the ERK (Whitmarsh et al, 1995; Xia et al, 1995). JNK, by contrast, participate in growth-factor signaling as well as responding to various stress events that also activate p38, including environmental stresses such as UVR and inflammatory cytokines (Raina and Kastan, 1996). Epidermal growth factor (EGF) and phorbol esters have been reported to be poor activators of p38 (Derijard et al, 1995; Rouse et al, 1994).

In comparison with the well-established function of ERK within the Ras/Raf/MEK/ERK signaling cascade, the mechanism of activation of p38 by UVB as well as its biologic role(s) remain to be elucidated. The focus of this investigation was to identify more
precisely the role of ROS, in particular H$_2$O$_2$, on the activation and regulation of ERK1/2 and p38 signal transduction pathways in normal human keratinocytes following exposure to physiologic doses of UVB radiation.

MATERIALS AND METHODS

Materials and chemicals The UVB source was a FS20 lamp (Westinghouse, Pittsburgh, PA) that emits an energy spectrum with high fluence in the UVB region and a peak at 313 nm (Peus et al., 1988). To block UVC emission a WG-295 long pass filter was used (Schott Glass Technologies, Duryea, PA). Experiments using a WG-295 long pass filter showed that differences in phosphorylated levels of p38 and ERK1/2 are only minimal when unfiltered and filtered UVB-radiation sources were compared. With longer incubation periods (30 min) a slightly stronger response was observed after exposure to unfiltered UVB radiation. The emitted dose was regularly quantitated by a UVB radiometer and photodetector (IL 443 and SEE 240, International Light, Newburyport, MA). Ascorbic acid (AA), butylated hydroxyanisole (BHA), and pyrrolidinecarboxamide (PDTC), were purchased from Sigma (St. Louis, MO). Dihydrorhodamine 123 (DHR), Amplex Red reagent and LIVE/DEAD Viability/Cytotoxicity Kit were obtained from Molecular Probes (Eugene, OR).

Culture and treatment of normal human keratinocytes Normal human keratinocytes were isolated from neonatal foreskin specimens, and primary cultures were initiated and maintained in a replicative state with complete, serum-free MCDB 153 medium. Keratinocytes from primary cultures were plated into secondary culture at 1–10 $\times$ 10$^5$ cells per cm$^2$. Complete medium was supplemented with 0.1 mM calcium, 0.2% bovine pituitary extract (BPE), EGF (10 ng per ml), insulin (5 $\mu$g per ml), hydrocortisone (5 $\times$ 10$^{-7}$ M), ethanolamine (1 $\times$ 10$^{-4}$ M), phosphoethanolamine (1 $\times$ 10$^{-4}$ M), and additional amino acids (Wille et al., 1984; Pittelkow and Scott, 1986). Cells were grown to confluence and fed with standard medium (without growth factors) for at least 48 h to obtain quiescent cells with low levels of activated ERK1/2 and p38 prior to experiments examining UVB irradiation or H$_2$O$_2$ treatment. All other assays were performed in confluent keratinocytes cultured in complete medium. No significant differences were observed whether cells were irradiated in phosphate-buffered saline or standard medium.

Detection of intracellular H$_2$O$_2$ by flow cytometric analysis Intracellular levels of H$_2$O$_2$ were analyzed using DHR as a specific fluorescent dye probe as described previously (Peus et al., 1998). Briefly, confluent keratinocytes in a 24 well tissue culture plate were loaded with DHR (5 $\mu$M) for 45 min and washed before treatment with UVB. In the intracellular release of H$_2$O$_2$, reduced DHR is irreversibly oxidized and converted to the red fluorescent compound rhodamine 123 (Wille et al., 1984; Pittelkow and Scott, 1986). Plates were read on a Cytofluor II, fluorescence plate reader (PerSeptives Biosystems, Framingham, MA) with an excitation wavelength of 485 nm and an emission wavelength of 530 nm. Each bar represents the mean value of triplicate wells ± SD.

