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Nitroxides are more efficient inhibitors of oxidative damage to calf skin collagen than antioxidant vitamins

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Abstract

Reactive oxygen species generated upon UV-A exposure appear to play a major role in dermal connective tissue transformations including degradation of skin collagen. Here we investigate on oxidative damage to collagen achieved by exposure to (i) UV-A irradiation and to (ii) AAPH-derived radicals and on its possible prevention using synthetic and natural antioxidants. Oxidative damage was identified through SDS-PAGE, circular dichroism spectroscopy and quantification of protein carbonyl residues. Collagen (2 mg/ml) exposed to UV-A and to AAPH-derived radicals was degraded in a time- and dose-dependent manner. Upon UV-A exposure, maximum damage was observable at 730 kJ/m^2 UV-A, found to be equivalent to roughly 2 h of sunshine, while exposure to 5 mM AAPH for 2 h at 50 °C lead to maximum collagen degradation. In both cases, dose-dependent protection was achieved by incubation with μM concentrations of nitroxide radicals, where the extent of protection was shown to be dictated by their structural differences whereas the vitamins E and C proved less efficient inhibitors of collagen damage. These results suggest that nitroxide radicals may be able to prevent oxidative injury to dermal tissues in vivo alternatively to commonly used natural antioxidants.

Keywords: Collagen; SDS-PAGE; UV-A; Alkyl and peroxyl radicals; Carbonyl groups; Nitroxide antioxidants

1. Introduction

Solar radiation has recently been recognized as the most important environmental health carcinogen, due mainly to the direct interaction of UV-B (290–320 nm) with cellular DNA leading to the formation of mutagenic cyclobutane pyrimidine dimers, 6-4 photoproducts and thymine glycols [1,2]. However, the UV-A component of sunlight (320–400 nm) also appears to be an important skin carcinogen via the generation of fingerprint mutations and of reactive oxygen species (ROS) which oxidatively modify DNA [3,4]. Besides this carcinogenic effect, over-exposure of human skin to UV radiation, especially to UV-A which is particularly efficient at generating ROS and which reaches deeper into the dermal layers of the skin [5], also leads to photoaging characterized by wrinkles, roughness, sagging, mottled dyspigmentation, loss of skin tone [6,7]. Histological and ultrastructural studies have shown that these cutaneous alterations are mainly found in the dermal connective tissue whose extracellular matrix is rich in type I collagen associated with a lower amount of type III collagen, elastin proteoglycans and fibronectin [8]. Solar elastosis for example, which is the most prominent histologic feature of photoaging, is characterized by the degradation of collagen and accumulation of abnormal elastin in the superficial dermis [9,10]. Although the skin is equipped with an intricate 'antioxidant network' consisting of both enzymatic and non-enzymatic antioxidants to counteract the harmful effects of ROS induced by UV-exposure, a disturbance in this pro-oxidant/antioxidant balance may result in oxidative damage of cellular targets [11,12]. Hence it is common practice nowadays to add low molecular weight antioxidants to the skin reservoir by applying antioxidants topically in order to protect the skin against photodamage/aging [13]. In fact, there is probably no greater focus of interest currently than the incorporation of vitamins and antioxidants in skin care products aimed at slowing down the aging process and reducing

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photodamage to skin, i.e. to improve not only the appearance of skin, but also its health [14]. The two most predominant antioxidants in skin are the vitamins C and E, therefore it is not surprising that these are the most widely used categories of actives formulated in topical applications including sunscreens [15,16]. Other than the interest on, and exploitation of, antioxidant vitamins, there is also growing interest on the efficacy of other antioxidants that could be used topically in cosmetics. These include polyphenolic compounds from various plants [17–20], lipoic acid [21], glycolic acid [22], and iron chelators such as 2-furildioxime [23], compatible solutes such as ectoin [24], and even essential minerals such as selenium and zinc [25].

