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UV Induces Phosphorylation of Protein Kinase B (Akt) at Ser-473 and Thr-308 in Mouse Epidermal Cl 41 Cells through Hydrogen Peroxide*

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The exposure of mammalian cells to UV irradiation leads to the activation of transcription factors and protein kinases, which are believed to be responsible for the carcinogenic effects of excessive sun exposure. The present study investigated the effect of UV exposure on reactive oxygen species (ROS) generation and protein kinase B (Akt) phosphorylation in epidermal cells and determined if a relationship exists between these UV responses. Exposure of mouse epidermal JB6 Cl 41 cells to UV radiation led to specific phosphorylation of Akt at Ser-473 and Thr-308 in a time-dependent manner. This phosphorylation was confirmed by the observation that overexpression of Akt mutant, Akt-T308/S473A, attenuated phosphorylation of Akt at Ser-473 and Thr-308. UV radiation also generated ROS as measured by electron spin resonance (ESR) in JB6 Cl 41 cells. The generation of ROS by UV radiation was measured further by H_2O_2 and $O_2^{\cdot-}$ fluorescence staining assays. The mechanism of ROS generation involved reduction of molecular oxygen to $O_2^{\cdot-}$, which generated H_2O_2 through dismutation. H_2O_2 produced $^{\cdot}OH$ via a metal-independent pathway. 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 Akt phosphorylation at Ser-473 and Thr-308, whereas the pretreatment of cells with sodium formate (an $^{\cdot}OH$ radical scavenger) or superoxide dismutase (an $O_2^{\cdot-}$ radical scavenger) did not show any inhibitory effects. Furthermore, treatment of cells with H_2O_2 increased UV-induced phosphorylation of Akt at Ser-473 and Thr-308. These results demonstrate that UV radiation generates a whole spectrum of ROS including $O_2^{\cdot-}$, $^{\cdot}OH$, and H_2O_2 and induces phosphorylation of Akt at Ser-473. Among the various ROS, H_2O_2 seems most potent in mediating UV-induced phosphorylation of Akt at Ser-473 and Thr-308. It is possible that Akt may play a role in the carcinogenesis effects by UV radiation.

UV irradiation is a major etiologic factor in the development of human skin cancers (1). Experimentally, UV irradiation acts both as a tumor initiator and a tumor promoter in animal models (2, 3). Although the mechanism for the tumor-promoting ability of UV is not well understood, it is believed that UV-initiated signal transduction pathways are responsible for its tumor promotion effects. It has been reported that exposure of mammalian cells to UV irradiation including short (UVC, 200–280 nM), long (UVA, 320–400 nM), and mid- (UVB, 280–320 nM) wavelengths leads to a large number of changes in cells such as activation of transcription factors and protein kinases (4–8). The UVC-induced signal transduction pathway is believed to originate at the cell membrane (9, 10), then the signal is transferred to the nucleus via a signaling cascade involving Src-like tyrosine kinase, Ras/Raf kinase, and mitogen-activated protein kinase, including c-Jun N-terminal kinase, resulting in the activation of transcription factors such as activated protein-1 (AP-1) and TCF/Elk-1 (11–14). Our previous studies have demonstrated that the pathway by which UV activates AP-1 activity requires atypical protein kinase C activation (15, 16). Inhibition of atypical protein kinase C blocks AP-1 activation via a suppression of Erks[1] activation but not through inhibition of c-Jun N-terminal kinases or p38 kinase activation (17).

c-Akt is the cellular homologue of the transforming oncogene of the AKT8 retrovirus (18, 19). Molecular study revealed that v-akt resulted from a recombination of viral gag sequences with the cellular *Akt* gene (20). There are three members of the Akt family, which were identified as Akt1, Akt2, and Akt3 (21). Akt family proteins contain a central kinase domain with specificity for serine or threonine residues in substrate proteins (19). The N terminus of Akt includes a pleckstrin homology domain, which can mediate lipid-protein and/or protein-protein interactions (21–23). The Akt C terminus includes a hydrophobic and proline-rich domain (21, 22). Akt was first found to be involved with signal transduction by the observation that Akt kinase activity is induced by growth factors such as platelet-derived and fibroblast growth factors (24, 25). Activation of Akt depends on its phosphorylation (21). Four phosphorylation sites on Akt have been identified that are phosphorylated *in vivo* including Ser-124, Thr-308, Thr-450, and Ser-473 (26). Thr-308 and Ser-473 are inducibly phosphorylated after treatment of cells with extracellular stimuli, whereas Ser-124 and Thr-450 appear to be basally phosphorylated (21, 26). Mutagenesis studies have demonstrated that the phosphorylation of Thr-308 and Ser-473 is required for Akt activation and that mimicking phosphorylation partially activates Akt (21, 27). In the present

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[1] The abbreviations used are: Erk, extracellular signal-regulated kinase; SOD, superoxide dismutase; Da, dichlorofluorescin diacetate; HE, dihydroethidium; FBS, fetal bovine serum; MEM, Eagle's minimal essential medium; ESR, electron spin resonance; DMPO, 5,5-dimethyl-1-pyrroline *N*-oxide; ROS, reactive oxygen species; PI, phosphatidylinositol.

