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Differential role of hydrogen peroxide in UV-induced signal transduction

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Abstract

The present study investigated the differential requirement of ROS in UV-induced activation of these pathways. Exposure of the mouse epidermal Cl41 cells to UV radiation led to generation of ROS as measured by electron spin resonance (ESR) and by H_2O_2 and $O_2^{\cdot-}$ fluorescence staining assay. Treatment of cells with UV radiation or H_2O_2 also markedly activated Erks, JNKs, p38 kinase and led to increases in phosphorylation of Akt and $p70^{S6k}$ in mouse epidermal JB6 cells. The scavenging of UV-generated H_2O_2 by N-acety-L-cyteine (NAC, a general antioxidant) or catalase (a specific H_2O_2 inhibitor) inhibited UV-induced activation of JNKs, p38 kinase, Akt and $p70^{S6k}$, while it did not show any inhibitory effects on Erks activation. Further, pretreatment of cells with sodium formate (an $^{\cdot}OH$ radical scavenger) or superoxide dismutase ($O_2^{\cdot-}$ radical scavenger) did not inhibit any of these pathways. These results demonstrate that H_2O_2 generation is required for UV-induced phosphorylation of Akt and $p70^{S6k}$, and involved in activation of JNKs and p38 kinase, but not Erks. (Mol Cell Biochem **234/235**: 81–90, 2002)

Key words: oxidative stress, antioxidants, oxyradical scavengers, MAP kinases

Abbreviations: PKB – protein kinase B; PKC – protein kinase C; UV – ultraviolet; AP-1 – activated protein-1; aPKC – atypical protein kinase C; MAP kinase – mitogen activated protein kinase; JNK – c-Jun N-terminal kinase; TPA – 12-*O*-tetradecanoyl phorbol-13-acetate; PH – pleckstrin homology; NAC – N-acety-L-cyteine; NADPH – β-nicotinamide adenine dinucleotide phosphate; SOD – superoxide dismutase; Di – DiOC6; Da – DCFH-DA; HE – Dihydroethidium; FBS – fetal bovine serum; MEM – Eagle's minimal essential medium; ROS – reactive oxygen species

Introduction

Skin cancer is one of the most common human cancers [1-3]. Ultraviolet radiation (UV[1]) from solar light elicits a number of biological effects in the skin, including pigmentation, erythema, cancer and cell death [1–6]. Of particular concern is the role of UV as a major etiologic factor in human skin cancer [1]. UV-induced epidermal tumors have been extensively studied in various animal models [3–7]. UV radiation is believed to not only act primarily at the initiation stage of tumor development due to its direct effects on cellular DNA [6, 7], but also acts as a tumor promoter by eliciting alternations of gene expressions [6, 7]. While the mechanism behind the tumor inductive ability of UV is not well understood, it is believed that UV-initiated signal transduction pathways, such as MAP kinase pathways, are involved in this process [8–11]. It is speculated that UV radiation can generate reactive oxygen species (ROS) near or within the cellular membrane, which subsequently elicit the protein kinase cascades, and in turn activate transcription factors and lead to gene expression [10, 11].

Akt and $p70^{S6k}$ as well as MAP kinase family, including Erks, JNKs, p38 kinase, are Ser/Thr kinases that are activated in response to stress and a variety of mitogens, such as TPA and growth factors [12–15]. The activation of these kinases is due to Ser/Thr residue phosphorylation on multiple sites. Four phosphorylation sites on Akt have been identified that are phosphorylated *in vivo*, including Ser^{124}, Thr^{308}, Thr^{450}, and

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Ser[473] [16]. Thr[308] and Ser[473] of Akt are inducibly phosphorylated after treatment of cells with extracellular stimuli, whereas Ser[124] and Thr[450] appear to be basally phosphorylated [16, 17]. Mutagenesis studies have demonstrated that phosphorylation of Thr[308] and Ser[473] is required for Akt activation, and that mimicking phosphorylation partially activates Akt [17, 18]. Phosphorylation sites of p70[S6k] include Thr[229], Thr[389], Ser[404], Ser[411], Ser[418], Ser[424], and Thr[421] [19–21]. Upon activation, p70[S6k] phosphorylates the S6 protein of the 40S ribosomal subunit [22]. Phosphorylated S6 directs the translational machinery toward increasing the production of translational machinery, such as ribosomal proteins and elongation factors [23]. In the present study, we investigated the differential role of H_2O_2 in UV-induced signal transduction pathways.

Materials and methods

Chemical reagents and antibodies

Deferoxamine, N-acety-L-cyteine (NAC), β-nicotinamide adenine dinucleotide phosphate (NADPH), superoxide dismutase (SOD), and sodium formate were purchased from Sigma (St. Louis, MO, USA); dichlorofluorescin diacetate (Da) and dihydroethidium (HE) were purchased from Molecular Probe (Eugene, OR, USA); fetal bovine serum (FBS) and Eagle's minimal essential medium (MEM) were from BioWhittaker (Walkersville, MD, USA); antibodies which specifically recognize phospho- and total-MAP kinase family members, including Erks, JNKs and p38 kinase, phospho-specific Akt (Thr[308]) antibody, phospho-specific Akt (Ser[473]) antibody and Akt antibody as well as phospho-specific p70[S6k] (Thr[389]) antibody, phospho-specific p70[S6k] antibody and p70[S6k] antibody were purchased from New England Biolabs (Beverly, MA, USA).

Cell culture

The JB6 P[+] mouse epidermal cell line, Cl 41, was cultured in monolayers at 37°C, 5% CO_2 using MEM containing 5% fetal calf serum (FBS), 2 mM L-glutamine, and 25 μg gentamicin per ml as described previously [24].

