Among the preventative and protective strategies against the harmful effects of ultraviolet radiation to the skin is the application of antioxidants. Ascorbic acid has been shown to protect against sunburn, delay the onset of skin tumors, and reduce ultraviolet-B–radiation–induced skin wrinkling. In this work, we sought to determine the antioxidative properties of a lipid-soluble derivative of ascorbic acid, ascorbic acid-6–palmitate. We found that ascorbic acid-6–palmitate reduced cellular levels of reactive oxygen species following ultraviolet B irradiation. Treatment of keratinocytes with ascorbic acid-6–palmitate inhibited ultraviolet-B–mediated activation of epidermal growth factor receptor, extracellular regulated kinases 1 and 2, and p38 kinase because of its ability to prevent reduced glutathione depletion and scavenge hydrogen peroxide. Ascorbic acid-6–palmitate strongly promoted ultraviolet-B–induced lipid peroxidation, c-Jun N-terminal kinase activation, and cytotoxicity, however. End products of lipid peroxidation, such as 4-hydroxy-2–none–enal, have been reported to mediate stress-activated protein kinase activation and cell toxicity in epithelial cells. The lipid component of ascorbic acid-6–palmitate probably contributes to the generation of oxidized lipid metabolites that are toxic to epidermal cells. Our data suggest that, despite its antioxidative properties, ascorbic acid-6–palmitate may intensify skin damage following physiologic doses of ultraviolet radiation. Key words: antioxidants/reactive oxygen species/signaling/skin. J Invest Dermatol 119:1103–1108, 2002

Exposure of the skin to ultraviolet B (UVB) radiation (290–320 nm), a minor but active constituent of sunlight, is the principal cause of sunburn, skin cancer, and many other effects on human skin (Pathak et al., 1999). UV radiation 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 in cancer and various inflammatory disorders (Cerutti, 1985; Darr and Fridovich, 1994). We previously reported that physiologic doses of UVB induced biologically relevant levels of H2O2 in primary human keratinocytes (Peus et al., 1998). In addition, we found that H2O2 is required for epidermal growth factor (EGF) receptor activation, thereby mediating downstream signaling (Peus et al., 1999b) and cell survival (Peus et al., 1999a; 2000b). Similarly, UVB-induced activation of extracellular receptor kinases 1 and 2 (ERK1/2) and the stress-activated protein kinase p38 are mediated by ROS. The activation of these mitogen-activated protein kinases is a key step in the sequential activation of cascades of cytoplasmic protein kinases that transmit signals from the membrane to the nucleus and play a major role in triggering and coordinating gene expression in response to various stimuli, including UV radiation (Karin, 1995). To diminish or prevent damaging effects of UVB, the regulation of antioxidant capacity in skin, including the maintenance of adequate levels of antioxidant compounds and enzymes, needs to be tightly controlled (Suzuki et al., 1989). Supplementation of the skin with antioxidants such as vitamin C (ascorbic acid) to enhance the skin’s antioxidant capacity is a valid approach, and the protective effect of topically applied antioxidants has been subject to intense investigation (Darr et al., 1992; Fryer, 1993; Jurkiewicz et al., 1995). As measured by erythema and sunburn cell formation, local application of ascorbic acid has been shown to protect animal skin against harmful effects of UV radiation (Bissett et al., 1990; Darr et al., 1992). Increased dietary intake of antioxidants, including ascorbic acid, reduced UV-induced tumors in mice (Black, 1987). Combined daily intake of ascorbic acid and α–tocopherol has recently been shown to be effective against sunburn (Eberlein-Konig et al., 1998), even though oral supplementation of vitamin C is not thought to increase skin levels of this vitamin significantly (Packer, 1994). Topical ascorbic acid and α–tocopherol delayed the onset of skin tumors and reduced UV-induced skin wrinkling (Bissett et al., 1987).