Based on this ongoing search for new, topical photoprotective/antiaging actives, the present investigation was undertaken to explore and compare the efficacy of a class of synthetic antioxidants, namely nitroxide radicals, with the natural antioxidants, vitamins C and E against the UV-A degradation of collagen. This is the most abundant protein constituent of the skin's extracellular matrix responsible for the mechanical strength of skin, and is characterized by three left-handed chains held together by hydrogen bonding and twisted into a right-handed triple helical structure [26]. Interest in studying these compounds stems from the fact that nitroxides can detoxify ROS in different ways, depending on the type of species they encounter, either through radical–radical coupling or electron transfer reactions: this latter mechanism lies at the basis of their SOD-mimic activity and their capacity to prevent metal-catalyzed free radical reactions [27–31]. Instead, the above-mentioned vitamins exert their antioxidant activity mainly *via* hydrogen donation or in some cases electron donation, depending on the environment where they are found [32–34]. In addition, the effects of alkyl and peroxyl radicals on collagen and the effect of the above mentioned antioxidants were also investigated, since these species are expected to be generated in abundance during UV-A induced peroxidation of skin barrier lipids and of cell membranes. Because of the different antioxidant properties of nitroxides and vitamins, their inhibitory effect is not expected to be the same, therefore it was of interest to study which could be more suitable for inhibiting oxidative damage to collagen. This damage was identified through SDS-PAGE electrophoresis, circular dichroism spectroscopy and quantification of protein carbonyl residues.

2. Materials and methods

2.1. Materials

Collagen (from calf skin) Type III, 4-Hydroxy TEMPO (Tempol), AAPH [2,2′-azobis(2-methylpropionamidine)dihydrochloride], vitamin E (α-tocopherol), vitamin C (L-ascorbic acid) and all other reagents and solvents were purchased from Sigma-Aldrich Chemical Co. (Milan, Italy). All the chemicals used for SDS-PAGE (sodium dodecyl sulphate polyacrylamide gel electrophoresis): sodium dodecyl sulphate, tris(hydroxylmethyl)aminomethane acrylamide, *N,N′*-methylene-bis-acrylamide, ammonium persulphate, *N,N,N′,N′*-tetramethylethylendiamine (TEMED), β-mercaptoethanol, and the electrophoresis kit were purchased from Bio-Rad (Milan, Italy). The indolinonic nitroxides N1 (1,2-dihydro-2-methyl-2-phenyl-3*H*-indol-3-phenylimino-1-oxyl) and N2 (1,2-dihydro-2-methyl-2-phenyl-3*H*-indol-3-one-1-oxyl) (Fig. 1) were synthesized as

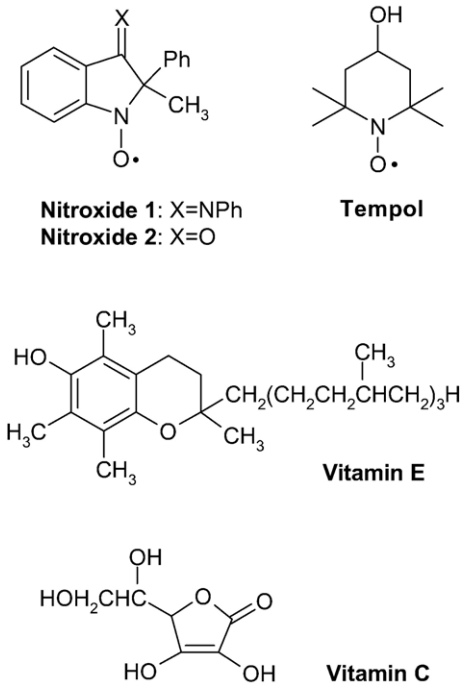

Fig. 1. Chemical structures of compounds studied.

described previously [35]. Their purity was checked by thin-layer chromatography, by mass spectroscopy on a Carlo Erba QMD 1000 spectrometer (Milan, Italy) in EI^+ mode, by infra-red spectroscopy (FT-IR) on a Nicolet Fourier Transform Infrared 20-XS spectrophotometer equipped with a Spectra Tech, and by electron spin resonance spectrosocopy on a Bruker EMX EPR spectrometer (Bruker, Karlsruhe, Germany) equipped with an XL microwave frequency counter, Model 3120 for the determination of *g* factors.