Extracellular H$_2$O$_2$ assay Measurement of extracellular H$_2$O$_2$ following UVB exposure was performed using Amplex a derivative of dihydrophenoxazine, which becomes highly fluorescent upon oxidation by H$_2$O$_2$. Measurement of extracellular H$_2$O$_2$ was performed using a microplate fluorometer. The excitation/emission wavelength filter was set at 590/615 nm. Fluorescence intensity of Amplex was measured within the linear region of the H$_2$O$_2$ dose–response curve. Wells without cells displaying the same substrates and exposed to the same treatment represented controls. The control fluorescent values and background fluorescence of untreated cells were monitored over time and subtracted from the relative fluorescence intensity of treated samples. These values were converted into absolute pmoles using an H$_2$O$_2$ standard curve. All H$_2$O$_2$ measurements were performed in 24-well plates containing confluent keratinocytes, 100 $\mu$M Amplex and 1 U per ml horseradish peroxidase (Mohanth et al., 1997). Each bar represents the mean value of triplicate wells ± SD.

Detection of activated ERK1/2 and p38 Normal human keratinocytes were extracted in a lysis buffer supplemented with protease and phosphatase inhibitors. Endogenous, activated forms of ERK1 and ERK2 were detected using 20 $\mu$g of total protein and the anti-ACTIVE MAPK antibody polyclonal antibody (Promega, Madison, WI) that selectively recognizes activated ERK1/2 kinase (Sonoda et al., 1997). For detection of activated p38, phospho-specific p38 polyclonal antibody was used (Wilhelm et al., 1997). A nonspecific band of higher molecular weight than p38 was also observed but was not detected with newer lots of the phospho-specific p38 antibody. Phospho-specific and total p38 antibodies were purchased from New England BioLabs (Beverly, MA). ERK1/ERK2 proteins were detected using the anti-ERK1 C-16 antibody (Santa Cruz Biotechnology, Santa Cruz, CA), and equivalent loading of ERK1/2 and p38 proteins was documented for each series of experiments. Samples were separated electrophoretically by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Proteins were electrotransferred to Immobilon-p (Millipore, Bedford, MA), and after blocking and incubation with secondary antibody coupled to peroxidase, bands were visualized using the ECL detection system (Amersham, Arlington Heights, IL).

Cell death assay Cell death was determined using the ethidium homodimer/calcein acetoxymethyl ester Viability/Cytotoxicity Kit (Molecular Probes, Eugene, OR) combination of vital dyes. Confluent keratinocytes in 24 well cell culture plates were treated with UVB, and 24 h later fluorescence was analyzed in the Cytofluor II microplate fluorometer. Results are representative of three independent experiments (mean ± SD).

RESULTS

Differential activation of ERK1/2 and p38 by UVB and H$_2$O$_2$. For selected cell types, ERK1/2 and p38 signaling pathways have been shown to be biochemically separate and distinct. To characterize specific cellular responses mediated by these pathways in keratinocytes, activation of ERK1/2 and p38 by UVB and H$_2$O$_2$ over time was evaluated. Levels of activated ERK1/2 and p38 were examined 5–120 min following treatment of keratinocytes with 200 J per m$^2$ UVB (Fig 1a) or 200 $\mu$M H$_2$O$_2$ (Fig 1b). Within 5 min of UVB treatment, ERK1/2 activation was maximally upregulated. By 30 min after irradiation, the signal declined and approached baseline level by 60 min (Fig 1a). In contrast, as shown in Fig 1a, activated p38 only reached a maximum level by 30 min with minimal decrease after 2 h. Interestingly, unlike UVB stimulation, H$_2$O$_2$ treatment showed similar profiles of ERK1/2 and p38 activation, with an increase by 5 min and maximum

Figure 1. Differential stimulation of ERK1/2 and p38 by UVB and H$_2$O$_2$. Confuent, resting human keratinocytes were exposed to (a) UVB (200 J per m$^2$) or (b) H$_2$O$_2$ (200 $\mu$M) and monitored for 5–120 min. Total cell protein was extracted at the indicated times, separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes and immunoblotted with anti-ACTIVE ERK1/2, anti-phospho-p38 antibody or total p38 antibody as described in Materials and Methods. One representative experiment is shown of three separate experiments.
phosphorylated levels reached by 15 min (Fig 1b). Decrease in p38 activation was observed within 45 min versus 60 min for ERK1/2 activation. Total ERK1/2 and p38 protein levels at all time points were equivalent as measured by immunoblot of the same stripped membranes using antibodies against total ERK1/2 (data not shown) and p38 (Fig 1b) as described in Materials and Methods. These results demonstrate marked differences in the activation of ERK1/2 and p38 following UVB exposure, but similar activation after H2O2 treatment in keratinocytes.

