Role of Ascorbic Acid in Stratum Corneum Lipid Models Exposed to UV Irradiation

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Purpose. The effects of ascorbic acid on Stratum corneum lipid models following ultraviolet irradiation were studied adding iron ions as transition metal catalysts.

Methods. Lipid peroxidation was quantified by the thiobarbituric acid assay. The qualitative changes were studied on a molecular level by mass spectrometry. To elucidate the nature of free radical involvement we carried out electron paramagnetic resonance studies. The influence of ascorbic acid on the concentration of hydroxyl radicals was examined using the spin trapping technique. Moreover, we checked the vitamin's ability to react with stable radicals.

Results. Ascorbic acid was found to have prooxidative effects in all lipid systems in a concentration dependent manner. The degradation products of ascorbic acid after its prooxidative action were detected. The concentration of the hydroxyl radicals in the Fenton assay was decreased by ascorbic acid. The quantification assay of 2,2-diphenyl-1-picrylhydrazyl hydrate showed reduced concentration levels of the stable radical caused by ascorbic acid.

Conclusions. Considering human skin and its constant exposure to UV light and oxygen, an increased pool of iron ions in irradiated skin and the depletion of co-antioxidants, the administration of ascorbic acid in cosmetic formulations or in sunscreens could unfold adverse effects among the Stratum corneum lipids.

KEY WORDS: ascorbic acid; thiobarbituric acid assay; mass spectrometry; EPR, oxidative stress.

INTRODUCTION

Ascorbic acid is a hydrophilic hexuronic acid lacton. Because humans have lost the ability to synthesize ascorbic acid, it must be provided as an essential micronutrient from food (1).

The most well-known biochemical function of ascorbic acid is the protection of the prolyl and lysyl hydroxylases. This seems to be essential for the synthesis of stable collagen molecules (2). Furthermore, ascorbic acid has been shown to be a neuromodulator (3), an antiviral substance and immunostimulant (4), and a radical scavenger (5).

Most of the chemical and biochemical properties of ascorbic acid are related to its oxoenediol structure and can be explained by its participation in biochemical redox processes. Ascorbic acid is a dibasic acid and in aqueous solution a strong reducing compound.

Despite the high number of publications dealing with the vitamin (1–13,18), and the relatively simple chemical structure of the molecule, other properties of the molecule are not yet fully understood. Conflicting data has lead to controversy in the literature (6,7), and at present even the daily human intake is still under debate (8). Further sources of controversy are the interactions of vitamin C with reactive oxygen species (ROS) (9), as both antioxidant and prooxidant properties have been reported (10,11). Recently, genotoxic effects were suggested to be a result of the ability of ascorbic acid to decompose lipid hydroperoxides to DNA damaging secondary products (12) raising the question why add ascorbic acid to drug formulations for topical application (13).

In the skin, ascorbic acid is part of the antioxidative network of the Stratum corneum (14) and therefore, along with lipophilic antioxidants (15), proteins (16), and other biomolecules, a target to the oxidative stressors of the environment (17). A decrease in the concentration of ascorbic acid was measured in murine Stratum corneum following ozone exposure (18).

In this paper, therefore, the mechanism of the redox behavior of ascorbic acid is studied using Stratum corneum lipid model systems to test its actions on exposure to UV light. As the biggest organ of the human body, the skin is constantly exposed to both ultraviolet radiation and oxygen. The degradation of ascorbic acid caused by UV-light was studied at a molecular level using electrospray ionization mass spectrometry (ESI-MS). The advantages of the soft electrospray ionization (ESI) avoiding early fragmentation and the ion trap possibilities of full scan mass spectrometry (MS), MS/MS, and multiple stage MS experiments (19) allowed the identification of the ascorbic acid decomposition products as well as the lipid peroxidation products.

Electron paramagnetic resonance (EPR) spectroscopy is being used more frequently in pharmaceutical research (20,21) because of the unique information which can be obtained by EPR spectrums of both *in vivo* and *in vitro* experiments (22). We used the spin trapping method by 5,5-dimethyl-1-pyrroline-1-oxide (DMPO) to detect the highly reactive hydroxyl radicals (23). The influence of vitamin C on the concentration of these free radicals generated by the Fenton system, as well as the vitamin's properties regarding the stable 2,2-diphenyl-1-picrylhydrazyl hydrate (DPPH) radical, were studied.

MATERIALS AND METHODS

Reagents

L-Ascorbic acid, α-Tocopherol, α-Linolenic acid (LLA), L-α-Dipalmitoylphosphatidylcholine (DPPC), Cholesterol (Chol), Ceramide IV (C IV; N-2-Hydroxyacyl-sphingosine from bovine brain), Ferrous sulfate, Ferrous chloride, Malondialdehyde-bis-(dimethylacetal), 2-Thiobarbituric acid, and Trichloroacetic acid for the thiobarbituric acid (TBA) assay (all of analytical grade) were obtained from Sigma (Deisenhofen, Germany) as well as 2,2-diphenyl-1-picrylhydrazyl

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hydrate (DPPH), 5,5-dimethyl-1-pyrroline-1-oxide (DMPO), Adenosine 5′-diphosphate sodium salt (ADP) as a chelating agent and 2,2,6,6-Tetramethyl-1-piperidyl-N-oxyl (TEMPO) for EPR investigations. Analytical grade Ceramide III (C III; N-Stearyl-phytosphingosine) was provided by Cosmoferm (Delft, Netherlands). Methanol of gradient grade and chloroform (LiChrosolv®) were purchased from Merck (Darmstadt, Germany).