2.2. Sample preparation

Before each experiment, collagen (2 mg/ml) was dissolved in acetic acid 0.04 M, pH 4.3 and maintained at 4 °C over night under gentle magnetic stirring in order to obtain a homogenous suspension. Stock solutions (11 mM) of the nitroxides and vitamin E were prepared in acetonitrile while vitamin C (11 mM) and AAPH (235 mM) were dissolved in distilled water and prepared freshly each time. Before exposure to UV-A or to AAPH, the appropriate amount of antioxidant was added to the collagen solution (0.5 ml) in an Eppendorf, in order to reach the final concentration desired and gently vortexed for thorough incorporation. The volume of acetonitrile used in the experiments never exceeded 2% v/v. When AAPH was used, this was added to the collagen solution after addition of the antioxidants.

2.3. UV-A exposure

Samples for UV-A exposure were transferred into a 24 multi-well plate for cell cultures (Orange Scientific, Cambrex BioScience, Walkerville, Inc.), covered with a 2-mm thick quartz slab to prevent any evaporation and placed on a brass block embedded on ice in order to maintain constant temperature. As UV-A irradiating source, a commercial sun lamp, Philips Original Home Solarium (model HB 406/A; Philips, Groningen, Holland) equipped with a 400-W ozone-free Philips HPA lamp, UV type 3, delivering a flux of 23 mW/cm^2 between 300 and 400 nm, at a distance of 20 cm was used. It was always pre-run for 10 min to allow the output to stabilize. The incident dose of UV-A received from above by the samples varied according to the length of exposure and the maximum time of exposure employed in most experiments, i.e. 40 min, corresponded to 730 kJ/m^2 as measured with a UV Power Pack Radiometer (EIT Inc, Sterling, USA). The emission spectrum of the UV-A lamp was further checked using a StellarNet portable spectroradiometer (Tampa, FL, USA) which

confirmed the manufacturer's declaration. The emission of UV light below 320 nm was <1.5% of the total emitted between 300 and 400 nm, hence the UV source is essentially a UV-A one. Non-illuminated controls were kept on ice in the dark for the same length of time as the UV-A exposure.

When natural sunlight was used as irradiation source, the same procedure as above was followed except that the collagen samples were exposed to direct natural sunlight, for 1 or 2 h between midday and 2.00 p.m., at sea level (Ancona, Italy) in the month of July 2006.

2.4. AAPH exposure

Samples for exposure to alkyl and peroxyl radicals generated by the water-soluble azo-initiator AAPH, were incubated in Eppendorfs in a water bath set at 50 °C for the desired amount of time. For the experiments in anoxic conditions, the eppendorfs containing the samples were first tightly sealed with overturned silicone rubber stoppers, placed on ice and purged thoroughly with nitrogen gas for 15 min using appropriate needles and then vortexed before incubation.

2.5. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

At the end of each exposure/incubation period, 15 μl of sample (corresponding to 30 μg of protein) were mixed with the same volume of sample buffer for qualitative assessment of collagen degradation using SDS-PAGE (in a 15% acrylamide running gel, 5% stacking gel) according to the method of Laemmli [36]. After the electrophoresis run at 200 V for 2 h, the gels were stained with Coomasie Brilliant Blue 0.5% for 45 min, and destained twice (10% acetic acid, 50% methanol) for 10 min. De-staining was subsequently completed in distilled water. Gels were then photographed using Kodak ID Image Analysis Software which also allowed for densitometric analysis of the relative collagen bands.