Cellular superoxide ($O_2^{\bullet-}$) and H_2O_2 staining assay

HE is a specific $O_2^{\bullet-}$ dye [25, 26], while Da has been frequently used to monitor H_2O_2 levels in cells [25, 26]. The cells were seeded in 6-well plates and cultured until 90% confluent. The cells were then treated with UVB (4 KJ/m^2) or UVC (60 J/m^2) radiation. After 30 min culturing, HE or Da (both dissolved in DMSO and diluted with PBS to final concentrations of 5 μM) was applied to the cells and incubated for another 15–20 min at 37°C. The cells were washed twice with PBS, harvested, and applied on slides for observation under fluorescence microscope.

Electron spin resonance (ESR) measurements

ESR spin trapping was used to detect short-lived free radical intermediates. This technique involves the addition-type reaction of a short-lived radical with a diamagnetic compound (spin trap) to form a relatively long-lived free radical product, the so-called spin adduct, which can be observed by conventional ESR. The intensity of the spin adduct signal corresponds to the amount of short-lived radicals trapped, and the hyperfine splittings of the spin adduct are generally characteristic of the trapped radical. ESR measurements were carried out using a Varian E9 ESR spectrometer and a flat cell assembly. Hyperfine couplings were measured (0.1 G) directly from magnetic field separation using potassium tetraperoxochromate (K_3CrO_8) and 1,1-diphenyl-2-picrylhydrazyl as reference standards. Cl 41 cells were seeded in 100 mm dishes and cultured until 90% of confluent. The cells were washed once with PBS and mixtures of 400 mM DMPO and 100 μM NADPH were added to each dish. The cells were then exposed to UVB radiation (4 KJ/m^2) for 4 min. The cells were harvested and transferred to a flat cell for ESR measurement as described previously [26, 27].

Western blot analysis

JB6 C1 41 cells were cultured in each well of 6-well plates or in 100 mm dishes to 90% of confluent with 5% FBS MEM medium. The cells were exposed to either UVB (4 KJ/m^2) or UVC (60 J/m^2). The cells were then washed once with ice-cold PBS and extracted with SDS-sample buffer. The cell extracts were separated on polyacrylamide-SDS gels, transferred and probed with one of the specific antibodies. The protein band specifically bound with primary antibodies

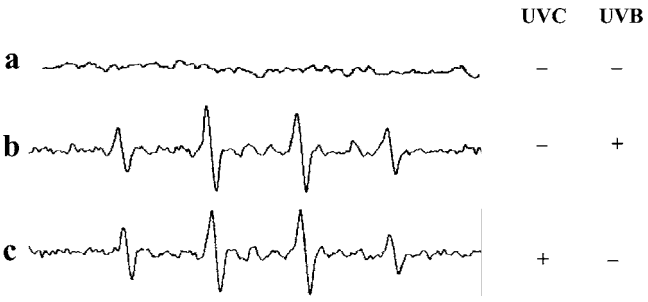

Fig. 1. Measurement of UV-induced ROS generation by ESR. ESR spectra were recorded 7 min after cells were exposed to UVB (4 KJ/m^2) or UVC (60 J/m^2) in a 100 mm dish with 90% confluent Cl41 cells, 400 mM DMPO, and 100 μM NADPH.

was detected by using an anti-rabbit IgG-AP-linked and ECF western blotting system [28].

Results

Generation of Reactive Oxygen Species (ROS) by UV radiation

To determine ROS generation directly in UV-radiated culturing cells, dye staining and ESR techniques were used. The results from ESR studies showed that cells without UV did not generate any detectable amount of free radicals (Fig. 1a). Exposure of cells to UV radiation generated a 1:2:2:1 ESR spectrum (Figs 1b and 1c) with hyperfine splittings of $a_H = a_N = 14.9G$, where a_N and a_H denote hyperfine splittings of the nitroxyl nitrogens and a-hydrogen, respectively. Based on these splittings and the 1:2:2:1 line shape, the spectrum was assigned to the DMPO/$^{\bullet}$OH adduct, which is evidence of $^{\bullet}$OH radical generation. Measurements using HE, a specific fluorescent dye for $O_2^{\bullet-}$, or Da, a fluorescent dye for H_2O_2, demonstrate that exposure of cells with UV radiation led to an increase in the generation of both $O_2^{\bullet-}$ and H_2O_2 (Fig. 2). These results provided direct evidence that UV radiation can