**UVB and H2O2 activate ERK1/2 and p38 in a concentration-dependent manner**

Human keratinocytes were irradiated with 50–800 J UVB per m2 and incubated for 15 or 30 min. Concentration-dependent increase in kinase phosphorylation was observed that was more marked for p38 than for ERK1/2 activation (Fig 2a). Increasing concentrations of H2O2 (10–400 µM) stimulated ERK1/2 and p38 activation in a concentration-dependent manner. ERK1/2 phosphorylation was enhanced at 30 µM H2O2, whereas significant p38 activation was observed only by 100 µM H2O2 (Fig 2b). Peak ERK1/2 activation was reached by 100 µM H2O2 versus 200 µM H2O2 for p38 activation. Further increase in ERK1/2 or p38 activation by higher concentrations of H2O2 was not observed (data not shown). Total p38 protein levels remained unchanged over the time course (Fig 2b). These results show that both ERK1/2 and p38 are activated by UVB or H2O2 in a concentration-dependent manner.

**Intracellular H2O2 production and H2O2 release induced by UVB is inhibited by ascorbic acid in a concentration-dependent manner**

We have previously shown that H2O2 is generated rapidly and in a concentration-dependent manner by human keratinocytes irradiated with UVB, and is inhibited by the anti-oxidants PDTC and BHA (Peus et al., 1998). To determine the effects of the anti-oxidant AA on intracellular H2O2 production in human keratinocytes, cells were loaded with DHR, pretreated with AA then irradiated with UVB and incubated for 30 min. The relative fluorescent intensity of cells exposed to 400 J UVB per m2 was specifically inhibited in a concentration-dependent manner by 30–300 µM AA (Fig 3a). In addition, levels of extracellular H2O2 produced and released in unstimulated human keratinocytes were also attenuated concentration-dependently by AA after UVB irradiation.
Antioxidants AA, PDTC, and BHA modulate ERK1/2 and p38 phosphorylation by UVB. Human keratinocytes pretreated for 30 min with 100 or 200 μM of AA prior to 200 J UVB per m² exposure resulted in potent inhibition of p38 and ERK1/2 phosphorylation (Fig 4a). Addition of 10 μM BHA to the medium 30 min before exposure to 200 J UVB per m² attenuated ERK1/2 and p38 activation (Fig 4b). Three micromoles of PDTC, however, potently inhibited ERK1/2 activation but failed to inhibit UVB-induced p38 activation. Both PDTC and BHA inhibit intracellular peroxide production but are less effective in blocking H₂O₂-induced ERK1/2 phosphorylation (data not shown). All three anti-oxidants AA and BHA attenuate levels of ROS induced in keratinocytes by UVB exposure and also down-regulate activation of ERK1/2 and p38, whereas PDTC failed to inhibit p38 activation. These findings provide further evidence for differential regulation of ERK1/2 and p38 activation in keratinocytes.

Specific inhibition of ERK1/2 by PD098059 after UVB or H₂O₂ exposure. PD098059 has been reported to inhibit specifically ERK1/2 activation in different cell types (Alessi et al., 1995b; Dudley et al., 1995). We determined whether this compound inhibits activation of ERK1/2 following pretreatment of human keratinocytes for 30 min and exposure to 400 J UVB per m² or 200 μM H₂O₂, PD098059 potently inhibited ERK1/2 activation stimulated by UVB (Fig 5a) and H₂O₂ (Fig 5b) in a concentration-dependent manner with maximum inhibition at 30 or 3 μM, respectively. No significant inhibition of p38 phosphorylation was observed. Therefore, UVB- or H₂O₂-induced activation of ERK1/2 can be potently and selectively blocked by PD098059 in keratinocytes.