Cellular lipids represent a major target for UV radiation. End products of lipid peroxidation (LPO), such as 4-hydroxy-2–none–enal, modulate cellular signaling and have been reported to
activate c-jun N-terminal kinase (JNK) and p38 kinase in rat liver epithelial cells (Uchida et al., 1999). 4-Hydroxy-2-nonenal is a highly toxic hydroxyaldehyde formed during LPO (Comporti, 1998). Preventing LPO therefore appears to be a promising strategy for reducing the harmful effects of UV radiation. In this study, we investigated the antioxidant effects of ascorbic acid-6-palmitate (AA6P), a synthetic lipophilic derivative of ascorbic acid located at cellular membranes. We show that, despite its antioxidant properties, AA6P promotes LPO, JNK activation, and cytotoxicity following UVB irradiation.

MATERIALS AND METHODS

Materials and chemicals A FS20 lamp (Westinghouse, Pittsburgh, PA) was used in these studies; it emits an energy spectrum with high fluence in the UVB region, peaking at 313 nm (Hedley and Chow, 1992). Experiments using a WG-295 long pass filter (Schott Glass Technologies, Duryea, PA) to block UVC emission did not affect experimental results (Peus et al., 1999b). The emitted dose was quantified with a UVB radiometer and photodetector (IL 443 and SEE 240, International Light, Newburyport, MA). AA6P and ascorbic acid were obtained from Sigma (St. Louis, MO). Fluorescent dyes were obtained from Molecular Probes (Eugene, OR).

Culture 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 133 medium. Keratinocytes from primary cultures were plated into secondary culture at 1–10 x 10^5 cells per cm^2. Complete medium was supplemented with 0.1 mM calcium, 0.2% (vol/vol) bovine pituitary extract, EGF (10 ng per ml), insulin (5 µg per ml), hydrocortisone (5 x 10^-5 M), ethanalamine (1 x 10^-4 M), and supplemented amino acids. Cells were grown to confluence and fed with standard medium (without growth factors) for at least 48 h to induce quiescence and basal levels of EGF receptor phosphorylation and ERK1/2 and p38 kinase activation (Peus et al., 1997).

LPO To quantify LPO, we initially used the fluorescent fatty acid analog cis-parinaric acid (cPA). cPA integrates into membranes, where it is readily oxidized because of its extensive unsaturation. As oxidized cPA loses its fluorescence, relative levels of LPO can be determined. Keratinocytes were loaded with cPA (10 µM) in complete medium for 1 h at 37°C (Hedley and Chow, 1992). Cells were then washed, irradiated, trypsinized, and fixed. Ten thousand cells were analyzed by flow cytometry using a multichannel argon UV laser, with excitation and emission wavelengths of 334–364 nm and 424 nm, respectively. The mean fluorescence values were used for further analysis (Peus et al., 2000a). To dismiss the possibility of UVB-induced cPA bleaching causing artificial results, we used the oxidation-sensitive fatty acid Ci1-BODIPY581/591 as an alternative probe to detect fatty acid oxidation. LPO shifts the excitation/emission spectra from red to green (580/590 nm) to green (500/510 nm) (Pap et al., 1999). To quantify LPO, cells were loaded with 5 µM BODIPY for 45 min, irradiated with 200 J per m² UVB for 60 min, trypsinized, and fixed. Ten thousand cells were analyzed for shifts in fluorescence by flow cytometry with excitation/emission wavelengths of 490/580 nm. The mean fluorescence values were used for further analysis. Increases in green fluorescence indicate LPO. For each experiment, we incubated keratinocytes with 300 µM cumene hydroperoxide for 45 min as a positive control. Results are representative of three independent experiments (mean ± SEM).

Intracellular ROS ROS levels were determined using the fluorescent probe dihydrodihomocarotene (DHR). DHR can be oxidized and converted to a fluorescent compound (rhodamine 123) by various ROS, including peroxynitrite, superoxide, and H_2O_2, but not nitric oxide. For flow cytometry, keratinocytes were loaded with DHR (10 µM) for 45 min at 37°C, washed, irradiated, fixed, and analyzed (excitation and emission wavelengths of 485 nm and 530 nm, respectively). Mean fluorescence values for 10,000 cells were used for further analysis (Peus et al., 1999b). Results are representative of three independent experiments (mean ± SEM).