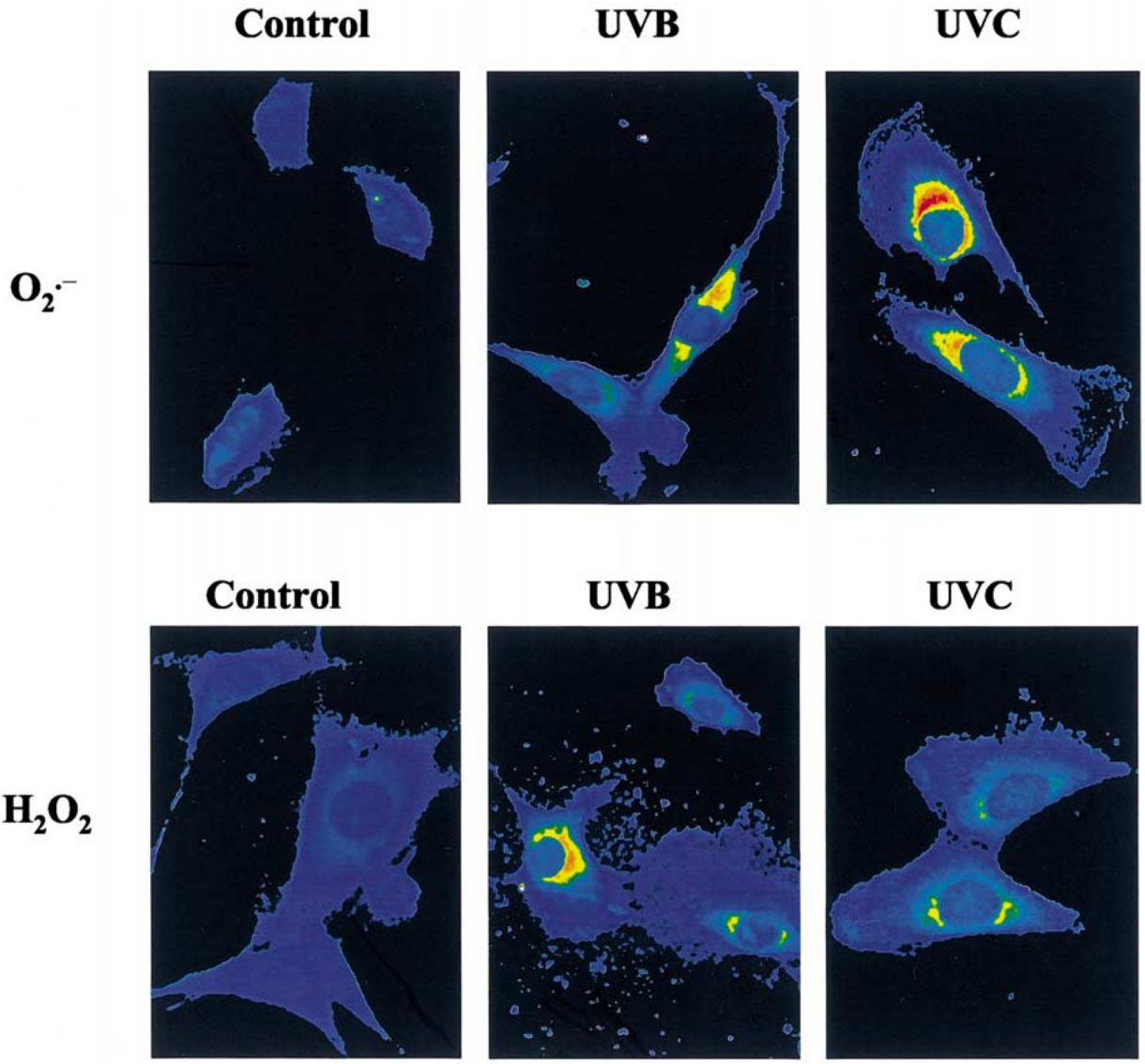

Fig. 2. Determination of $O_2^{\bullet-}$ and H_2O_2 by HE and Da staining. Cl41 cells were seeded in 6-well plates and cultured until 90% confluent. The cells were then exposed to UVB (4 KJ/m^2) or UVC (60 J/m^2) radiation for 30 min. HE or Da was applied to the cells and incubated for another 15–20 min at 37°C. The cells were washed twice with PBS. The cells then were harvested, applied on slides for observation under a fluorescence microscope.

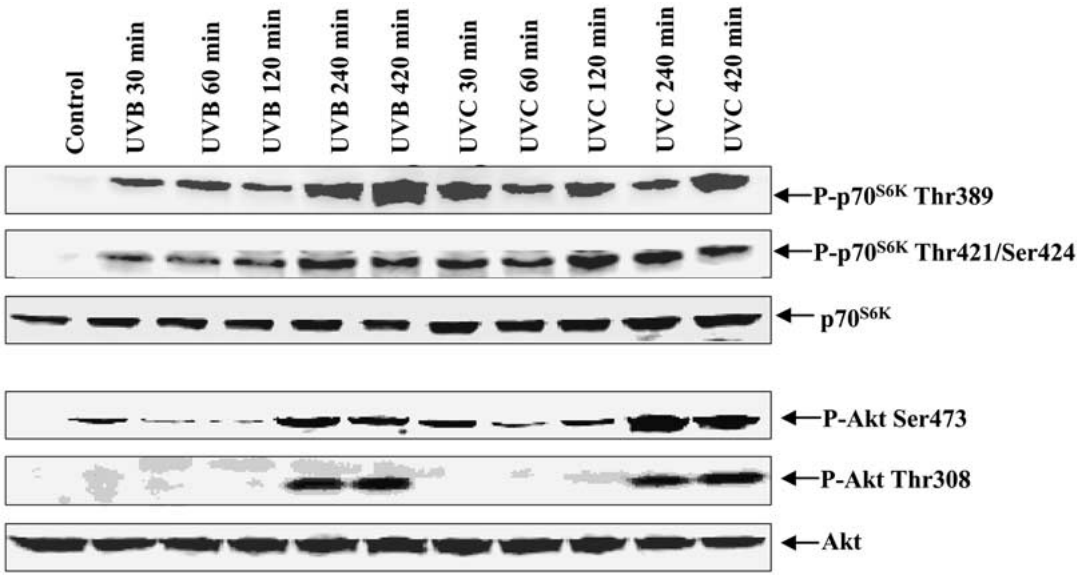

Fig. 3. Induction of phosphorylation of Akt and $p70^{S6k}$ by UV radiation. Subconfluent (90%) monolayers of JB6 C1 41 cells in 100 mm dishes were subjected to either UVC (60J/m^2) or UVB (4 KJ/m^2) and cultured for time points as indicated. The cells were then washed once with ice-cold PBS and extracted with SDS-sample buffer. The cell extracts were separated on polyacrylamide-SDS gels, transferred and probed with one of the antibodies. The protein band specific bound with primary antibodies was detected by using an anti-rabbit IgG-AP-linked and ECF Western blotting system [47].

induce generation of a whole spectrum of ROS in mouse epidermal cells.