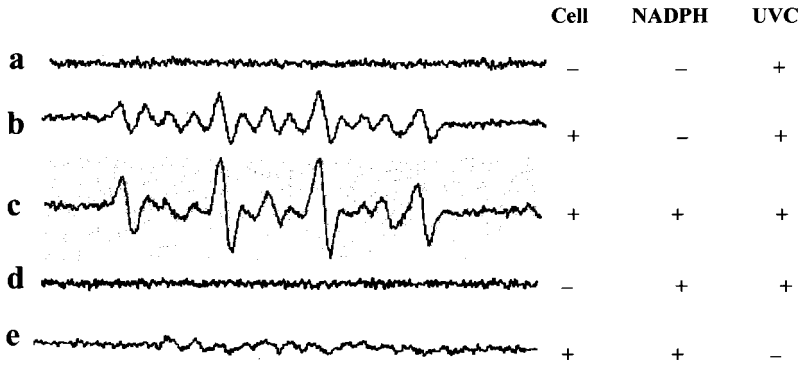

FIG. 1. **Measurement of UV-induced ROS generation by ESR.** Cl 41 epidermal cells were grown to 90% confluence in 100-mm dishes. Cells (2×10^6) were then incubated in PBS containing 400 mM DMPO with or without 100 μM NADPH. ESR spectra were recorded 7 min after the cells were exposed to UVC (60 J/m^2). *a*, UVC; *b*, cells + UVC; *c*, cells + NADPH + UVC; *d*, NADPH + UVC; *e*, cells + NADPH.

study, we found that UV radiation induces Akt phosphorylation at Ser-473 and Thr-308, and this phosphorylation of Akt is mediated by the H_2O_2 generated by UV radiation.

MATERIALS AND METHODS

Plasmids and Reagents—the Akt mutant plasmid, SRα-Akt-T308A/S473A, and its vector control plasmid, SRα, were a generous gift from Dr. Bing-Hua Jiang (West Virginia University) (27). The SRα-Akt-T308A/S473A construct has a C-terminal influenza virus hemagglutinin epitope tag, which is detected easily in transfected cells using a specific hemagglutinin-tagged antibody (27). Deferoxamine, *N*-acety-L-cyteine, β-nicotinamide adenine dinucleotide phosphate (NADPH), superoxide dismutase (SOD), and sodium formate were purchased from Sigma; dichlorofluorescin diacetate (Da) and dihydroethidium (HE) were purchased from Molecular Probes (Eugene, OR); fetal bovine serum (FBS) and Eagle's minimal essential medium (MEM) were from BioWhittaker (Walkersville, MD); the phospho-specific Akt (Thr-308) antibody, phospho-specific Akt (Ser-473) antibody, and Akt antibody as well as hemagglutinin-tagged antibody were purchased from New England Biolabs (Berverly, MA).

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% FBS, 2 mM L-glutamine, and 25 μg of gentamicin/ml as described previously (28).

Cellular Superoxide ($O_2^{\cdot-}$) and H_2O_2 Staining Assay—HE is a specific $O_2^{\cdot-}$ dye (29), whereas Da has been used frequently to monitor H_2O_2 levels in cells (29). The cells were seeded in 6-well plates and cultured until 90% confluent. The cells were then treated with UVC radiation (60 J/m^2) for 1 min. After a 30-min incubation, HE or Da (both dissolved in Me_2SO 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 a 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 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% 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 UVC radiation (60 J/m^2) for 1 min. The cells were harvested and transferred to a flat cell for ESR measurement as described previously (30).

Oxygen Consumption Measurements—Oxygen consumption was measured from UVC-exposed cells, because molecular oxygen is the potential source of $O_2^{\cdot-}$. Oxygen consumption was measured by oxygen equipped with a Clark microelectrode, model 516 (Gilson Medical Electronics, Middleton, WI). Cells (1×10^6/ml) were preincubated at 37 °C for 10 min, and resenting steady-state oxygen consumption was monitored over a period of 10 min. The oxygraph was calibrated using medium equilibrated with gases of known oxygen content.

Transient Transfection—Cl 41 cells were cultured in a 6-well plate until they reached 85–90% confluence. 15 μl of LipofectAMINE reagent with 15 μg of Akt-T308A/S473A plasmid DNA were used to transfect each well in the absence of serum. After 10–12 h, the medium was replaced with 5% FBS MEM. Approximately 24–30 h after the beginning of the transfection, the cells were exposed to either UVB (4 KJ/m^2) or UVC (60 J/m^2). The cells then were washed once with ice-cold PBS and extracted with SDS sample buffer. The cell extracts were used for Western blot.

Western Blot Analysis—2×10^4 of JB6 C1 41 cells were cultured in each well of 6-well plates to 90% confluence with 5% FCS MEM medium. The cells were exposed to either UVB (4 KJ/m^2) or UVC (60 J/m^2) and incubated for different time points as indicated in the figure legends. 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 including rabbit phospho-specific Akt (Thr-308) antibody, phospho-specific Akt (Ser-473) antibody, and Akt antibody. The Akt protein bands specifically bound to primary antibodies were detected using an anti-rabbit IgG-AP-linked and eosinophil chemotactic factor Western blotting system (31).