2.6. Quantification of collagen-bound carbonyl groups after UV-A and AAPH exposure

Protein carbonylation was quantified spectrophotometrically according to the method described by Davies and Hawkins which uses the reaction of 2,4-dinitrophenylhydrazine (DNPH) with the carbonyl groups of oxidized proteins [37]. Briefly, after UV-exposure or treatment with AAPH, 0.5 ml of 1 mM DNPH in 1 M HCl was added to 0.5 ml of each sample; blank samples lacked DNPH. Following 30 min incubation at 50 °C the samples were cooled and 1.5 ml NaOH 1 M were added, and the absorbance of the solution at 370 nm recorded 5 min later on a UV Kontron 941 spectrophotometer. An extinction coefficient of 22,000 M^{-1} cm^{-1} was used to quantitate carbonyl formation after subtraction of the appropriate blanks.

2.7. Circular dichroism (CD) analysis of UV-treated collagen

Collagen (0.25 mg/ml) in acetic acid 0.04 M, pH 4.3, was transferred to a 0.1-mm path quartz CD cuvette, placed horizontally on a brass block embedded on ice and exposed to UV-A as described above. At regular intervals, its far-UV CD spectra (from 260 nm to 200 nm) was recorded on a Jasco 500 spectropolarimeter under constant nitrogen flux.

2.8. EPR measurements

1 mM acetonitrile solution of nitroxide 2 was exposed to 40 min UV-A under the same conditions as described above for UV-A exposure. After exposure, the solution was diluted ten times, transferred to a closed glass capillary tube (1 mm i.d.) and then placed in the EPR cavity of a Varian E4 spectrometer containing a ruby as reference, for spectral measurement.

Appropriate controls were carried out throughout all the experiments described above. The data represent average values from at least three independent experiments each performed in duplicate. The SDS-PAGE gels are representative of at least three independent experiments each performed in duplicate.

3. Results

In order to mimic and simplify oxidative damage to an important constituent of the extra-cellular matrix, degradation of collagen was achieved by two independent means: exposure to (i) UV-A irradiation and to (ii) alkyl and peroxyl radicals thermally generated by the water-soluble radical generator, AAPH. Once the conditions for collagen degradation were established, its possible prevention using synthetic and natural antioxidants was investigated.

Fig. 2A shows the SDS-PAGE patterns of collagen at different UV-A exposure times, where maximum exposure corresponds to 730 kJ/m^2. The first lane at time point 0, shows the major bands starting from the high-end of the gel: γ, β, and α bands typical of native, β-mercaptoethanol-reduced collagen type III corresponding, respectively, to approximately 340 kDa, 170 kDa and 97.4 kDa [38]. Up to 20 min of UV-A exposure, no substantial differences can be observed with respect to native untreated collagen. However, at 30 min there is an appreciable reduction of the native γ band which totally disappears at 40 min where there is also a marked reduction in the β and α bands. In concomitance with these changes, one can also observe a continuum of dye along the length of the gel, an index of progressive