Sample Preparation

Lipid model systems were chosen for our experiments to allow investigations on a molecular level. There were two standards the systems should meet. They should be simple to allow mechanistic investigations and avoid overlapping of effects which would make data interpretation difficult or impossible. Despite this simplicity the main properties of the Stratum corneum intercellular lipid matrix should be present. The lipids for the experiments were chosen according to the lipid composition of the horny layer lipid matrix. Cholesterol, ceramide III, and ceramide IV as sphingolipid models, linolenic acid and DPPC as a liposome generator, were used.

The simple system was an oil in water dispersion of linolenic acid obtained by shaking the system for 120 min using an laboratory flask shaker (GFL 3006, Gesellschaft für Labortechnik, Burgwedel, Germany).

The complex systems were prepared as liposomes using the thin layer method. The lipids were dissolved in 0.5 ml chloroform. The solvent was removed using a rotation vaporizer (Labo-Rota C-311, resona technics, Switzerland) and a vacuum pump (PIZ 100 Mini-Tower-MPC 050-Z, Saskia Hochvakuum- and Labortechnik, Ilmenau, Germany). To obtain a thin and homogeneous lipid film a fast rotation was chosen (150 rpm). The remaining lipid film was dispersed in 20 ml of double distilled water by shaking intensively for 360 min. The remaining double distilled water was added to achieve the final concentration. The concentrations of the ingredients in the liposome stock solutions were: DPPC 1.0 mM, Chol, LLA, C III, and C IV 0.5 mM.

To obtain vesicles of a uniform size the stock solution was passed through a 400 nm polycarbonate filter (Costar, Cambridge, United Kingdom) at 20°C under nitrogen pressure using an extruder device (Lipex Biomembranes, Vancouver, British Columbia, Canada). This step was repeated five times. The exact composition of the samples as well as an overview of the manufacturing processes are shown in Table I.

Ferrous sulfate (10 μM) was added to the samples as an electron donator and catalyst of the Haber-Weiss reaction to initiate ROS creation via a Fenton type reaction. Previous investigations have shown that there is no effect when irradiating lipid model systems without transition metal catalysts (24). All the liposome suspensions and fatty acid dispersions were freshly prepared just before use.

Particle Size Determination

To control the vesicle size and the success of extrusion, the particle diameters were determined via photon correlation spectroscopy using the Malvern Instruments Autosizer 2c equipped with a series 7032 Multi-8 Correlator (Malvern, Worcester, United Kingdom).

Ultraviolet Irradiation

UV-B irradiation experiments were carried out using a special irradiation equipment (Dr. Gröbel UV-Elektronik, Ettlingen, Germany) enabling a selective exposure to UV-B because of the special lamp F15/T8 15 W with a main emission range of 290–320 nm (Sankyo Co., Tokyo, Japan). Before irradiation, the samples were transferred to 55 mm open glass dishes. Thereby, the optical pathlength was 2.1 mm and a homogeneous exposure was assumed despite light scattering. The samples were treated with an UV-B dose of 0.25 J/cm^2 which corresponds approximately with the 2–3 fold of the minimal erythemal dose (MED) of humans. This high dosage was required to test under high stress conditions.

Thiobarbituric Acid Assay

The thiobarbituric acid (TBA) test is a quantitative assay for the detection of malondialdehyde (MDA), and is the most widely used technique to determine lipid peroxidation products.

In this study, the Buege-Aust method was used (25). Briefly, 2 ml of a stock TBA reagent containing 15% (w/v) trichloroacetic acid in 0.25 M HCl and 0.37% (w/v) thiobarbituric acid in 0.25 M HCl was added to 1 ml of the UV-B treated sample. After heating at 90°C for 15 min and cooling, the red TBA:MDA-complex (2:1) appears allowing fluores-

Table I. The Systems Studied (Additionally 10 μM $FeSO_4$ Were Added to Each Sample)

System	Lipids	Concentration	Manufacturing Process
1. Simple system (System A)	α-Linolenic acid	100 μM	Shaking for 120 min
2. Complex system (System B)	α-Linolenic acid	100 μM	Liposomes prepared by the thin film method
	DPPC	200 μM	
	Cholesterol	100 μM	
3. Complex system with ceramides			
a) Ceramide III (System C)	α-Linolenic acid	100 μM	Liposomes prepared by the thin film method
	DPPC	200 μM	
	Cholesterol	100 μM	
	Ceramide III	100 μM	
b) Ceramide IV (System D)	α-Linolenic acid	100 μM	Liposomes prepared by the thin film method
	DPPC	200 μM	
	Cholesterol	100 μM	
	Ceramide IV	100 μM	

cence measurement. A HPLC system (Merck-Hitachi, Darmstadt, Germany) equipped with an auto sampler AS-4000A, interface D-6000A, pump L-6200A, UV-VIS-Detector L-4250, fluorescence detector F-1080 was used to quantify the pigment. A reversed phase column (LiChrospher® 100, RP 8, particle size 5 μm) was used with a mobile phase methanol/water 30:70 for HPLC procedure. The wavelength for excitation was 515 nm and the emission measurement was performed at 555 nm.

A calibration curve was generated by preparing an aqueous solution of malondialdehyde-bis-(dimethylacetal). Under acidic conditions the acetal groups were hydrolyzed and malondialdehyde was formed.

Mass Spectrometry

The mass spectrometric operations were carried out using a LCQ ion trap mass spectrometer with ESI interface and integrated syringe pump (Finningan MAT, San Jose, California). Electrospray mass spectrometry was performed in the negative ion mode with an ESI voltage of –4.5 kV and a heated capillary temperature of 200°C. The aqueous samples were mixed with methanol to obtain a stable ESI spray. Then the solution was injected via syringe pump (10 μl/min).