RESULTS

Generation of Reactive Oxygen Species (ROS) by UV Radiation—To determine ROS generation by UV radiation directly in culturing cells, the well characterized mouse epidermal cell line Cl 41 and dye staining as well as ESR techniques were used. The results show that UV radiation without cells did not generate any detectable amount of free radicals (Fig. 1*a*). Exposure of cells to UVC radiation generated a 1:2:2:1 ESR spectrum (Fig. 1*b*) with hyperfine splittings of $a_H = a_N = 14.9$ G, where a_N and a_H denote hyperfine splittings of the nitroxyl nitrogens and α-hydrogen, respectively. Based on these splittings and the 1:2:2:1 line shape, the spectrum was assigned to the DMPO/$^{\cdot}$OH adduct, which is evidence of $^{\cdot}$OH radical generation. The addition of NADPH, which enhanced the reduction of molecular oxygen to ROS by NADPH oxidase, enhanced the $^{\cdot}$OH generation (Fig. 1*c*), whereas NADPH with UV radiation or cells with NADPH did not generate any detectable DMPO/$^{\cdot}$OH signal (Fig. 1, *d* and *e*). Time course studies show that the radical generation reached a maximum within 7 min after 1 min of exposure (Fig. 2). The addition of catalase, a scavenger of H_2O_2, inhibited $^{\cdot}$OH radical generation (Fig. 3*b*), indicating that H_2O_2 was produced in the UV-treated cells and served as a precursor of $^{\cdot}$OH generation. The addition of sodium formate, an $^{\cdot}$OH radical scavenger, decreased the DMPO/$^{\cdot}$OH signal intensity and generated a new spin adduct signal (Fig. 3*c*). The hyperfine splittings of this signal are $a_N = 18.7$ G and $a_N = 15.7$ G. These splittings are typical of those DMPO/$^{\cdot}COO^-$ adducts. The generation of these new radicals is caused by the abstraction of a hydrogen atom by $^{\cdot}$OH from formate. The $^{\cdot}COO^-$ was trapped by DMPO to generate DMPO/$^{\cdot}COO^-$ adducts. These results confirm the generation of $^{\cdot}$OH in UV-irradiated cells. Incubation of the mixture with deferoximine, a metal chelator, did not affect the signal intensity (Fig. 3*d*). It may be noted that a previous study has shown that UV photolysis of aqueous H_2O_2 is able to generate the $^{\cdot}$OH radical (32).

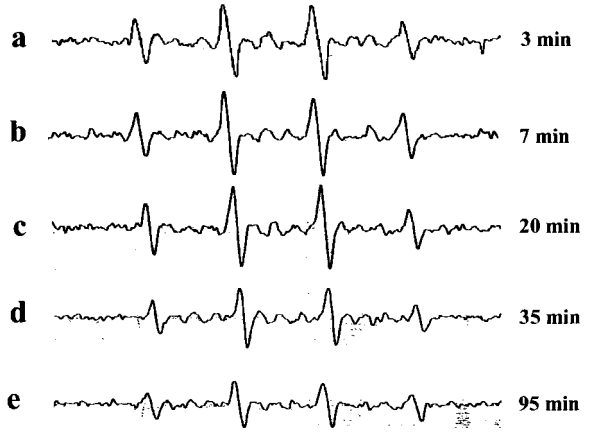

FIG. 2. **Time course studies on UVC-induced ROS generation.** Cl 41 epidermal cells were grown to 90% confluence in 100-mm dishes. Cells (1×10^6) then were incubated in PBS containing 400 mM DMPO with 100 μM NADPH. ESR spectra were recorded at different time points as indicated after cells were exposed to UVC (60 J/m^2).

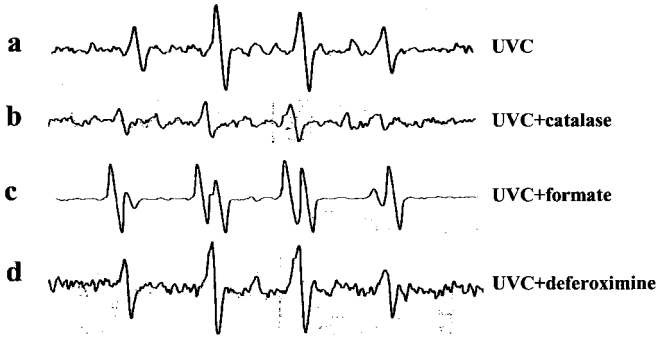

FIG. 3. **Effects of free radical modifiers on UVC-induced ROS generation.** Cl 41 epidermal cells were grown to 90% confluence in 100-mm dishes. Cells (1×10^6) were then incubated in PBS containing 400 mM DMPO and 100 μM NADPH with or without different free radical modifiers as indicated. The final concentrations of these scavengers were: catalase, 2000 units/ml, and sodium formate, 100 mM. Deferoxamine, 2 mM, was used as a metal chelator. ESR spectra were recorded 7 min after the cells were exposed to UVC (60 J/m^2).