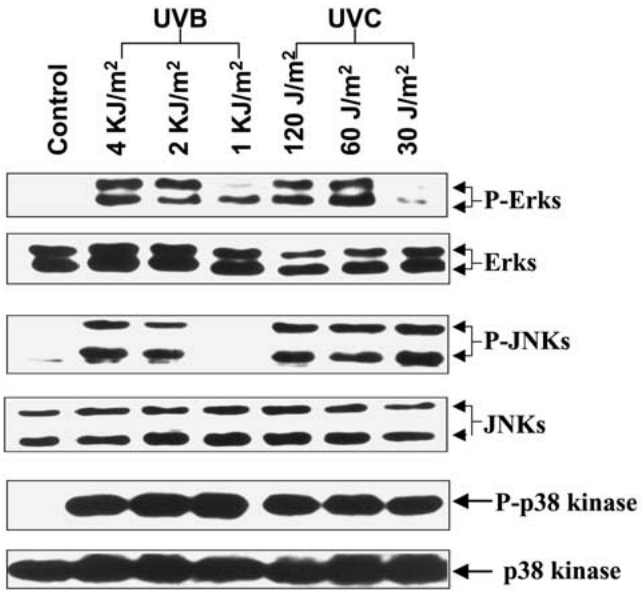

Fig. 4. Activation of Erks, JNKs and p38 kinase by UV radiation. Subconfluent (90%) monolayers of JB6 C1 41 cells in 100 mm dishes were subjected to different doses of either UVC or UVB. The cells were then washed once with ice-cold PBS and extracted with SDS-sample buffer. The cell extracts were separated on polyacrylamide-SDS gels, transferred and probed with one of the antibodies. The protein band specific bound with primary antibodies was detected by using an anti-rabbit IgG-AP-linked and ECF Western blotting system [47].

UV Radiation induces phosphorylation of Akt at Ser^{473} and Thr^{308} and $p70^{S6k}$ at Thr^{389} and Thr ^{421}/Ser^{424}

To investigate the induction of phosphorylation of Akt and $p70^{S6k}$ in the UV response, we exposed the Cl 41 cells to either UVB or UVC radiation. The results show that UV radiation markedly induced phosphorylation of Akt at Ser^{473} and Thr^{308} and $p70^{S6k}$ at Thr^{389} and Thr ^{421}/Ser^{424} in a time-dependent manner (Fig. 3). The maximum phosphorylation induction of Akt and $p70^{S6k}$ in response to either UVB or UVC radiation occurred between 4–7 h after exposure (Fig. 3).

Activation of Erks, JNKs and p38 kinase by UV radiation

Exposure of cells to UV radiation also induced significantly activation of MAP kinase, including Erks, JNKs and p38 kinase (Fig. 4).

Effects of H_2O on activation of Erks, JNKs, p38 kinase and phosphorylation of Akt and $p70^{S6k}$

The results from above studies demonstrated that UV radiation can generate ROS and induces increases in phosphorylation of Akt, $p70^{S6k}$ and MAP kinase. If ROS are involved in UV-induced activation of signaling, exposure of cells to H_2O_2 may lead to the activation of these signal transduction pathways. As shown in Fig. 5, treatment of cells with H_2O_2