Electron Paramagnetic Resonance

Electron paramagnetic resonance studies were carried out in the X-Band using a Bruker ESP 380 E FT-EPR spectrometer (Bruker, Rheinstetten, Germany). The ascorbic acid radical was detected by use of the cylindrical resonator ER 4103 TM and the aqueous sample flat cell ER 166 FCTMVT (Bruker, Rheinstetten, Germany). The instrument settings for the cylindrical resonator were the following: microwave frequency 9.75 GHz, microwave power 3.6 mW, modulation frequency 100 kHz, modulation amplitude 0.1 mT, sweep width 2 mT, conversion time 20.48 ms, time constant 5.12 ms, sweep time 20.97 s. Quantitative investigations were performed using the double resonator technique in the rectangular cavity ER 4105 DR with corresponding aqueous sample cells ER 160 FC (Rototec Spintec, Spektroskopie Vertriebs-GmbH, Remshalden).The double resonator settings were: microwave frequency 9.78 GHz, microwave power 13.6 mW, modulation frequency 100 kHz, modulation amplitude 0.3 mT, sweep width 15 mT, conversion time 40.96 ms, time constant 10.24 ms, sweep time 41.94 s. For evaluating the hydroxyl radical scavenging activity, the spin trapping method was used. Therefore, the reactive hydroxyl radicals were produced via the Fenton system consisting of hydrogen peroxide and iron (II) ions and trapped by DMPO. Before transferring the specimen into the cavity 1 ml of each stock solution was added in the following order: (I) ADP (2 mM), (II) Ferrous sulfate (0.5 mM), (III) Test substance or double distilled water as a blank control, (IV) The spin trap DMPO (40 mM), (V) H_2O_2 (0.3%).

Exactly 120 s after the addition of hydrogen peroxide the recording of the spectrums was started measuring the specimen in the resonator 1 of the cavity and TEMPO (50 μM in methanol) as the reference in the resonator 2 of the double resonator.

The double resonator technique is a method to study aqueous samples in a reproducibly quantitative manner. Every measured signal was set in proportion to a reference signal of TEMPO to avoid analytical artifacts due to changes in the Q factor of the resonator.

DPPH (0.1 mM in methanol) was chosen as a stable free radical. We mixed different concentrations of ascorbic acid (a range from 1–100 μM; 2.0 ml of each specimen) with 3.0 ml of the DPPH stock solution and started the spectrum recording again after 120 s against TEMPO as a reference sample in the double resonator. All signals are the results of 5 time accumulations. The quantitative analysis of the EPR spectrums were carried out by double integration of the EPR signals using the WinEPR software (Bruker, Rheinstetten, Germany).

RESULTS AND DISCUSSION

Thiobarbituric Acid Assay

Figure 1 shows the results of the thiobarbituric acid assay of the Stratum corneum lipid systems. The effects of 1–100 μM ascorbic acid on a linolenic acid dispersion are shown in Fig. 1A. Secondary lipid peroxidation products (measured as malondialdehyde units) are significantly increased in a concentration dependent manner. The TBA reaction product concentration level is increased by ascorbic acid from 250 ng/ml for 1 μM ascorbic acid to 1100 ng/ml for 100 μM vitamin C.

Figure 1B shows the effects of the vitamin measured using the complex system including the phospholipid DPPC, cholesterol, and linolenic acid. Ascorbic acid is acting prooxidatively as well, but the level of peroxidative damage is increased. Only linolenic acid is responsive to UV light, transition metals, and ROS because it is the only compound containing allylic double bonds. The effects of the enhanced sensitivity of unsaturated compounds when encapsulated into liposomes has been reported (24).

The addition of ceramide III and ceramide IV to the lipid systems made the models more complex, making them more similar to the lipid matrix of the human Stratum corneum (Fig. 1C and 1D). The addition of the sphingolipids caused no additional effect on the TBA levels of the complex system. This can be explained by the lack of conjugated or allylic double bonds, and the relative stability against oxidative attack arising from the sphingolipid structure.

Ascorbic acid increases the amount of lipid peroxidation products measured as malondialdehyde content in all skin lipid systems in a concentration dependent manner. The level of the thiobarbituric acid assay reaction products is higher in lipid systems that are more complex. This provides evidence supporting the EPR results of Buettner and Jurkiewicz: Catalytic metals, ascorbate and free radicals are combinations to avoid. (26).

Figure 1E shows that α-Tocopherol protects lipids from UV induced oxidative damage catalyzed by iron ions, while ascorbic acid was found to increase oxidative damage. A mixture of both vitamins is able to decrease the amount of lipid peroxidation caused by ascorbic acid, UV and transition metal catalysts. This may reflect the redox cycling abilities of ascorbic acid; as a molecule with strong reducing properties it is able to regenerate used α-Tocopherol from its oxidized radical form.