Thus, it appears that UV-generated $^{\cdot}OH$ requires H_2O_2 but through a metal-independent mechanism. Measurements using HE, a specific fluorescent dye for $O_2^{\cdot-}$, or Da, a fluorescent dye for H_2O_2, demonstrate that exposure of cells to UV radiation led to an increase in the generation of both $O_2^{\cdot-}$ and H_2O_2 (Fig. 4). Importantly, preincubation of cells with SOD or catalase reduced the fluorescent intensities for $O_2^{\cdot-}$ and H_2O_2, respectively (Fig. 4). These observations confirm that UV-induced fluorescence staining by HE and Da are $O_2^{\cdot-}$ and H_2O_2, respectively. Exposure of cells to UV radiation resulted in an increased rate of oxygen consumption as comparing with control cells (Fig. 5). These results provide direct and strong evidence that UV radiation can induce the generation of ROS in epidermal cells.

Induction of Akt Phosphorylation at Ser-473 and Thr-308 by UV Radiation—To investigate the possible induction of Akt phosphorylation in the UV response, we exposed the Cl 41 cells to either UVB or UVC radiation. The results show that UV radiation markedly induced Akt phosphorylation at Ser-473 and Thr-308 in a time-dependent manner (Fig. 6*a*). The maximum induction of Akt phosphorylation at Ser-473 and Thr-308 in response to either UVB or UVC radiation occurred between 4 and 7 h after exposure (Fig. 6*a*). To confirm these findings, we transiently transfected the SRα-Akt-T308A/S473A plasmid into Cl 41 cells. The results show that the UV-induced increases of Akt phosphorylation at Ser-473 and Thr-308 were

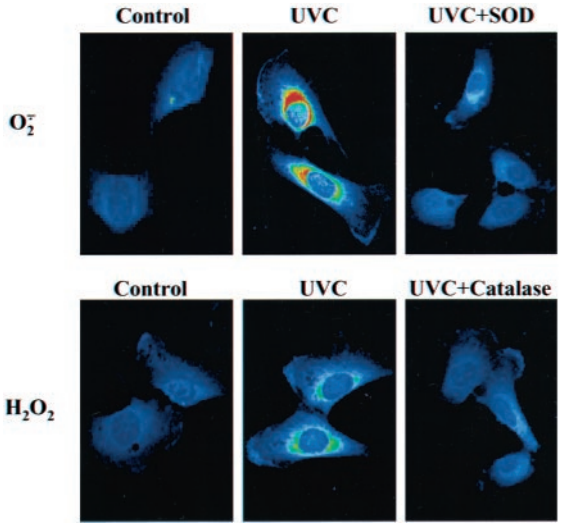

FIG. 4. **Determination of $O_2^{\cdot-}$ and H_2O_2 by HE and Da staining.** Cl 41 cells were seeded in 6-well plates and cultured until 90% confluent. The cells were or were not pretreated with SOD (250 units/ml) or catalase (5×10^4 units/ml) for 30 min and then exposed to UVC radiation (60 J/m^2) and cultured 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 were harvested and applied on slides for observation under a fluorescence microscope.

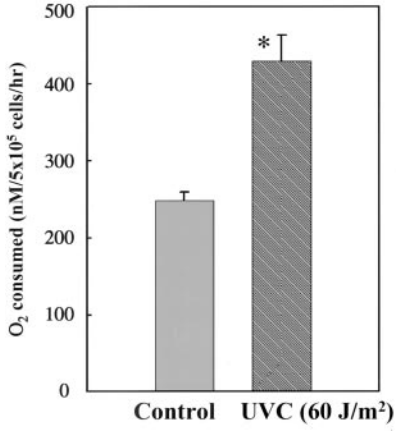

FIG. 5. **Increase of cell oxygen consumption after cells were exposed to UVC radiation.** Subconfluent (90%) monolayers of JB6 C1 41 cells in 100-mm dishes were subjected to UVC (60 J/m^2). The cells were then washed once with ice-cold PBS and harvested for an oxygen consumption assay. Oxygen consumption was measured by oxygen equipped with a Clark microelectrode, model 516 (Gilson Medical Electronics) as described under "Materials and Methods." The *asterisk* indicates a significant increase from control (*, $p < 0.05$).

reduced markedly by the overexpression of Akt protein with Thr-308 and Ser-473 double point mutation (Fig. 6*b*). These results demonstrate that UV radiation is a potent stimulus for induction of Akt phosphorylation at Ser-473 and Thr-308.

Induction of Akt Phosphorylation at Ser-47 and Thr-308 by H_2O_2—The results from the studies discussed above demonstrate that UV radiation can generate ROS in Cl 41 cells. Considering findings from other groups that exogenous H_2O_2 can induce activation of Akt activity (21, 33) and the phosphorylation of Akt at Ser-473 in the human glioblastoma cell line (34), we determined the induction of Akt phosphorylation at Ser-473 and Thr-308 by H_2O_2 in mouse epidermal JB6 cell line. The results show that, similar to UV radiation, treatment of cells with H_2O_2 resulted in an increase in Akt phosphorylation at Ser-473 and Thr-308 in JB6 cells (Fig. 7). These data suggest that the Akt phosphorylation induced by H_2O_2 is consistent with the phosphorylation induced by UV radiation. Thus, it is