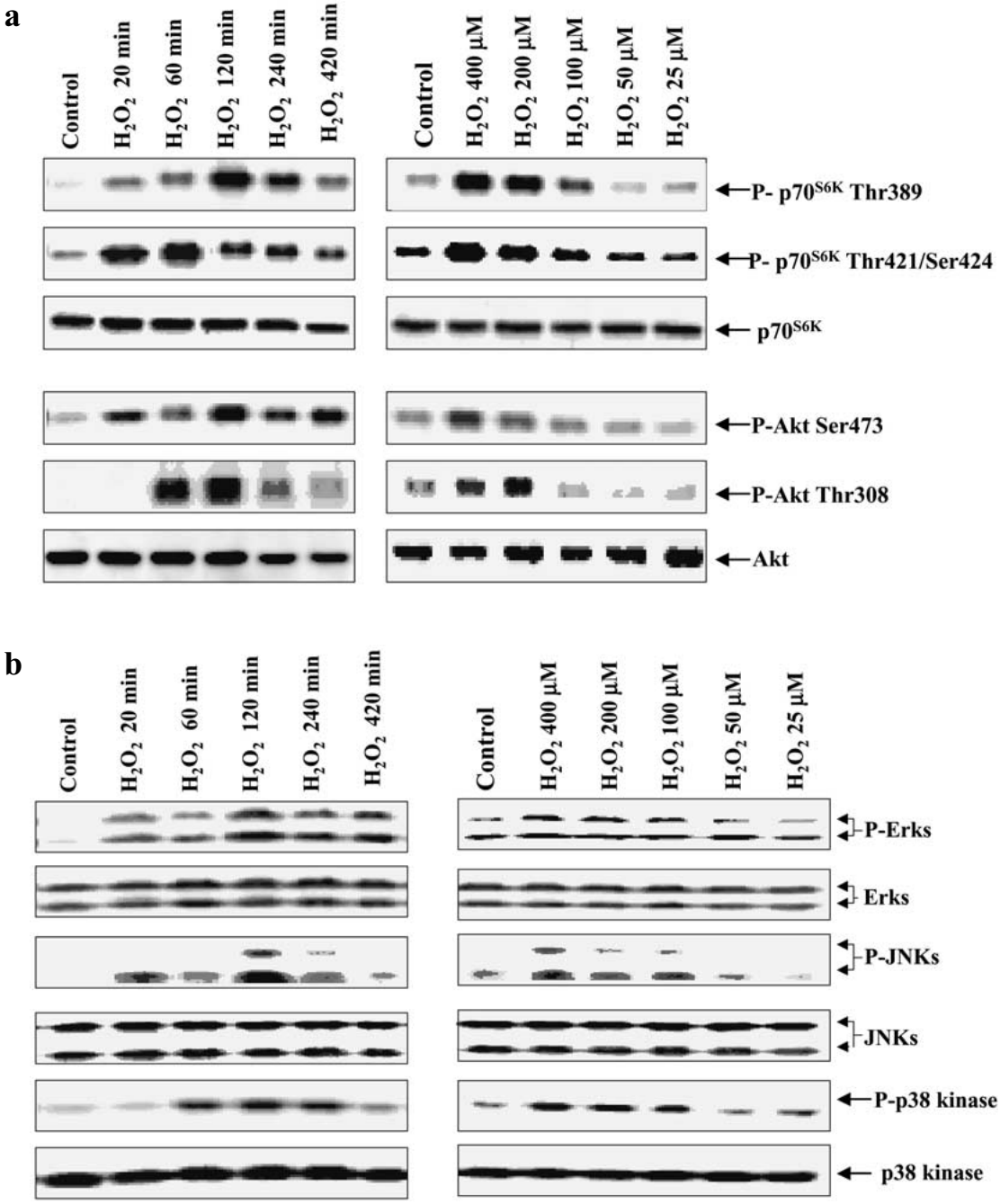

Fig. 5. Induction of phosphorylation of Akt and p70^{S6k} (a) and activation of Erks, JNKs and p38 kinase (b) by H_2O_2. Subconfluent (90%) monolayers of JB6 C1 41 cells in 6-well plates were subjected to different doses of H_2O_2 for 120 min.; or 200 µM of H_2O_2 for time points as indicated. The cells were then washed once with ice-cold PBS and extracted with SDS-sample buffer. The cell extracts were separated on 8% polyacrylamide-SDS gels, transferred and probed with one of the antibodies. The protein band specific bound with primary antibodies was detected by using an anti-rabbit IgG-AP-linked and ECF Western blotting system [28].

resulted in increases in phosphorylation of Akt and p70^{S6k} (Fig. 5a) and activation of Erks, JNKs and p38 kinase (Fig. 5b) in a time- and dose-dependent manner. The results revealed that H_2O_2 might be involved in UV-induced activation of signal transduction pathways.

Generation of H_2O_2 is required for phosphorylation of Akt and p70^{S6k} induced by UV radiation

To obtain direct evidence for the involvement of ROS in phosphorylation of Akt and p70^{S6k} in UV responses, the ef-

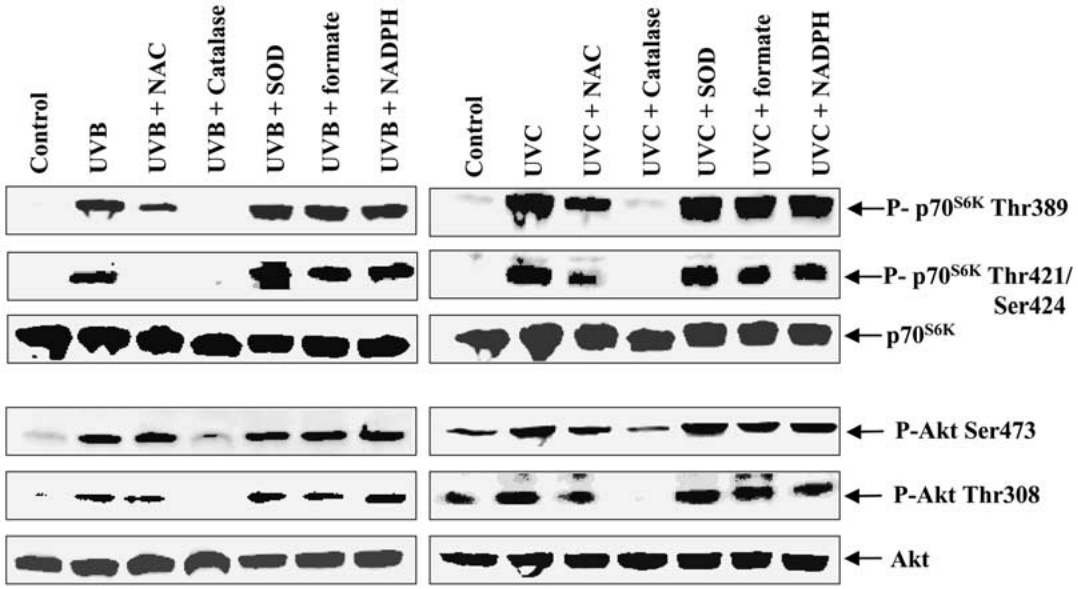

Fig. 6. Effects of free radical modifiers on phosphorylation Akt and $p70^{S6k}$ induced by UV radiation. JB6 C1 41 cells, suspended in 5% FBS MEM, were added to each well of 6-well plates and cultured overnight. The cells were pretreated with different free radical modifiers as indicated. The cells were then exposed to (right panel) UVC (60J/m^2) or (left panel) UVB (4 KJ/m^2). The cells were harvested and the Western blotting was carried out as described in Fig. 5.

fects of specific ROS modifiers on UV-induced phosphorylation of Akt and $p70^{S6k}$ were investigated respectively. Pretreatment of cells with NAC, a general antioxidant, or catalase, a specific H_2O_2 catalyst, inhibited UV-induced phosphorylation of Akt at Ser^{473} and Thr^{308} or $p70^{S6k}$ at Thr^{389} and Thr^{421}/Ser^{424}, respectively (Fig. 6). Treatment of cell with sodium formate, an $^{\cdot}OH$ radical scavenger, did not inhibit UV-induced response (Fig. 6). These data suggest that H_2O_2 generation by UV radiation is involved in UV-induced phosphorylation of Akt and $p70^{S6k}$.