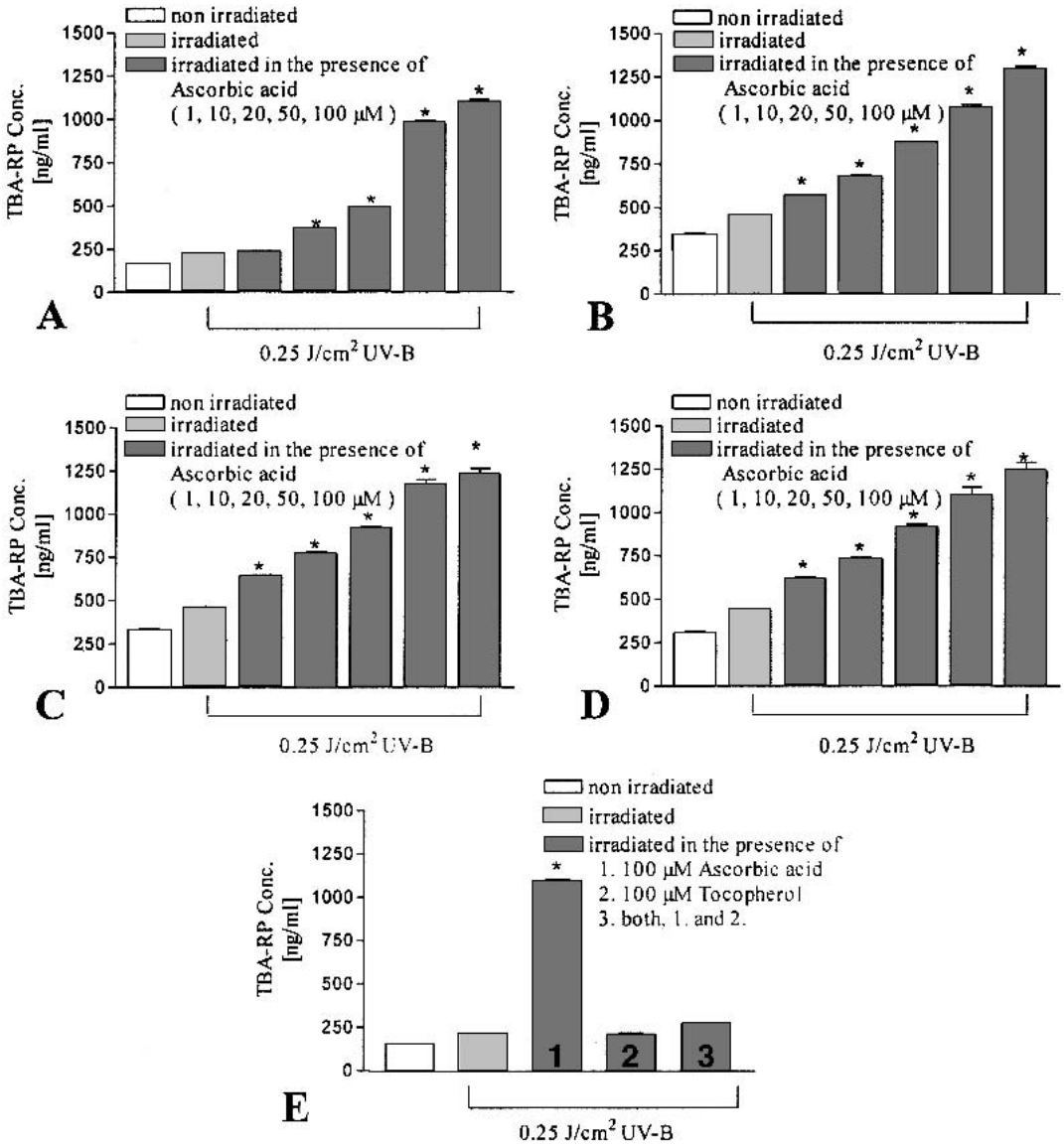

Fig. 1. Concentration of the thiobarbituric acid reaction products (TBA-RP Conc.) and influence of ultraviolet irradiation and ascorbic acid in the systems used. (A) Simple system, (B) Complex system, (C) Complex system with ceramide III, (D) Complex system with ceramide IV, (E) Effect of ascorbic acid on the TBA-RP concentration levels of system A compared with that of tocopherol and a mixture of both. $^*P < 0.05$, UV exposed in the presence of ascorbic acid vs. irradiated without ascorbic acid (light grey columns), two-tailed test. All data represent the mean values ± SD of sextuple measurements (n = 6).

Mass Spectrometry

To elucidate the mechanism of degradation of ascorbic acid at a molecular level, mass spectrometric investigations were performed using an ESI-MS device. Figure 2A shows the negative ion mode spectrum of ascorbic acid and iron ions exposed to 0.25 J/cm^2 UV-B.

A degradation scheme is also shown (Fig. 2B). In the m/z range 70–270, 7 main peaks were detected. The ascorbic peak at m/z = 175 disappeared completely. Dehydroascorbate, the corresponding redox partner and oxidation product could be detected at m/z = 173. The main peak of the irradiated system appeared at m/z = 191. This peak was assigned to the open chained compound diketogulonate as the main degradation product of ascorbic acid. However, it could also represent the monohydrate of dehydroascorbic acid. The addition of an H_2O molecule leads to a m/z of 209. This peak was identified as the dihydrate of DHAA or the open chained monohydrated diketogulonic acid. Another H_2O addition brings the diketogulonic acid dihydrate, the only open ring form possible. Decarboxylation of diketogulonic acid leads to trihydroxy-keto-valeraldehyde and its tautomers. The peaks at m/z = 129 and m/z = 111 can be explained by a twice running water loss.

Figure 3A shows the qualitative changes which occur when linolenic acid is exposed to 0.25 J/cm^2 of UV-B light. For a better illustration the potential pathways of linolenic acid peroxidation are shown (Fig. 3B).

Assuming a proton abstraction in the bis-allylic position as the initiating step of lipid peroxidation there are several possibilities for the generation of oxygenated compounds according to the presence of two cis-cis pentadiene elements.