Phosphorylated EGF receptor and active ERK1/2 and p38 kinase Cell lysates were extracted in a 1% Triton X-based lysis buffer supplemented with protease and phosphatase inhibitors. Immunoprecipitated EGF receptor was separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), transferred to Immobilon-P membrane (Millipore, Bedford, MA), and blotted for phosphotyrosine. Active ERK1/2 and p38 kinase were detected by immunoblotting. Whole cell lysates were separated by 12% SDS-PAGE and transferred to Immobillon-P membrane. Active forms of ERK1/2 and p38 kinase were detected by using phosphospecific antibodies (New England Biolabs, Beverly, MA).

JNK activity JNK activity was determined with recombinant GST-c-Jun (1–223) as ligand and substrate (Hibi et al., 1993). Endogenously expressed JNK was affinity purified using GST-c-Jun (1–223) bound to glutathione-agarose. The kinase reaction was performed at 30°C for 30 min in kinase buffer (20 mM HEPES pH 7.6, 20 mM MgCl_2, 10 mM β-glycerophosphate, 20 mM p-nitrophenylphosphate, 0.5 mM Na_2VO_3, 2 mM dithiothreitol, 50 µM ATP, and 5 µg [γ^32P]ATP). Phosphorylated proteins were separated by 11% SDS-PAGE and analyzed by autoradiography (Peus et al., 2000a).

Cell viability Live cells were distinguished by enzymatic conversion of the nonfluorescent cell-permeant calcein AM to the intensely fluorescent calcine. Cells were exposed to 200 J per m² UVB. 24 h later, cells were loaded with 0.2 µM calcein AM for 30–45 min at room temperature, trypsinized, fixed, and analyzed by flow cytometry (10,000 cells per sample, with excitation and emission wavelengths of 485 nm and 530 nm, respectively). Mean fluorescence values were used for further analysis, and the percentage of dead cells was calculated according to the protocol provided by Molecular Probes (cat L-3224). Results are representative of three independent experiments (mean ± SEM).

RESULTS

Lipid component of AA6P promotes UVB-induced LPO

UVB-induced LPO was determined by using cPA. This fluorescent fatty acid analog integrates into membranes, where it is readily oxidized because of its extensive unsaturation. As oxidized cPA loses its fluorescence, relative levels of LPO can be determined. Here, cPA-loaded keratinocytes were exposed to 400 J per m² UVB. Fluorescence intensities were determined by flow cytometry (Fig La) or differential UVB-induced LPO (Fig LB). Both techniques revealed a substantial loss of fluorescence. Keratinocytes treated with 10–100 µM AA6P prior to UVB irradiation showed increased loss of fluorescence. At the 100 µM dose, there was significant loss of cPA fluorescence versus UVB-irradiated cells not exposed to AA6P (p<0.05) with little residual fluorescence detectable. In contrast, similar concentrations of unconjugated ascorbic acid did not affect UVB-induced LPO levels.

cPA absorbs in the UV range at 303 nm wavelength, is prone to oxidation, and may therefore be photobleached. To confirm our results, we used the oxidation-sensitive fatty acid Ci1-BODIPY581/591 as an alternative probe for LPO detection. BODIPY has been previously demonstrated to quantify fatty acid oxidation and antioxidant activity in living cells (Pap et al., 1999). Lipid oxidation induced by a 200 J per m² dose of UVB is relatively small, representing a fraction of that generated by micromolar concentrations of cumene hydroperoxide. We found BODIPY a sensitive and reliable probe for LPO detection secondary to UVB irradiation. UVB-induced increases in LPO in the absence of AA6P were readily detected in three independent experiments (p<0.05 versus nonirradiated cells) and arbitrarily defined as a 1-fold increase in fluorescence (Fig 1C). Cells pretreated with 100 or 300 µM AA6P for 30 min prior to irradiation showed dose-dependent increases in mean fluorescence values. At the 300 µM dose, AA6P pretreatment resulted in significant increases in LPO versus UVB only, no AA6P.