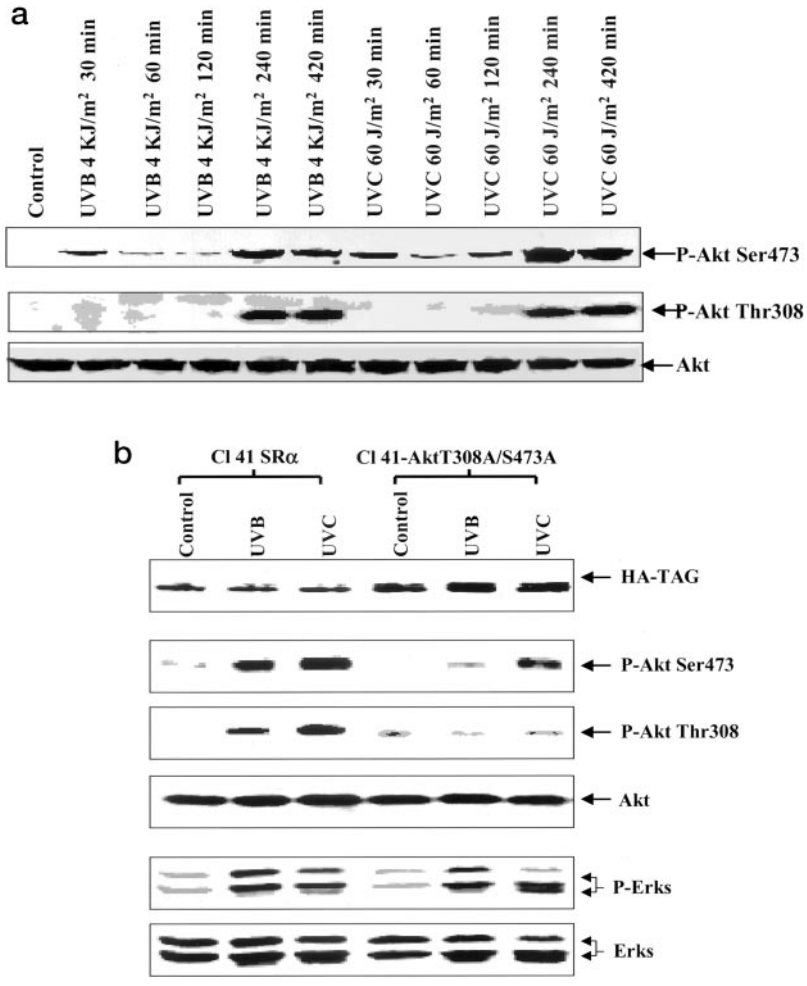

FIG. 6. **Induction of Akt phosphorylation at Ser-473 and Thr-308 by UV radiation.** *a*, subconfluent (90%) monolayers of JB6 C1 41 cells in 100-mm dishes were subjected to either UVC (60 J/m^2) or UVB (4 KJ/m^2) and cultured for the time points indicated. The cells were then washed once with ice-cold PBS and extracted with SDS sample buffer. The cell extracts were separated on polyacrylamide-SDS gels, transferred, and probed with one of the antibodies including rabbit phospho-specific Akt (*P-Akt Thr308*) antibody, phospho-specific Akt (*P-Akt Ser473*) antibody, and Akt antibody. The Akt protein band specifically bound to the primary antibody was detected by using an anti-rabbit IgG-AP-linked and eosinophil chemotactic factor Western blotting system (31). *b*, Cl 41 cells were cultured in a 6-well plate until they reached 85–90% confluence. LipofectAMINE reagent with Akt-T308A/S473A plasmid DNA was used to transfect each well as described under "Materials and Methods." The cells were exposed to either UVB (4 KJ/m^2) or UVC (60 J/m^2) and then extracted with SDS sample buffer. The cell extracts were used for Western blot with the specific antibodies indicated. *HA*, hemagglutinin.

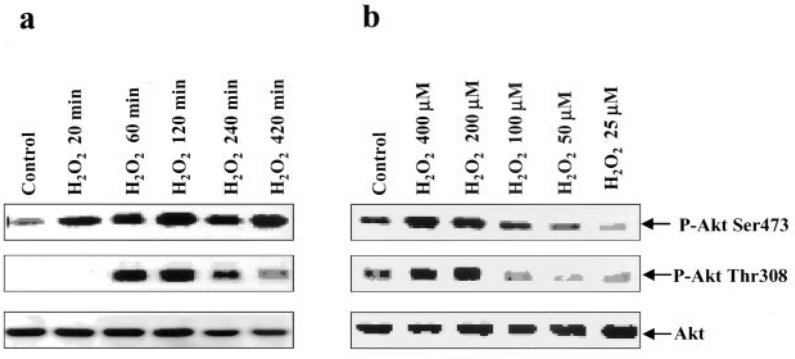

FIG. 7. **Induction of Akt phosphorylation at Ser-473 and Thr-308 by H_2O_2.** Subconfluent (90%) monolayers of JB6 C1 41 cells in 100-mm dishes were subjected to different doses of H_2O2 for 120 min (*a*) or 200 μM H_2O_2 (*b*) for the various times indicated. The cells were then washed once with ice-cold 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 including rabbit phospho-specific Akt (*P-Akt Thr308*) antibody, phospho-specific Akt (*P-Akt Ser473*) antibody, and Akt antibody. The Akt protein band specifically bound to the primary antibody was detected by using an anti-rabbit IgG-AP-linked and eosinophil chemotactic factor Western blotting system (31).

possible that H_2O_2 generated by UV radiation may be involved in Akt phosphorylation induced by UV radiation.