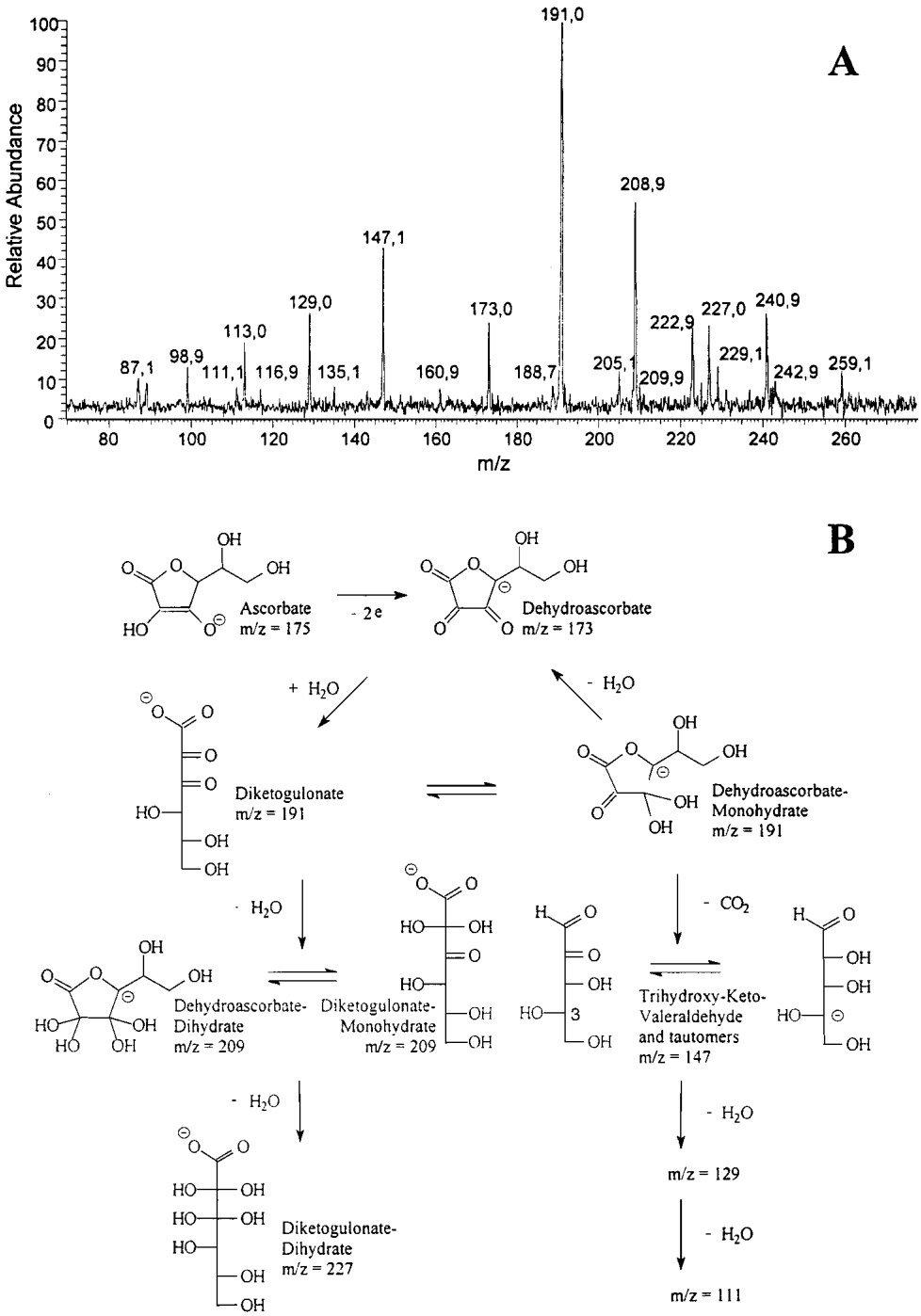

Fig. 2. (A) Negative ion mode ESI mass spectrum of ascorbic acid irradiated with 0.25 J/cm^2 UV-B in the presence of 10 μM Fe(II). (B) Degradation scheme.

After initiation, the system converts its double bonds from allylic to conjugated as a molecular rearrangement. Molecular oxygen and hydrogen are added to the alkyl radical and a hydroperoxide is formed which can be stabilized by reduction to the hydroxide. Because of the 3 allylic double bonds, there are several possibilities for the formation of primary and secondary peroxidation products, only a few of which are shown in Fig. 3B.

Figure 4 shows an ESI-MS spectrum of the system linolenic acid/ascorbic acid and iron ions exposed to 0.25 J/cm^2 UV-B. The peaks described in the Figs. 2 and 3 are detected. The spectrum shows the degradation of the ascorbic acid molecule after having enhanced the oxidative change of the unsaturated fatty acid. The peroxidation products of linolenic acid discussed above are also present.

Electron Paramagnetic Resonance

Using EPR spectroscopy we were able to elucidate the radical processes involved in the studied systems. First, the