AA6P reduces intracellular levels of ROS

To determine intracellular levels of ROS, cells were loaded with DHR. DHR can be oxidized and converted to the fluorescent compound rhodamine 123, which localizes mostly to the mitochondria and other negatively charged intracellular compartments. Peroxynitrite as well as higher oxides of nitrogen (such as N_2O_3) can directly oxidize DHR. Superoxide, H_2O_2 alone, or physiologic levels of nitric oxide, however, do not oxidize...
DHR. Oxidation requires the presence of metal, cytochrome c, or peroxidase (Ischiropoulos et al., 1999), all of which are present in human keratinocytes. Here, cells were loaded with 1^25 pretreated with 1–25 μM AA6P, and exposed to 200 J per m^2 UVB. Fluorescence intensities were determined by flow cytometry. AA6P effectively inhibited DHR oxidation in a dose-dependent manner, indicating its antioxidant potential (Fig 2A). In addition, AA6P-pretreated cells blocked H_2O_2-mediated ERK1/2 and p38 kinase activation and reduced the basal activity of these kinases (Fig 2B).

AA6P inhibits UVB-induced EGF receptor, ERK1/2, and p38 kinase activation but activates JNK. We have previously shown that H_2O_2 is required for UVB-induced EGF receptor and ERK1/2 activation (Peus et al., 1999a). Here, we show that because of the ability of AA6P to effectively scavenge various ROS and inhibit H_2O_2-mediated signaling events, AA6P also effectively inhibited UVB-induced EGF receptor activation (Fig 3B) and ERK1/2 activation (Fig 3B). Surprisingly, we found that p38 kinase and JNK-two stress-activated protein kinases—are differentially modulated by AA6P. Whereas AA6P reduced p38 kinase activation, JNK activity was significantly elevated (Fig 3B, C).

AA6P promotes UVB cytotoxicity. End products of LPO have been shown to be toxic to epithelial cells (Fukuda et al., 1996). Various cytotoxic oxidized lipid compounds have been identified, and 4-hydroxy-2-nonenal has been implicated as a prototypic molecule of oxidative stress. Here, cells were exposed to 200 J per m^2 UVB and a viability assay was performed. Interestingly, UVB cytotoxicity was increased in AA6P-treated keratinocytes (Fig 4) in a dose-dependent manner. Lower AA6P doses (3–30 μM) seemed to have no significant protective effect on viability. Higher doses, ranging from 100 to 300 μM, significantly increased cytotoxicity. These findings correlate with the observed levels of LPO (Fig 1).

DISCUSSION

ROS generation plays an important role in UV-radiation-induced skin damage and can cause injury by reacting with various molecules such as lipids, proteins, and nucleic acids and also by depleting the skin of endogenous enzymatic and nonenzymatic antioxidants (Shindo et al., 1993; Darr and Fridovich, 1994). Nonenzymatic scavengers of free radicals include vitamins C and E, and enzymatic scavengers include superoxide dismutase, catalase, and glutathione peroxidase. In contrast to the inherent instability of ascorbic acid, AA6P, an amphipathic molecule with a polar head and a long hydrophobic tail, not only possesses greater.