Inhibition of ERK1/2 results in enhanced cell death after UVB. To examine the biologic significance of ERK1/2 blockade, we analyzed the effect of PD098059 on cell survival after UVB exposure. Using the sensitive Viability/Cytotoxicity Kit (Molecular Probes), we observed that 24 h after irradiation with 400 J UVB per m² cell death increased significantly in cells treated with PD098059 compared with irradiated control without the ERK1/2 inhibitor (Fig 6). In separate experiments the ratio of apoptotic to necrotic cells was determined by using DAPI and propidium iodide staining and enumeration using fluorescence microscopic visualization. Twenty-four hours after UVB irradiation, the ratio of apoptotic to necrotic cells was ≈2:3, and remained unchanged with or without PD098059 (data not shown). The results indicate that ERK1/2 activation following UVB exposure plays an important part in mediating keratinocyte survival in the UVB response.

DISCUSSION
The biologic mediators of various cellular responses to UVR are only beginning to be identified and characterized. Our observations provide new evidence for the regulation of ERK1/2 and p38 signal transduction pathways in keratinocytes exposed to UVB or H₂O₂. Previous studies have used UVC irradiation, largely at supraphysiologic doses, and tested immortalized or carcinoma cell lines where intracellular signal transduction pathways are frequently perturbed as part of the transformation process. Therefore, this investigation was designed to examine doses of 50–800 J UVB per m² that are in the range of a minimal erythema dose (MED) under standard skin testing conditions for skin types I–III and typically induce the physiologic response of solar erythema (Norris et al., 1993). As a
UVB-induced H$_2$O$_2$ may mediate ERK1/2 activation through a concentration-dependent manner, and this reduction closely parallels with inhibition of EGFR phosphorylation (Peus et al., 1998) and ERK1/2 activation (Fig 4a). Therefore, we suggest that UVB-induced H$_2$O$_2$ may mediate ERK1/2 activation through upstream substrates such as ras, raf, and MEK1 and 2 as a result of EGFR phosphorylation. In addition, the distinct levels and kinetics of activation and inhibition of ERK1/2 and p38 strongly implicate independent mechanisms of regulation of each pathway in keratinocytes.

result, the dose range of UVB that induces ERK1/2 and p38 pathway activation in cultured keratinocytes has physiologic relevance for epidermis and skin.

In addition to characterizing the concentration- and time-dependent activation of ERK1/2 and p38 pathways following UVB or H$_2$O$_2$ treatment, we examined potential mechanisms by which these pathways are activated and regulated. Recently, it has been shown that H$_2$O$_2$ induces ERK activation in different immortalized cell lines (Guyton et al., 1996; Rao, 1996; Zhao et al., 1996). It is not clear, however, how cellular stress selectively triggers p38, and to a lesser extent ERK1/2, pathway activation in the cytoplasm. It has been shown that MAPK are activated by dual phosphorylation on threonine and tyrosine within protein kinase subdomain VIII (Davis, 1994). ERK are activated by MEK1 and MEK2 (Cobb and Goldsmith, 1995), and p38 by MKK3, MKK4, and MKK6 (Derijard et al., 1995; Raingeaud et al., 1996). Upon stimulation, these kinases translocate into the nucleus where they selectively activate several different transcription factors and are then rapidly inactivated by dual specific phosphatases (reviewed in Keyse, 1995).