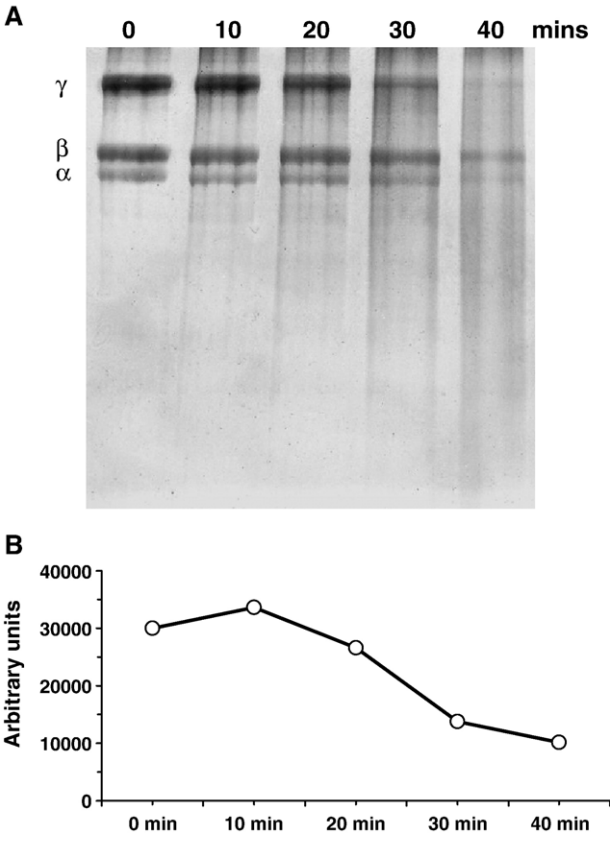

Fig. 2. Collagen damage as a result of UV-A illumination. Acid-soluble type III collagen (2 mg/ml) from calf skin was exposed to increasing doses of UV-A irradiation (up to 40 min corresponding to 730 kJ/m^2 UV-A) and protein integrity was examined by SDS-PAGE analysis with Coomassie Brilliant Blue stain (A). The migration positions of collagen α(III) monomer, β(III) dimer and γ(III) trimer are indicated. (B) Densitometry of gel's γ bands showing a decrease in intact α-chains.

fragmentation of collagen's three α-chains. The resulting population of chain lengths and peptides of lower molecular mass are distributed along the length of the gel leading to the characteristic protein smear which becomes more intense after 40 min UV-A exposure. These observations can be accounted for by considering that UV-A light generates free radicals in the water molecules surrounding the collagen molecule and these radicals react with the collagen destabilizing it [39,40]. However, recent reports also point to the fact that collagen itself is a photosensitizer molecule which generates superoxide radical and hydrogen peroxide when exposed to UV-A [41,42]. Hence in our system, at least one of these reactions mediated by photosensitized collagen-derived radicals and/or those generated from the water molecules, can cause chain scission which can occur randomly at many sites along the length of the molecule. The number of intact α-chains in the population of collagen molecules therefore declines and the cut α-chain matter smears out along the whole length of the gel. As irradiation proceeds, the number of undamaged molecules falls while the number of damaged ones rises. Therefore one can deduce that collagen degradation is UV-A dose-dependent with maximum degradation in our system observable at 730 kJ/m^2. Densitometric analysis of the gel's γ bands (Fig. 2B) shows a progressive decrease in intact α-chains with a marked drop occurring between 10 and 30 min of UV-A exposure.

At this point it was of interest to find if a correspondence existed between the collagen damage observed with artificially generated UV-A irradiation and that obtained upon exposure to natural sunlight. Fig. 3 shows that collagen exposed to 60 min of natural sunlight is significantly damaged as deduced by the smear in the corresponding lane and that this is more pronounced after 120 min of sunlight exposure. Hence 2 h of exposure to natural sunlight during the month of July at sea level leads to approximately the same amount of collagen degradation as that produced by exposure to 730 kJ/m^2 UV-A.

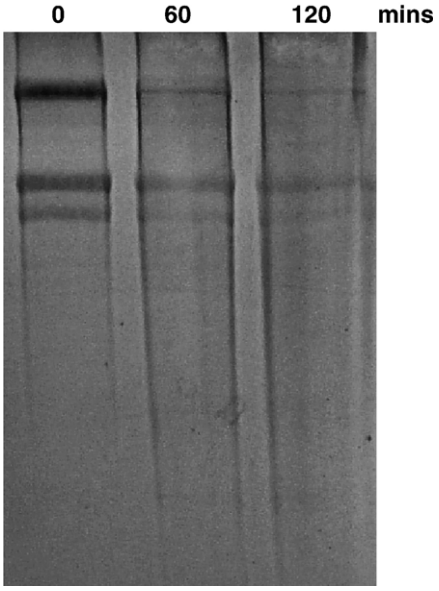

Fig. 3. Collagen damage as a result of exposure to natural sunlight. Acid-soluble type III collagen (2 mg/ml) from calf skin was exposed to 60 and 120 min of natural sunlight (July 2006 between 12.00 and 14.00 h) and protein integrity was examined by SDS-PAGE analysis with Coomassie Brilliant Blue stain.