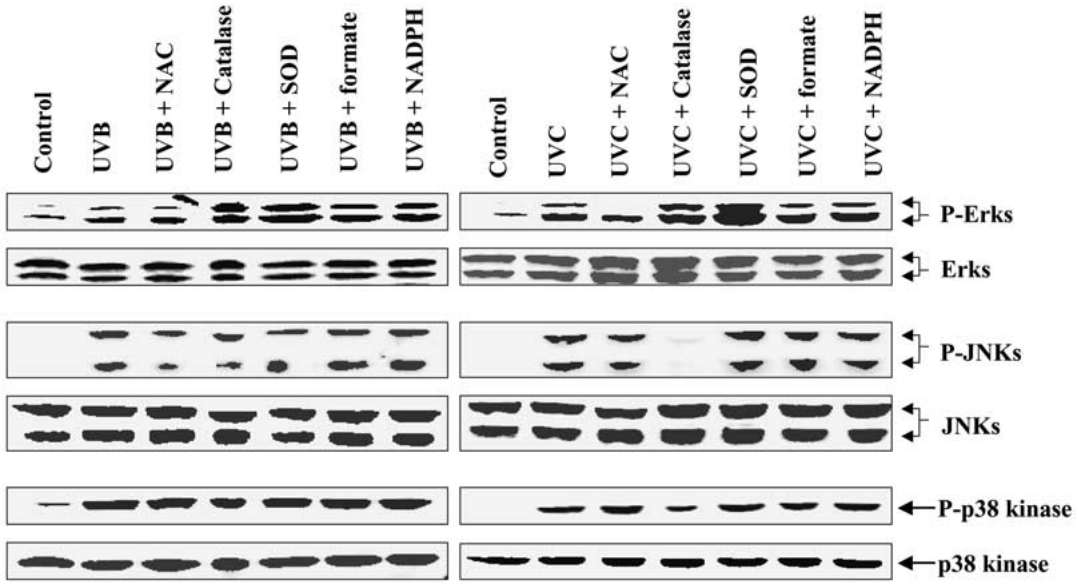

Fig. 7. Effects of free radical modifiers on activation of Erks, JNKs and p38 kinase induced by UV radiation. Subconfluent (90%) monolayers of JB6 C1 41 cells in 6 well plates were pretreated with different free radical modifiers as indicated. The cells were then exposed to (right panel) UVC (60J/m^2) or (left panel) UVB (4 KJ/m^2). The cells were harvested and the Western blotting was carried out as described in Fig. 5.

Generation of H_2O_2 is involved in UV-induced activation of JNKs and p38 kinase, but not Erks

To investigate the possible involvement of ROS in UV-induced activation of MAP kinase family, we also observed the effects of specific ROS modifiers on UV-induced activation of Erks, JNKs and p38 kinase. The results indicated that treatment of cells with catalase inhibited UV-induced activation of JNKs and p38 kinase to some extend, while it did not exhibit any inhibition on Erks activation (Fig. 7). Treatment of cell with sodium formate or SOD resulted in a slight increase in UV-induced response (Fig. 7). These data suggest that H_2O_2 generation by UV radiation is involved in UV-induced activation of JNKs and p38 kinase, but not Erks.

Inhibition of Erks by PD98059 reduces UV-induced phosphorylation of $p70^{S6k}$, but not Akt

To study the possible cross talk among UV-induced signaling pathways, we used PD98059, a specific Erks pathway inhibitor. Pre-treatment of cells with PD98059 specifically blocked UV-induced activation of Erks, but not JNKs and p38 kinase (Fig. 8a). Blockade of UV-induced Erks activation exhibited a partial inhibition of UV-induced $p70^{S6k}$ phosphorylation at Thr^{389} and Thr^{421}/Ser^{424} (Fig. 8b), while slight increases in UV-induced Akt phosphorylation at Ser^{473} and Thr^{308} were observed (Fig. 8b). The data suggest that activation of Erks is specific for UV-induced $p70^{S6k}$ phosphorylations at Thr^{389} and Thr^{421}/Ser^{424}.

Discussion

In the present study, it was found that: (a) exposure of cells to UV radiation lead to H_2O_2 generation by fluorescence staining assay and $^{\bullet}OH$ radical production using H_2O_2 as a precursor by ESR spin trapping measurements; (b) catalase, a specific H_2O_2 decomposer, inhibited UV-induced phosphorylation of Akt or $p70^{S6k}$ and activation of JNKs, p38 kinase, but not Erks, while sodium formate, which scavenges $^{\bullet}OH$ radicals, exhibited no inhibitory effects, suggesting that $^{\bullet}OH$ radicals are not involved in UV-induced phosphorylation of Akt or $p70^{S6k}$ and activation of JNKs and p38 kinase; (c) pretreatment of cells with SOD did not inhibit these signal transduction pathways, revealing that $O_2^{\bullet-}$ itself did not cause activation of MAP kinase and phosphorylation of Akt and $p70^{S6k}$; (d) H_2O_2 alone was able to induce activation of these signal transduction pathways. Thus, we concluded that, among ROS, H_2O_2 appears to be an important mediator involved in UV-induced phosphorylation of Akt or $p70^{S6k}$ and activation of JNKs and p38 kinase.