Figure 1. The lipid component of AA6P promotes UVB-induced LPO. (A) Cells were loaded with 10 μM cPA and exposed to 400 J per m^2 UVB. Pictures were taken before and 5 min after UVB irradiation using a Zeiss 410 confocal microscope. The loss of fluorescence intensity demonstrates an increase in LPO. (B) Cells were loaded with 10 μM cPA, pretreated with 10, 30, or 100 μM AA6P or ascorbic acid (AA) for 30 min, and exposed to 200 J per m^2 UVB for 30 min. Loss of fluorescence was determined by flow cytometry. Controls are cPA-loaded untreated cells (C = 100% fluorescence) and unstained cells (0% fluorescence). Results are representative of three independent experiments (mean ± SEM). For each UVB-irradiated and AA6P/AA-pretreated experimental sample, a control sample was run that had been treated with AA6P/AA only (all stained with cPA). Baseline fluorescence changes induced by AA6P/AA alone compared to untreated cPA-stained cells were insignificant, and all UVB-irradiated samples were normalized to the corresponding baseline levels. Whereas AA6P promoted UVB-induced LPO, unconjugated ascorbic acid did not affect LPO levels. (C) Cells were loaded with 5 μM BODIPY, pretreated with AA6P as indicated, and exposed to 200 J per m^2 UVB for 60 min. LPO shifts the excitation/emission spectra from red (581/591) to green (500/510) (Pap et al., 1999). Increases in green fluorescence were detected by flow cytometry (excitation/emission: 490/580). Non-irradiated, AA6P only controls were run for all AA6P concentrations and base line fluorescence was subtracted from UVB-irradiated samples. UVB-induced increases in LPO in the absence of AA6P were readily detected (p < 0.05 versus nonirradiated cells) in three independent experiments (±SEM). The mean UVB-induced increase in fluorescence was arbitrarily defined as a 1-fold increase in fluorescence. 30 min 100 μM AA6P pretreatment resulted in increased mean fluorescence values post UVB (p > 0.05). 300 μM AA6P significantly increased LPO post UVB versus UVB only, no AA6P (mean ± SEM, three independent experiments).
stability but also has the commercial advantage of aqueous solubility—hence its widespread use in numerous over-the-counter topical skin care products and sunscreens (Darr et al., 1997). These products contain concentrations of AA6P as high as 15% (360 mM), thus exceeding the dose range used in our experiments by a factor of 1000. Although ascorbyl palmitate is also available as an oral supplement, most of the compound is probably hydrolyzed to ascorbic acid and palmitic acid in the digestive tract before absorption (DeRitter, 1951).

AA6P, a synthetic lipophilic derivative of ascorbic acid, appears to intercalate into the cellular membrane with the ascorbyl group situated more proximal to the membrane surface (May et al., 1996). Even though the antioxidant effect of AA6P has been recognized, its interaction with cellular lipids and its effect on intracellular signaling and cell viability following UV irradiation have not been studied in keratinocytes.

We found that AA6P reduced intracellular levels of ROS dose-dependently and at very low concentrations (Fig. 2A). Interestingly, the membrane-bound phosphatase that regulates EGF receptor phosphorylation is glutathione dependent; in addition, sulfhydryl-group-protecting agents such as reduced glutathione effectively prevent EGF receptor phosphorylation. Although not proven, the recently characterized family of dual-specificity mitogen-activated protein kinase phosphatases that are involved in dephosphorylating activated ERK1/2 and p38 kinase (Haneda et al., 1999) may also be sensitive to glutathione or other sulfhydryl-group-protecting agents. Conversely, n-ethylmaleimide-induced depletion of reduced glutathione in cultured keratinocytes activates EGF receptor, ERK1/2, and p38 kinase (Meves et al., 2001). AA6P might therefore inhibit UVB-induced EGF receptor phosphorylation by scavenging ROS and blocking cellular reduced glutathione depletion. In concordance with these results, a recent report showed that ascorbic acid induces an increase in glutathione concentration in untreated and UVA-irradiated keratinocytes (Tebbe et al., 1997), findings that confirm earlier
results where ascorbic acid protected endogenous glutathione from oxidation (Meister, 1994).

H$_2$O$_2$ itself has been described as exerting its stimulatory effect on receptor tyrosine kinases by potently inhibiting protein tyrosine phosphatases (Gross et al., 1999), perhaps because of the depletion of sulfhydryl groups. The active site of tyrosine phosphatase containing a cysteine residue essential for biologic activity has been shown to be a direct target for H$_2$O$_2$, which can oxidize specific cysteine residues (Caselli et al., 1998). It can therefore be suggested that UVB-induced H$_2$O$_2$ formation directly leads to increased phosphorylation of EGF receptor and that AA6P inhibits its phosphorylation by scavenging H$_2$O$_2$.