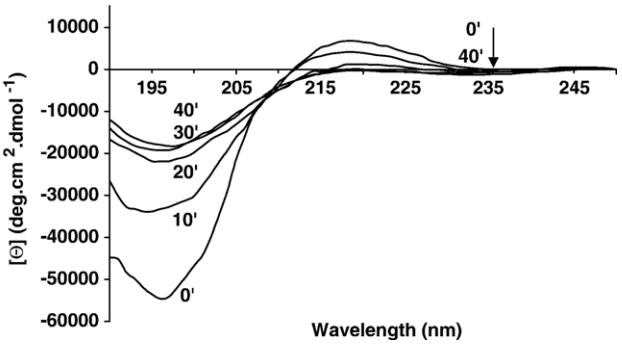

Fig. 4. Collagen damage as a result of UV-A illumination. Acid-soluble type III collagen (0.25 mg/ml) from calf skin was exposed to increasing doses of UV-A irradiation (up to 40 min corresponding to 730 kJ/m^2 UV-A) and protein integrity was examined by CD dichroism spectroscopy. See Materials and methods for details.

Fig. 4 at time point 0′ shows the CD spectrum of native, untreated collagen which is typical of the intact molecule, i.e. with a positive peak centred at about 218 nm and a more pronounced negative peak at 197 nm. Upon UV-A exposure, the positive peak gradually disappears while the negative peak becomes less profound suggestive of a UV-A induced conformational change in the triple helix of collagen [43,44]. This may likely occur through breakage of the hydrogen bonds which contribute to stabilization of the helical structure of collagen. These results correlate with the progressive increase in collagen fragmentation observed with SDS-PAGE.

Since 40 min exposure to UV-A of native collagen induced significant structural alterations, this exposure time was subsequently used for the study of the protective effects of the antioxidants shown in Fig. 1. As can be observed in Fig. 5A, at equimolar concentration (100 μM) all the compounds studied with the exception of nitroxide 1 were unable to prevent collagen fragmentation. In fact, the profile in the presence of nitroxide 1 is very similar to that of unexposed collagen while in the other cases the profiles are comparable to that obtained after 40 min UV-A exposure. This is also clear from the densitometric analysis of this gel's γ bands reported in Fig. 5B.

In order to confirm that the exposure to UV-A is inducing collagen damage and that this appears to be prevented by the presence of nitroxide 1, the generation of carbonyl groups which are a known product of oxidant damage was investigated [45]. The results reported in Fig. 6 are generally consistent with the SDS-PAGE. Thus, an increase in the level of protein carbonyl residues is observed in collagen after 40 min UV-A exposure, an increment which is almost totally abolished when nitroxide 1 is present. However, vitamins C and E also appear to inhibit the formation of carbonyl group residues by approximately 60% even though they were unable to inhibit protein fragmentation (see Fig. 5A). Since protein carbonyls may be formed either by oxidative cleavage of proteins, or by direct oxidation of certain amino acid residues, such as lysine, arginine, proline and threonine residues [46,47], it may be envisaged that these two vitamins protect only against the UV-A induced oxidation of