$p70^{S6k}$ plays important role in the cell growth, transformation and transition of cell cycle in mammalian cells. For this reason, the signal transduction pathways leading to activation of $p70^{S6k}$ have attracted considerable attention in the last few years [13–15, 19–23]. It is believed that phosphotidylinositol 3-kinase (PI-3K) and its structurally related enzyme, mTOR (also termed FRAP or RAFT), are involved in the regulation of phosphorylation of $p70^{S6k}$ [23]. It was found recently that H_2O_2 could induce activation of $p70^{S6k}$ through the upstream rapamycin-sensitive component FRAP/RAFT and wortmannin-sensitive PI-3K [29]. Very recently, Parrott *et al*. reported that UV radiation also induced activation of $p70^{S6k}$ in kidney 293 cells and CV1 cells [30]. However, the molecular mechanisms and signal transduction pathways by which UV causes the increased phosphorylation of $p70^{S6k}$ remain unclear. ROS are important determinants in the regulation of cell functional pathways involved in proliferation, apoptosis and transformation [26, 31–36]. Intercellular levels of ROS are influenced by a number of endogenous and exogenous processes and regulated by several radical scavenger enzymes [34]. Exogenous agents that induce ROS generation include chemical and physical carcinogens and various cytokines [26, 34]. It is generally believed that these extracellular stimuli generate and/or require reactive free radicals or derived oxidant species to successfully transmit their signals to the nucleus [35, 36]. Accumulating data suggest a vital role of ROS in mediating cellular responses to various extracellular stimuli [31–36]. It has been reported that free radicals are involved in the production of cytokines, growth factors and hormones, in the activation of nuclear transcription factors, in gene transcription, in neuromodulation, and in apoptosis [31-36]. Recently we demonstrated that increased intracellular H_2O_2 levels and activation of p53 activity were detected upon incubation of cells with vanadate [26]. The important role of ROS in regulating signal transduction pathways is further supported by the data that ROS, such as H_2O_2, induce the expression of signal components, including c-fos, c-myc and c-jun [37, 38]. Exposure of cells to H_2O_2 causes NFκB translocation from the cytoplasm into the nucleus and HIV-LTR transactivation [39]. The cells over expressing catalase were unable to activate NFκB in response to TNF-α and okadaic acid [40]. The catalase inhibitor, aminotriazole, restored the NFκB response [40]. In contrast, over expressing cytosolic superoxide dismutase, which causes cytosolic H_2O_2 accumulation, potentiated the NFκB response [40]. The results presented here demonstrate that increased levels of intracellular H_2O_2 and $p70^{S6k}$ phosphorylation at Thr^{389} and Thr^{421}/Ser^{424} were detected upon exposure of cells to UV radiation. Pretreatment of cells with NAC or catalase prevented the increase in H_2O_2, and resulted in inhibition of increased $p70^{S6k}$ phosphorylation at Thr^{389} and Thr^{421}/Ser^{424}. These data suggest that UV radiation can generate H_2O_2 in cultured cells, and this UV-generated H_2O_2 plays an essen-