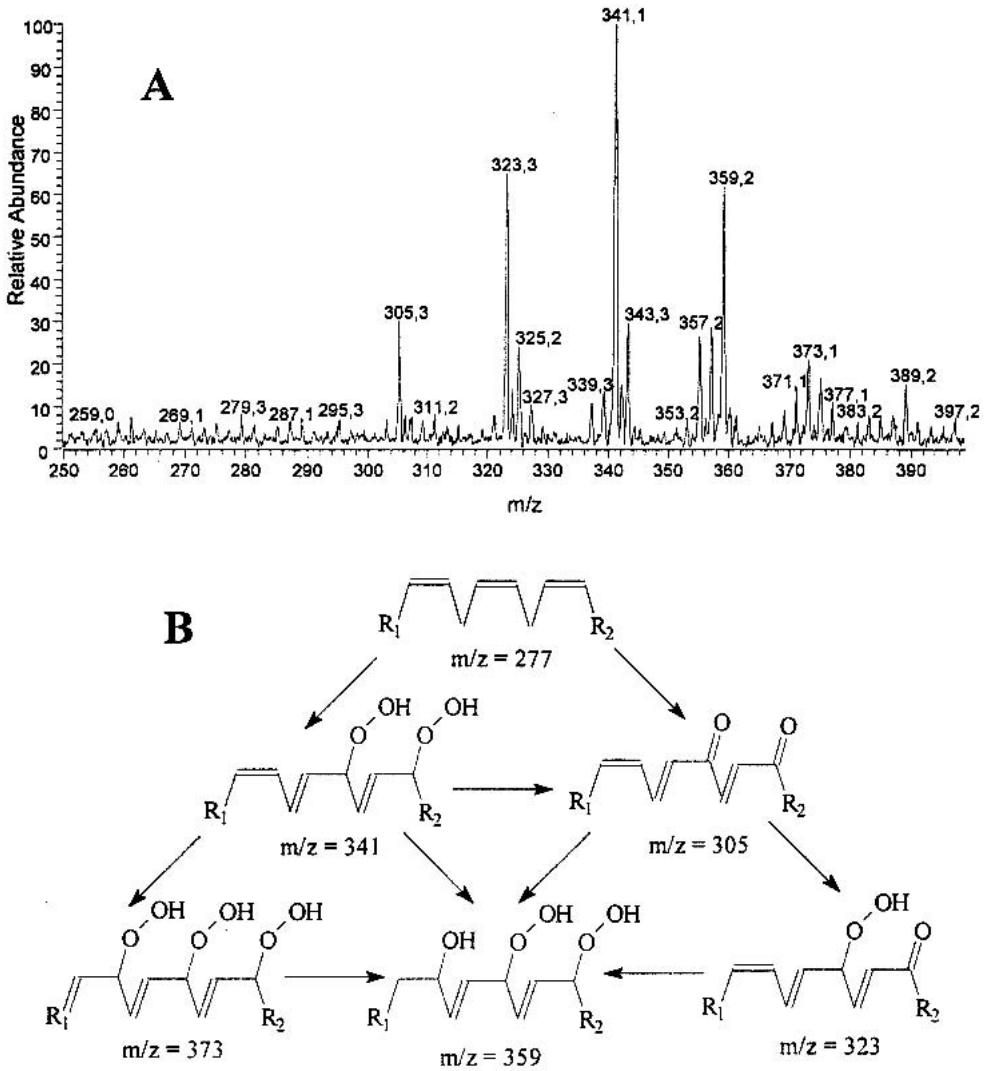

Fig. 3. (A) Negative ion mode ESI mass spectrum of linolenic acid irradiated with 0.25 J/cm^2 UV-B in the presence of 10 μM Fe(II). (B) Scheme of some of the possible lipid peroxidation products of linolenic acid.

ascorbic acid radical (Fig. 5A) was detected as an intermediate of the corresponding redox partners ascorbic acid and dehydroascorbic acid with its typical proton hyperfine splitting (hfs) of $a^H = 0.18$ mT and a g factor of $g = 2.0052$ (27). This radical was present in a freshly prepared solution of 100 μM ascorbic acid in double distilled water at room temperature.

Furthermore, experiments with hydroxyl radicals using the spin trapping technique with DMPO as a trap for the reactive oxygen species were carried out. Hydroxyl radicals are the most reactive oxygen species, hence it is impossible to detect them directly even by EPR because of their high reaction constants. An indirect determination can be achieved by the reaction with spin traps such as DMPO. The decrease in the signal intensity is regarded as a measure of the antioxidative potential of compounds. Figure 5B shows the EPR spectrum of the DMPO-OH adduct with its characteristic spin Hamiltonian hfs parameter $a^H = a^N = 1.49$ mT (28).

In the Fenton assay, there were no radicals to quantify even after adding ascorbic acid in the lowest concentration. This could be due to the ability of ascorbic acid to scavenge Fenton system generated hydroxyl radicals faster than DMPO as shown for ascorbyl palmitate in various *in vitro* models (29). In the study by Perricone *et al.* the vitamin C derivate was found to be one order of magnitude faster in radical scavenging than the spin trap. Another explanation could be the reduction of the already generated paramagnetic OH-spin adduct to the corresponding hydroxylamine as concluded by Haberland *et al.* (30). However, both interpretations provide evidence to the role of the vitamin as a terminal small molecule antioxidant with reducing properties in biological systems which is due to its position at the bottom of the one-electron reduction potentials list for radical couples.