ROS have been implicated as potential mediators of signaling from the membrane to nucleus, including phosphorylation of EGF receptors (EGFR) (Peus et al., 1998), association of SOS, Gab2, and shc with EGFR (Huang et al., 1996), and ERK1/2 activation following UV exposure (Assafa et al., 1997). UVB-induced EGFR phosphorylation, similar to ligand-induced autophosphorylation of EGFR (Peus et al., 1997) was inhibited by anti-oxidants and catalase, and these effects were mimicked by H$_2$O$_2$, which was found to be generated within keratinocytes after UVB exposure (Peus et al., 1998). H$_2$O$_2$ is generated in the cell following UVB irradiation, stimulates phosphorylation of EGFR, and therefore plays an important part as a cellular mediator. This hypothesis is supported by the observations that structurally different anti-oxidants including PDTC, BHA, and AA inhibit UVB-induced H$_2$O$_2$ formation in a concentration-dependent manner, and this reduction closely correlates with inhibition of EGFR phosphorylation (Peus et al., 1998) and ERK1/2 activation (Fig 4). Therefore, we suggest that UVB-induced H$_2$O$_2$ may mediate ERK1/2 activation through upstream substrates such as ras, raf, and MEK1 and 2 as a result of EGFR phosphorylation. In addition, the distinct levels and kinetics of activation and inhibition of ERK1/2 and p38 strongly implicate independent mechanisms of regulation of each pathway in keratinocytes.

The finding that PDTC and BHA differently inhibit UVB- and H$_2$O$_2$-induced ERK1/2 and p38 activation (Fig 4b) as well as the dose disparity of other anti-oxidants required for inhibition (Fig 4a) indicate that the regulation of these pathways by ROS is distinct, and their responsiveness may differ depending on the types of ROS involved. Within 30 min of UVB exposure, ERK1/2, but not p38, activation is markedly downregulated (Fig 1a), whereas both ERK1/2 and p38 activation induced by H$_2$O$_2$ are attenuated within 60 and 30 min after treatment, respectively (Fig 1b). Dephiroshorylation of activated ERK1/2 and p38 may be mediated by the recently isolated and characterized family of dual-specificity (Thr/Tyr) MAP kinases (MKP), comprised mainly of MKPs-1–4 (Chu et al., 1996; Muda et al., 1996) which are inducible by cellular stress and rapidly localize to the nucleus (Keyse, 1995). ERK, however, are more likely than p38 to be inactivated by protein phosphatase 2A and a protein tyrosine phosphatase distinct from MKPs (Alessi et al., 1995a), as MKP-1, 2, and 4 predominantly inactivate JNK and p38 MAPK (Muda et al., 1996; Hirsch and Stork, 1997). Further investigation is required to delineate more precisely the mechanisms and specific regulators of stress-induced MAPK inactivation. Our observations suggest that both the ERK1/2 and p38 pathways are not only distinct and differentially regulated by oxidative stress in keratinocytes, but different types of ROS may preferentially mediate UVB-induced activation of ERK1/2 or p38 signaling pathways.

The role of ERK1/2 activation in cell survival remains controversial. In cardiac myocytes, inhibition of ERK increased apoptotic cell death (Aikawa et al., 1997) and cardiotoxin 1 promotes survival via activation of a signaling pathway requiring ERK (Sheng et al., 1997). In hematopoietic cells, activation of ERK partially prevents apoptotic death (Kinoshita et al., 1997). By contrast, ERK1/2 inhibition had no significant effect on the insulin- or brain-derived neurotrophic factor-induced survival of cerebellar granule cells (Gunn-Moore et al., 1997). In this study with keratinocytes, PD098059 specifically inhibits ERK1/2 (Fig 5) as has been reported for other cell types (Alessi et al., 1995b; Dudley et al., 1995). Here we show that inhibition of ERK1/2 increases UVB-induced cell death, indicating that ERK1/2 plays an important part in protecting keratinocytes from cytocytotoxicity following oxidative stress.

Based on these findings, we speculate that the balance between pro-oxidative and anti-oxidative mechanisms within the keratinocyte will determine the levels of activation of downstream signaling cascades such as EGFR/ERK1/2 and p38 that are differentially triggered by UVB irradiation. Collectively, these results provide strong evidence that ROS, in particular H$_2$O$_2$, act as mediators to activate ERK1/2 and p38 pathways and induce cellular responses following UVB exposure. These observations will be useful in focusing future investigations on specific UVR-related intracellular signaling pathways of keratinocytes and the epidermis. Early activation of signaling pathways in response to UVR may be involved in the erythema and inflammatory reactions, photoaging various photodermatoses, and carcinogenesis.