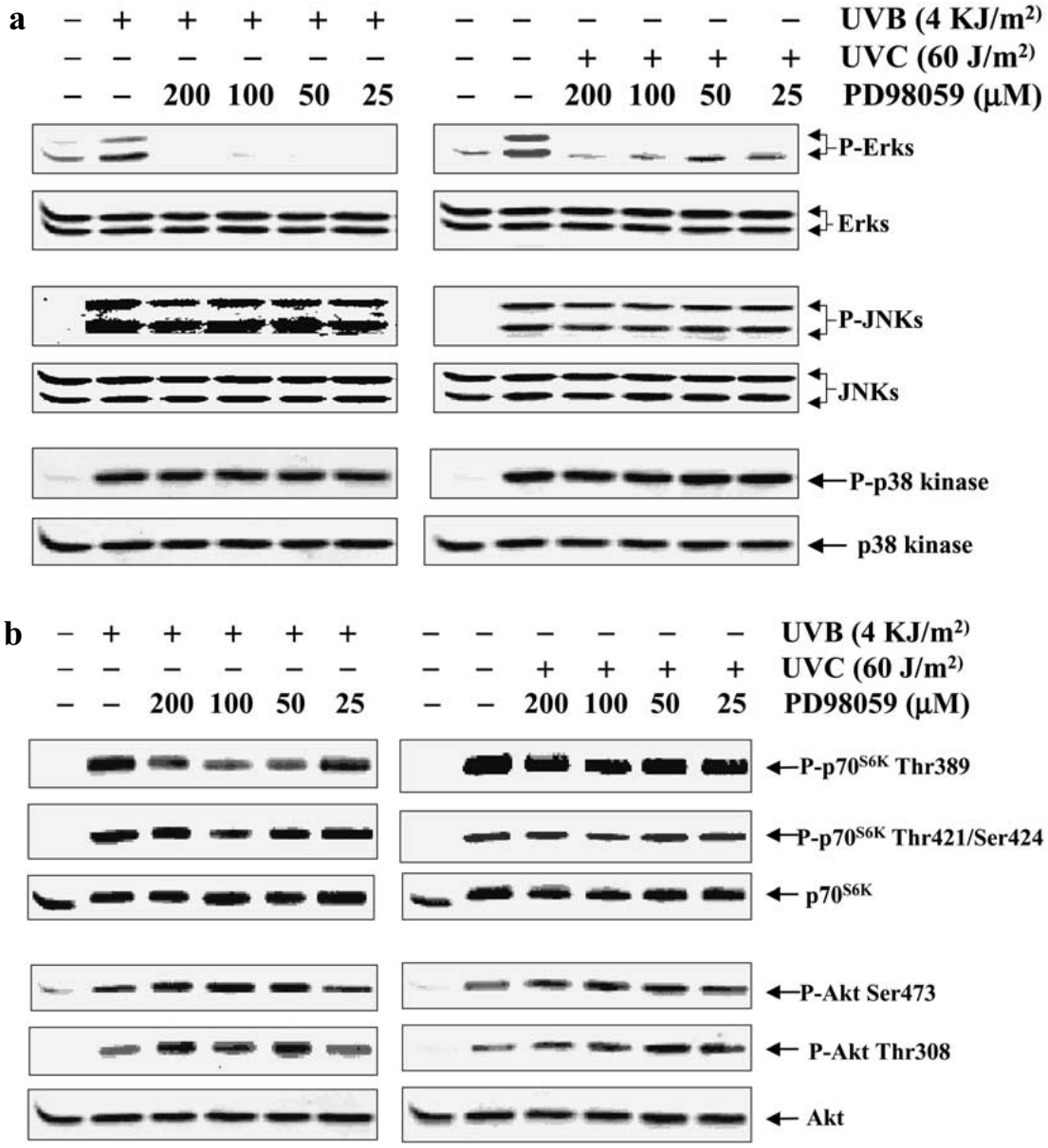

Fig. 8. Effects of PD98059 on the phosphorylation of Akt and $p70^{S6k}$ induced by UV radiation. Subconfluent (90%) monolayers of JB6 C1 41 cells in 6-well plates were pretreated with PD98059 at various concentrations as indicated. The cells were then exposed to UVC (60J/m^2) or UVB (4 KJ/m^2). The cells were harvested and the Western blotting was carried out as described in Fig. 5.

tial role in UV-induced $p70^{S6k}$ phosphorylation at Thr^{389} and Thr^{421}/Ser^{424}.

It has been reported that physiologic stimuli are capable of inducing Akt kinase activity involving PI-3K [17]. PI-3K-generated phospholipids act by multiple mechanisms that co-operate to regulate Akt activation [17]. One of them is through the direct binding of phosphoinositides to the Akt PH domain [41–43]. *In vivo* study has shown that this binding is necessary for Akt activation [42, 44, 45]. A consequence of Akt binding by phospholipids is the translocation of Akt from the cytoplasm to the inner of the plasma membrane [45, 46]. This translocation is necessary for activation of Akt because c-Akt is constitutively activated when it is specifically targeted to inner surface of the plasma membrane [45]. Akt activity is regulated by phosphorylation [16, 17]. There are four phosphorylation sites identified, including Ser^{124}, Thr^{450}, Thr^{308} and Ser^{473} [16, 17]. Mutagenesis studies have suggested that phosphorylation of Ser^{473} and Thr^{308} is required for Akt activity [16, 17], whereas Ser^{124} and Thr^{450} appear to be basally phosphorylated [16, 17]. Because the kinases that phosphorylate

Akt are PI3,4,5P dependent, they were termed 3-phosphoinositide-dependent protein kinases (PDKs) [17]. The second mechanism by which phospholipids regulate Akt activation is by regulating the PDK1/PRK-2 complex, which can phosphorylate Akt at both Ser^{473} and Thr^{308} [17]. Some studies have indicated that binding of phospholipids to the Akt PH domain induces a critical conformational change that renders Akt competent for phosphorylation by PDKs [17].

The results in the present study demonstrate that UV radiation led to increases in Akt phosphorylation at Ser^{473} and Thr^{308}. Although the details of the molecular mechanism for involvement of signal transduction pathways are not clear, the role of PKA and CaMKK in H_2O_2-mediated Akt phosphorylation at Ser^{473} and Thr^{308} by UV radiation need to be further studied. This hypothesis is supported by our previous findings that UV radiation induced a rapid increase of intracellular free calcium and transactivation of NFAT, which is believed to be dependent on Ca^{2+}/calmodulin complex formation and activation of CaMKK [46].

The role of ROS in AP-1 induction is not only supported by data that ROS mediates expression of *c-fos* and *c-jun*, two major components of AP-1 [37, 38], but also supported by inhibition of AP-1 activation by ROS scavengers [40]. The results from present study demonstrated that H_2O_2 plays a crucial role in UV-induced activation of JNKs and p38 kinase, but not Erks.

UV radiation generates ROS and induces activation of Erks, JNKs and p38 kinase as well as phosphorylation of Akt and $p70^{S6k}$. Among ROS generated by UV radiation, H_2O_2 is a mediator for phosphorylation of Akt, $p70^{S6k}$, JNKs and p38 kinase, but not Erks. Considering the important role of $p70^{S6k}$, Akt, JNks and p38 kinase in regulation of cell growth, apoptosis and the cell cycle, the present study suggests that H_2O_2-mediated activation of signal transduction pathways in 'UV responses' may play a role in UV-induced carcinogenesis. Further study on the precise mechanism by which UV radiation and H_2O_2 triggers signal transduction cascades should help us to understand the basis of UV-induced skin diseases such as cancer and aging.

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