The DPPH radical was used as an example of stable organic free radicals. The X-Band EPR spectrum of 0.1 mM solution is shown in Fig. 5C. Often, the antioxidative properties of compounds are referred to their ability to reduce the concentration of the DPPH free radical. TEMPO is a stable free radical and was used as a reference specimen in the double resonator for both DMPO-OH-determinations and DPPH studies. Figure 5D illustrates the EPR spectrum of a 50 μM methanolic solution with a characteristic splitting of

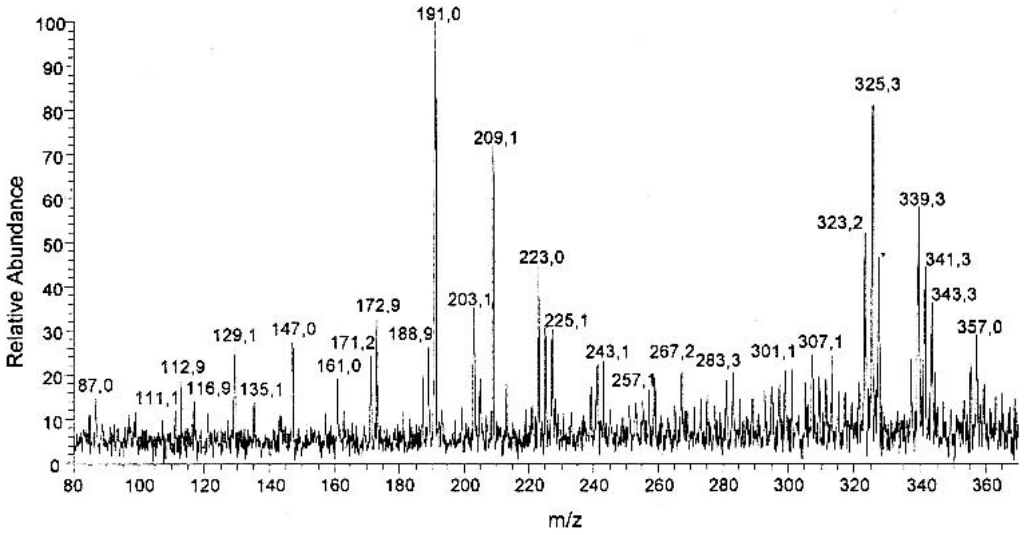

Fig. 4. Negative ion mode ESI mass spectrum of a mixture of ascorbic acid and linolenic acid irradiated with 0.25 J/cm^2 UV-B in the presence of 10 μM Fe(II).

a^N = 1.74 mT. By adding ascorbic acid to a DPPH solution in several concentrations (Fig. 6), a decrease of the EPR signal intensities of the stable radical in relation to the TEMPO reference could be observed. The reducing potential of ascorbic acid could be the reason for this result. The molecule can easily provide electrons to other compounds to be oxidated itself.

We have shown in this article that ascorbic acid exhibits prooxidative effects on lipid model systems of differing complexity. These effects are concentration dependent and transition metal ions such as iron ions (Fe^{II}) are required. The molecular mechanism of the ascorbic acid degradation after its lipid damaging action was demonstrated by mass spectrometry. The results of EPR investigations were useful for

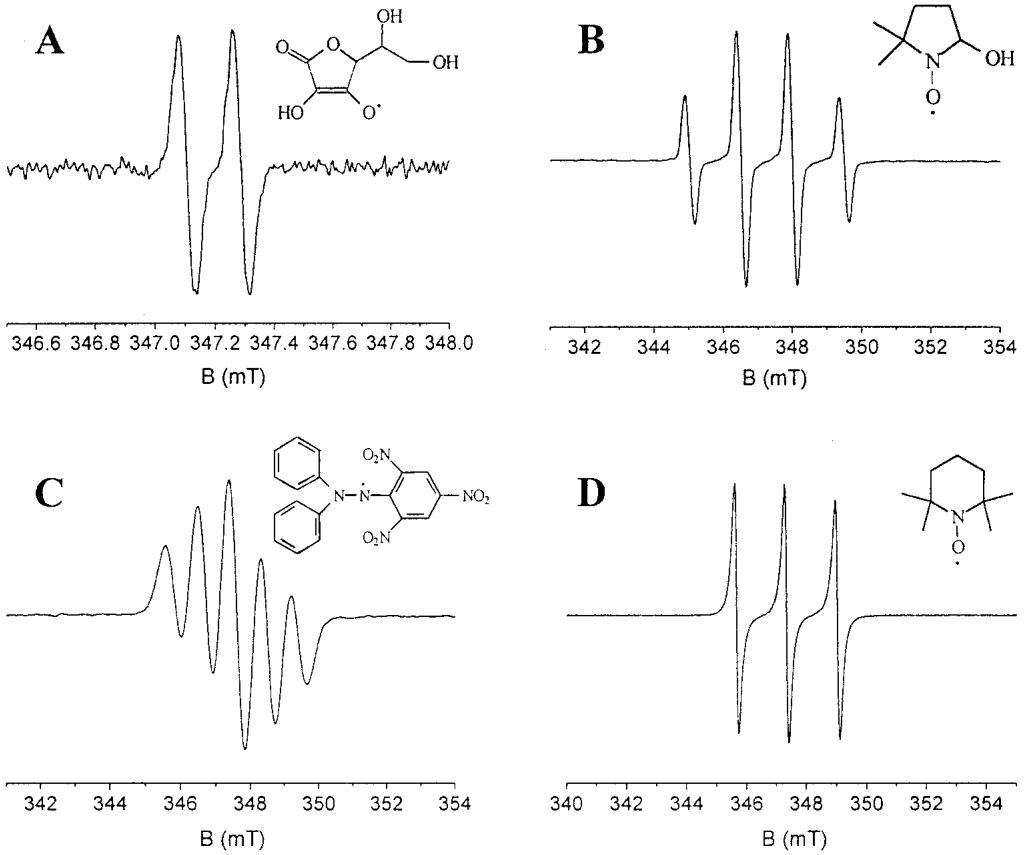

Fig. 5. X-Band EPR spectra. (A) The ascorbic acid radical, (B) Hydroxyl radicals trapped by DMPO, (C) The DPPH radical, (D) The spin probe TEMPO (instrument settings are described in the Materials and Methods section).