The UVB-induced signaling events are probably part of an antioxidant defense strategy in keratinocytes. It has been reported that the activation of selective growth factor receptors, signal transduction pathways, and other transcription factors such as c-fos (Schreiber et al., 1995) and egr-1 (Huang et al., 1996) have a protective function against harmful consequences of UV radiation and are thereby able to modulate cell survival. We have recently reported that pretreatment with EGF receptor inhibitor PD153035 followed by UVB irradiation diminishes keratinocyte clonogenicity and increases cell death (Peus et al., 2000b). AA6P abrogates UVB-induced EGF receptor phosphorylation through the inhibition of reduced glutathione depletion and ROS formation. The lipid component of AA6P, however, promotes UVB-induced LPO and formation of potentially toxic oxidized lipids at higher concentrations exceeding 30 $\mu$M. Abrogating antioxidant signaling but promoting oxidative stress through the formation of cytotoxic compounds potentially induces substantial cellular damage (Fig 5).

Both antioxidant and oxidant properties of AA6P are reflected in the activation pattern of mitogen-activated protein kinases. The ascorbyl group has antioxidant properties and seems to be responsible for the blockage of UVB-induced ERK1/2 and p38 kinase activation. The conjugated lipid has oxidant properties and probably mediates JNK activation. It has been reported that JNK is activated by oxidized lipid metabolites formed during LPO (Uchida et al., 1999). In earlier studies, we have also observed that the level of LPO correlates with JNK activation (Peus et al., 2000a). It is not yet clear whether JNK signaling induces proapoptotic responses in keratinocytes. As higher AA6P concentrations promote UVB cytotoxicity, however, the activation pattern of all three mitogen-activated protein kinases clearly does not seem to effectively protect keratinocytes from cell death.

In summary, at concentrations exceeding 100–300 $\mu$M the potential damage induced by the oxidant properties of AA6P probably outweighs the benefits of its antioxidant potential. Lower AA6P doses (3–30 $\mu$M) seemed to have no significant protective effect on keratinocyte viability although the ascorbyl group was effective in scavenging intracellular ROS. At higher concentrations, the lipid component promoted UVB-induced LPO and the formation of cytotoxic oxidized lipid compounds (Fig 5). AA6P may effectively protect against ROS and oxidant agents.

Figure 4. AA6P promotes UVB cytotoxicity. Live cells were distinguished by enzymatic conversion of calcein AM to fluorescent calcein. Cells were treated with various concentrations of AA6P and exposed to 200 J per m$^2$. After 24 h, cells were loaded with calcein AM, trypsinized, fixed, and analyzed by flow cytometry. Results are representative of three independent experiments (mean ± SEM). For each UVB-irradiated and AA6P-pretreated experimental sample, a control sample was run that had been treated with AA6P only. Background cell death changes induced by AA6P alone compared to untreated cells were insignificant, and all UVB-irradiated samples were normalized to the corresponding baseline levels. *$p<0.05$ versus control (UVB-irradiated, no AA6P).

Figure 5. A model of AA6P cytotoxicity in response to UVB. UVB triggers LPO and ROS generation, thereby mediating JNK, p38 kinase, and ERKs activation. AA6P promotes LPO and inhibits ROS-mediated antiapoptotic signaling. This signaling constellation results in massive cell death in response to UVB (Fig 4). 4-HNE, 4-hydroxynonenal; p38, p38 kinase.
that do not exploit the oxidant properties of AA6P by targeting cellular lipids. Oxidative-stress-induced signaling pathway activation, however, which underlies acute and chronic skin responses to UV radiation, including solar erythema, skin aging, and carcinogenesis, may be negatively influenced by the vitamin C derivative ascorbyl palmitate.

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REFERENCES