Generation of Reactive Oxygen Species Is Required for Akt Phosphorylation at Ser-473 and Thr-308 Induced by UV Radiation—To obtain direct evidence for the involvement of H_2O_2 in Akt phosphorylation at Ser-473 and Thr-308 in UV responses, the effects of specific modifiers of ROS on UV-induced Akt phosphorylation at Ser-473 and Thr-308 were determined. Pretreatment of cells with *N*-acetyl-L-cyteine, a general antioxidant, or catalase, a specific H_2O_2 scavenger, inhibited UV-induced phosphorylation of Akt at Ser-473 and Thr-308 (Fig. 8). In contrast, treatment of the cell with sodium formate, an $^{\cdot}OH$ radical scavenger, did not inhibit UV-induced Akt phosphorylation at Ser-473 and and Thr-308 (Fig. 8). It should be noted that increasing H_2O_2 generation with the addition of SOD or NADPH did not show an increase of UV-induced Akt phosphorylation (Fig. 8). The reason for these observations may be that the generation of H_2O_2 by UV radiation is strong enough to induce maximum Akt phosphorylation at Ser-473 and Thr-308 in Cl 41 cells. These data suggest that H_2O_2 generation by UV radiation is involved in UV-induced Akt phosphorylation at Ser-473 and Thr-308.

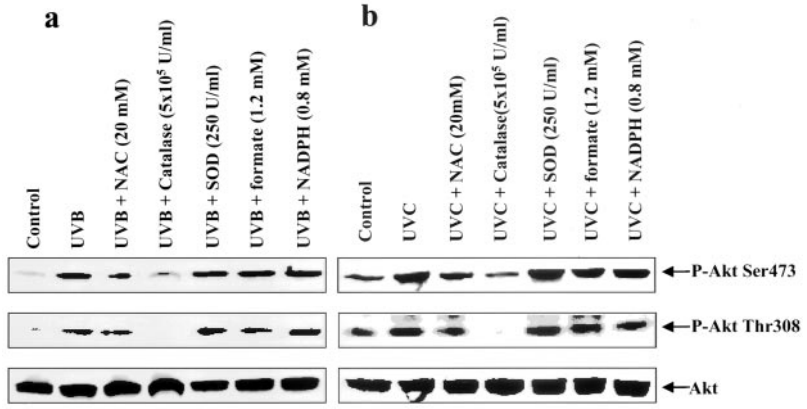

FIG. 8. **Effects of free radical modifiers on Akt phosphorylation at Ser-473 and Thr-308.** JB6 C1 41 cells suspended in 5% FBS MEM were added to each well of 96-well plates and cultured overnight. The cells were pretreated with different free radical modifiers as indicated. The cells were then exposed to UVB (4 KJ/m^2) (*a*) or UVC (60 J/m^2) (*b*). The cells were harvested, and the Western blot was carried out as described in the Fig. 7 legend. *P-Akt Thr308*, rabbit phospho-specific Akt antibody; *P-Akt Ser473*, phospho-specific Akt antibody; *Akt*, Akt antibody.

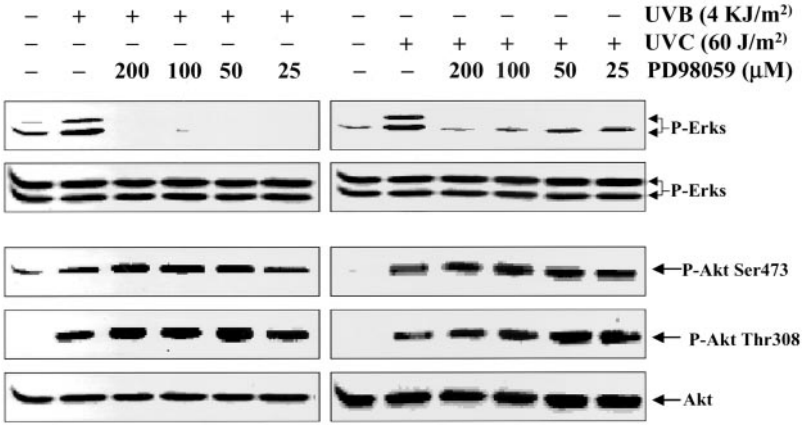

FIG. 9. **Effects of PD98059 on Akt phosphorylation at Ser-473 and Thr-308.** JB6 C1 41 cells suspended in 5% FBS MEM were added to each well of 96-well plates and cultured overnight. The cells were pretreated with different concentrations of PD98059 as indicated. The cells then were exposed to UVB (4 KJ/m^2) or UVC (60 J/m^2). The cells were harvested, and the Western blot was carried out as described in the Fig. 7 legend. *P-Erks*, phospho-specific Erks; *P-Akt Thr308*, rabbit phospho-specific Akt antibody; *P-Akt Ser473*, phospho-specific Akt antibody; *Akt*, Akt antibody.