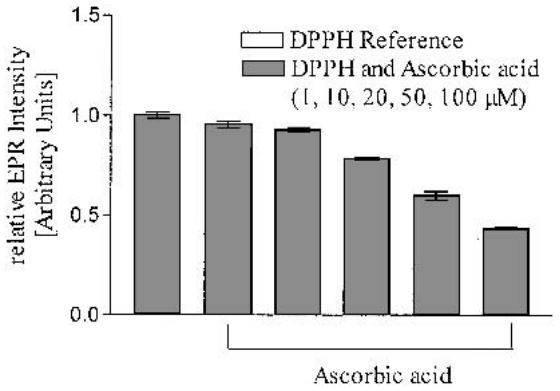

Fig. 6. Effect of ascorbic acid on the concentration of the stable radical DPPH measured using the EPR double resonator technique in relation to the TEMPO reference. The error bars represent the standard deviation (± SD) obtained by quadruple measurements (n = 4) which was always below 5% (SD < 5%).

detailed studies regarding the redox properties of ascorbic acid.

Normally, in the human body there is an antioxidative network where several antioxidative systems influence and renew themselves. Ascorbic acid plays an important role in the collagen production and in the recycling process of these physiologically present co-antioxidants. Kitazawa *et al.* confirmed these relationships between skin antioxidants by EPR spectroscopy. The addition of hydrophilized short chain vitamin E homologues to murine skin homogenates exposed to UV irradiation prevented the oxidation of ascorbic acid (31).

Under physiological conditions there is the chance for an interplay of the nonenzymatic small molecule antioxidants with the enzymatic antioxidant system of the skin (32). *In vivo* studies of topically administered vitamin C, therefore, mostly cause no harmful response but modest protective effects (33). Sunburn cell number determinations and erythema measurements by laser Doppler velocimetry after aqueous vitamin C application to animal skin of the porcine model showed protective effects of the vitamin after UV irradiation (34).

Damage of biomolecules only occurs when all antioxidative systems such as the vitamin E redox system are exhausted or reduced in function. The whole reductive potential of vitamin C would then be available for the reduction of Fe^{III} ions. This would lead to ascorbyl radicals and Fe^{II} ions as catalysts of the Haber-Weiss-reaction generating more damaging hydroxyl radicals. Our results are evidence for the jeopardizing synergistic effects of the combination of ascorbic acid, iron ions, and ultraviolet irradiation. The iron concentration used in this study when finding the harmful effects (10 µM) was relatively high towards the lipids. However, Green *et al.* demonstrated that human skin is a significant side of iron excretion (35). In a collaborative study a total iron content in the epidermal skin of 22.5 (±17.8) mg was calculated from material obtained from eccrine areas. The involvement of iron ions in UV irradiation induced free radical formation in the skin was confirmed by Buettner and Jurkiewicz (26). The treatment of skin samples with the iron chelator Desferal® resulted in a significant reduction of a spin adduct after irradiation suggesting a key role for iron in UV mediated free radical formation.

UV radiation is able to increase the skin pool of non-heme iron (Fe^{II}) in dermis and epidermis significantly. Authors have measured 18 ppm/dry weight of unexposed body parts (buttocks, thigh) vs. 53 ppm/dry weight in epidermis biopsies of exposed parts such as cheek, forehead, and neck (36). Additional to direct damage of biomolecules by UV, this is an indirect pathway leading to dangerous oxygen radicals.

Recently, Lee *et al.* (12) found ascorbic acid to decompose lipid hydroperoxides to endogenous genotoxins even without transition metal ion catalysis. This point is to take into consideration when discussing our results. The formation of DNA reactive bifunctional electrophiles could be an additional damaging effect of ascorbic acid in our experiments. First, ascorbic acid reduces Fe^{III}. More Fe^{II} ions are available and the Fenton reaction can run on a higher level leading to more peroxidized lipids. Following the results of Lee *et al.*, more substrate for ascorbic acid to decompose is in the system. The amount of cell toxic aldehydes is increased which could be the reason for the increased levels of the TBA assay. Considering the presence of high concentrations of ascorbic acid in many cosmetic formulations and even in sunscreens our results appear to be alarming under the conditions discussed above. However, *in vivo* models are required to confirm this assumption.

CONCLUSION

Several analytical techniques were applied to investigate the complex and controversial discussed matter of ascorbic acid redox behavior using *in vitro* systems. The TBA assay was applied to quantify lipid peroxidation products. It could be shown that ascorbic acid exerts prooxidative effects on the models.

To elucidate the mechanism of these prooxidative effects, mass spectrometric studies were carried out and have shown that the vitamin is degraded after acting prooxidatively. The degradation products of ascorbic acid as well as the peroxidation products of linolenic acid damaged this way were detected.

To learn more about the radical involvement during these processes EPR experiments were applied. Ascorbic acid lowered both the concentration of spin trapped hydroxyl radicals and the content of the stable organic radical DPPH. The first observation results from ascorbic acid acting as a key small molecule antioxidant in biological systems. This fact keeps undisputable and refers to its thermodynamically position at the bottom of the rank order of oxidizing free radicals (37). The behavior in the presence of DPPH can be explained by the reducing properties of the vitamin. Considering human skin and its constant exposure to UV light and oxygen, combined with the increased iron content of the exposed skin, exclusive topical administration of ascorbic acid in cosmetic and pharmaceutical semisolid formulations could be rather adverse than protective for the lipids within the largest human organ. This could be the case under the circumstances that all other substances belonging to the antioxidative network of the horny layer are depleted when regarding the Stratum corneum as a key player in the antioxidant protection of the skin and assuming the presence of ascorbic acid at the place where the damage can occur.

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