No Cross Talks between UV-induced Erks Cascade and Akt Pathway—Because the Akt and Erks pathways all are associated with cell growth, apoptosis, cell differentiation, and transformation, it is reasonable to ask whether there is any cross talk between the two pathways. To answer this question, we used PD98059, a specific Erks pathway chemical inhibitor, and a dominant negative Akt mutant, Akt-T308A/S473A. We observed that pretreatment of cells with PD98059 did not show any inhibitory effect on UV-induced Akt phosphorylation at Ser-473 and Thr-308, but it did block UV-induced Erks phosphorylation (Fig. 9). Furthermore, overexpression of a dominant negative Akt mutant, Akt-T308A/S473A, did not affect UV-induced Erks activation, whereas it attenuated increases of Akt phosphorylation at Ser-473 and Thr-308 induced by UV radiation (Fig. 6*b*). All the data suggest that there is no observed cross talk between the Erks cascade and Akt pathway in "UV responses."

DISCUSSION

The results presented in the present study demonstrate that UV radiation leads to the generation of ROS. Among them, H_2O_2 is responsible for an increase in Akt phosphorylation at Ser-473 and Thr-308 in mouse epidermal Cl 41 cells. This conclusion is based on the following observations: (*a*) a fluorescence staining assay shows that exposure of cells to UV radiation lead to H_2O_2 generation, and ESR spin trapping measurements show that UV-radiated cells generate the $^{\cdot}OH$ radical using H_2O_2 as a precursor; (*b*) UVC radiation led to an increase of the rate of cell oxygen consumption; (*c*) catalase, a specific scavenger of H_2O_2, inhibited UV-induced Akt phosphorylation at Ser-473 and Thr-308, whereas sodium formate, which scavenges $^{\cdot}OH$ radicals, exhibited no inhibitory effects, suggesting that $^{\cdot}OH$ radicals are not involved in UV-induced Akt phosphorylation at Ser-473 and Thr-308; (*d*) pretreatment of cells with SOD did not inhibit UV-induced Akt phosphorylation at Ser-473 and Thr-308; instead, it slightly enhanced the Akt phosphorylation, revealing that $O_2^{\cdot-}$ itself did not cause Akt phosphorylation at Ser-473 and Thr-308; and (*e*) H_2O_2 alone was able to induce Akt phosphorylation at Ser-473 and Thr-308. Thus, among ROS, H_2O_2 appears to be the one involved in UV-induced Akt phosphorylation at Ser-473 and Thr-308. It is possible that H_2O_2-mediated phosphorylation and activation of Akt may be involved in UV-induced carcinogenesis. With regard to the mechanism of ROS generation from mouse epidermal Cl 41 cells under UV radiation, it appears that molecular oxygen is reduced to $O_2^{\cdot-}$ as measured by the oxygen consumption assay. NADPH oxidase is likely to be responsible for the reduction of molecular oxygen, because the addition of NADPH enhanced the ROS generation. $O_2^{\cdot-}$ generated H_2O_2 through dismutation. H_2O_2 produced $^{\cdot}OH$ via a metal-independent pathway as indicated by the failure of deferoxamine to inhibit $^{\cdot}OH$ generation.

Increased exposure to the UV radiation of sunlight has been associated with an increased incidence of skin cancer (1). UV radiation not only acts by inducing DNA damage and cell mutagenicity but also can modulate the expression of several genes at dose levels relevant to natural solar exposure (35). The solar UV radiation includes the wavelengths UVA, UVB, and UVC. The doses that reach the surface of the earth are composed of 90–99% UVA, 1–10% UVB, and a small fraction of UVC as a result of atmospheric ozone depletion (4). The intensity of UVA and UVB in natural sunshine is such that an individual can receive as much as 1000 KJ/m^2 and 10 KJ/m^2 of radiations in a single day at the beach, respectively (4). Also UVA has been shown to promote UVB carcinogenesis in animal experiments (4). Thus, the doses of UVB used in this study are relevant to natural solar exposure.

Some UV-induced genes are believed to be regulated by an oxidative mechanism (35). Naturally occurring free radicals typically include ROS and reactive nitrogen species (36). In addition to inducing cellular injury such as DNA damage and lipid peroxidation, free radicals also function as intracellular messengers (36, 37). Data are accumulating to indicate a vital role of ROS in mediating cellular responses to various extracellular stimuli (35–37). 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, gene transcription, neuromodulation, and apoptosis (35–37). For example, it has been reported that the generation of H_2O_2 is required for platelet-derived growth factor signal transduction (38). The results presented here demonstrate that increased levels of intracellular H_2O_2 and Akt phosphorylation at Ser-473 and Thr-308 were detected upon exposure of cells to UV radiation. Pretreatment of cells with *N*-acetyl-L-cyteine or catalase prevented the increase in H_2O_2 and resulted in an inhibition of UV-induced Akt phosphorylation at Ser-473 and Thr-308. These data suggest that UV radiation can generate a high level of H_2O_2 in cultured cells, and this UV-generated H_2O_2 plays an essential role in UV-induced Akt phosphorylation at Ser-473 and Thr-308. It may be noted that UV-induced ROS peaks appear from 7 to 20 min, whereas UV-induced Akt phosphorylation at Ser-473 occurs at 30 min and reaches the maximum at 4–7 h after cells were exposed to UV radiation. The lag time of Akt phosphorylation at Ser-473 as compared with ROS generation suggests that H_2O_2 initiates the Akt phosphorylation through indirect pathways. This hypothesis was supported by the finding that the pretreatment of cells with the PI3-kinase inhibitor, wortmannin, or expression of dominant negative p85 prevents the H_2O_2-induced Akt activation (33).

It has been reported that physiologic stimuli are capable of inducing Akt kinase activity involving PI3-kinase (21). PI3-kinase-generated phospholipids act by multiple mechanisms that cooperate to regulate Akt activation (21). One of them is through the direct binding of phosphoinositides to the Akt pleckstrin homology domain (39–41). An *in vivo* study has shown that this binding is necessary for Akt activation (40, 42, 43). A consequence of Akt binding by phospholipids is the translocation of Akt from the cytoplasm to the inner of the plasma membrane (43, 44). This translocation is necessary for the activation of Akt, because c-Akt is constitutively activated when it is specifically targeted to the inner surface of the plasma membrane (44). Akt activity is regulated by phosphorylation (21, 26). There are four phosphorylation sites identified including Ser-124, Thr-450, Thr-308, and Ser-473 (21, 26). Mutagenesis studies have suggested that the phosphorylation of Thr-308 and Ser-473 is required for Akt activity (21, 26), whereas Ser-124 and Thr-450 appear to be basally phosphorylated (21, 26). Because the kinases that phosphorylate Akt are PI3,4,5P-dependent, they were termed 3-phosphoinositide-dependent protein kinases (21). The second mechanism by which phospholipids regulate Akt activation is by regulating the 3-phosphoinositide-dependent protein kinase-1/protein kinase C-related protein kinase-2 complex, which can phosphorylate Akt at both Thr-308 and Ser-473 (21). Some studies have indicated that binding of phospholipids to the Akt pleckstrin homology domain induces a critical conformational change that renders Akt competent for phosphorylation by 3-phosphoinositide-dependent protein kinases (21).

Recently, it has been reported that Akt could be activated in a PI3-kinase-independent manner (43, 44). An agonist of the protein kinase A pathway can activate Akt by increasing cytoplasmic calcium levels (43, 44). The increased calcium binds to calmodulin, and the Ca^{2+}/calmodulin complex activates the calcium/calmodulin-dependent kinase kinase, which then activates Akt by directly phosphorylating Akt at Thr-308 (45). 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 protein kinase A and calcium/calmodulin-dependent kinase kinase in H_2O_2-mediated Akt phosphorylation at Ser-473 and Thr-308 by UV radiation need to be studied further. This hypothesis is supported by our previous findings that UV radiation induced rapid increase of intracellular free calcium and transactivation of nuclear factor of activated T cells, which is believed to depend on Ca^{2+}/calmodulin complex formation and activation of calcium/calmodulin-dependent kinase kinase (46).

The identification of Akt as a key regulator of cellular survival has been associated with current models of oncogenesis. A number of oncogenes and tumor suppressor genes that function upstream of Akt have been found to affect cancer development by regulating Akt activity (21). The cells transformed by v-PI3-kinase exhibit constitutive Akt activation, and the transfection of such cells with dominant negative *Akt* can revert oncogenic transformation (47, 48). Cotransfection of bone marrow precursors with kinase-inactive Akt suppresses the ability of wild-type Bcr/Abl to promote leukemogenesis *in vivo* (49). Overexpression of wild-type Akt may also cause oncogenesis (50, 51). A large number of studies has demonstrated that transfection of a variety of cell types with constitutively active Akt blocks apoptosis induced by apoptotic stimuli such as UV radiation, DNA damage, and the anti-fas antibody (21). This is one of the mechanisms by which Akt plays a role in promoting oncogenesis. Akt may also mediate tumor promotion by inducing cell cycle progression (21). In light of the role of Akt in oncogenesis and tumor promotion and considering the activation of Akt by UV radiation, it is reasonable to hypothesize that UV-induced phosphorylation of Akt at Ser-473 and Thr-308 may play an important role in the promotor effects of UV in skin cancer development.

In summary, UV radiation generates ROS and induces the phosphorylation of Akt at Ser-473 and Thr-308. Among ROS generated by UV radiation, H_2O_2 is responsible for the phosphorylation of Akt at Ser-473 and Thr-308. Considering the important role of Akt in oncogenesis, the present study suggests that H_2O_2-mediated phosphorylation of Akt at Ser-473 and Thr-308 in UV responses may play a role in UV-induced carcinogenesis. It should be noted that by using ESR spin trapping and fluorescence staining, the present study has shown that exposure of cells to UV radiation generates a whole spectrum of ROS including H_2O_2, $^{\cdot}OH$, and $O_2^{\cdot-}$. Because these ROS are reported to be involved in many biological effects and signal transduction pathways, it is reasonable to speculate that many of the biological responses to UV exposure such as DNA damage, lipid peroxidation, and the induction of apoptosis and transcriptional activation may be mediated by ROS.

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UV Induces Phosphorylation of Protein Kinase B (Akt) at Ser-473 and Thr-308 in Mouse Epidermal Cl 41 Cells through Hydrogen Peroxide

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