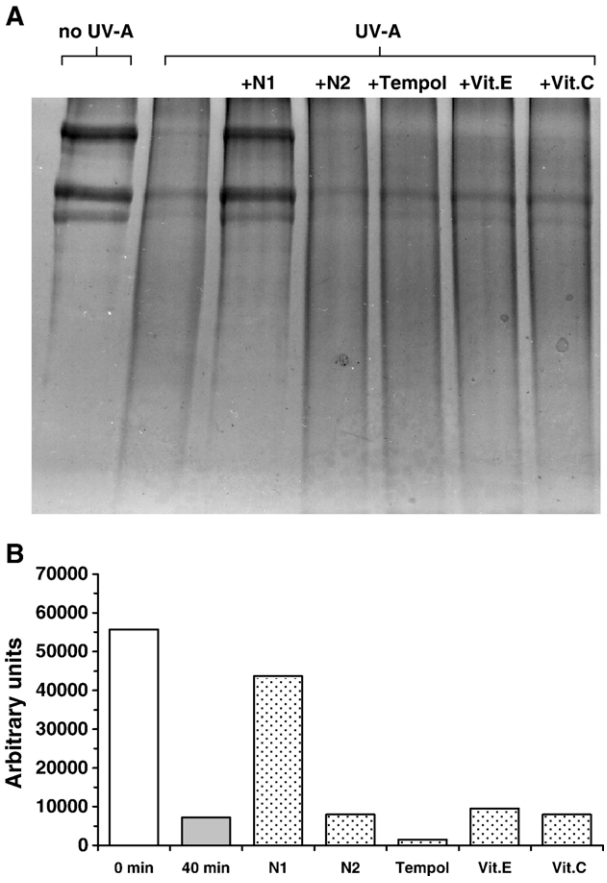

Fig. 5. Effects of antioxidant compounds on UV-A-induced collagen damage. Acid-soluble type III collagen (2 mg/ml) from calf skin was exposed to 40 min UV-A (730 kJ/m^2) in the absence or presence of antioxidants (100 μM) and protein integrity was examined by SDS-PAGE analysis with Coomassie Brilliant Blue stain (A). (B) Densitometry of gel's γ bands showing a remarkable decrease in intact α-chains after UV-A illumination, except in the case of nitroxide 1 (N1).

certain amino acid residues and not against UV-A oxidative cleavage of the collagen backbone which leads to fragmentation of the protein [47]. Nitroxide 1 on the other hand, appears to be capable of inhibiting both oxidative processes associated with protein carbonyl formation. Interestingly, the presence of the other two nitroxides (nitroxide 2 and Tempol) lead to an even greater increment in carbonyl residues compared to the irradiated control, although this is statistically significant only for nitroxide 2. This finding could likely be explained by invoking photosensitization reactions. In fact, the carbonyl group in position 3 of nitroxide 2 may be responsible for photoexcited states upon UV-A irradiation, since compounds bearing carbonyl groups are usually photosensitive as has been previously observed [48–50]. In general, compounds in photoexcited states are known to act as stronger electron transfer oxidants (or reductants) than the parent compound thus inducing oxidative damage either through hydrogen abstraction, 2+2 cycloaddition, cis–trans isomerization or photofragmentation. This could plausibly explain the large increment in carbonyl residues in collagen when nitroxide 2 was present, a hypothesis confirmed further on in this study when collagen was exposed to a non-photoinduced source of oxidative damage (see later). To test whether nitroxide 2 undergoes oxidation or degradation upon exposure to UV-A, an experiment was carried out in which a 1-mM acetonitrile solution of this nitroxide was exposed to 40 min UV-A under the same conditions of exposure as described in UV-A exposure. Thin Layer Chromatography (TLC) analysis of this solution after exposure (eluting with cyclohexane/ethyl acetate 4:1) showed no additional coloured or fluorescent spots compared to the unexposed solution. Furthermore, the intensity of the EPR signal of the exposed solution compared to the unexposed one was also the same. This therefore excludes the possibility of photodegradation or photooxidation of nitroxide 2 in the experimental conditions of this study, and strongly suggests that nitroxide 2 acts by photosensitizing molecules in its surroundings and that it returns to its ground state after energy transfer.

Since nitroxide 1 appeared to be the most efficient protectant against UV-A induced collagen damage, experiments were carried out to determine whether this protective effect was dose-dependent. Fig. 7 shows that at 50 μM, this compound is

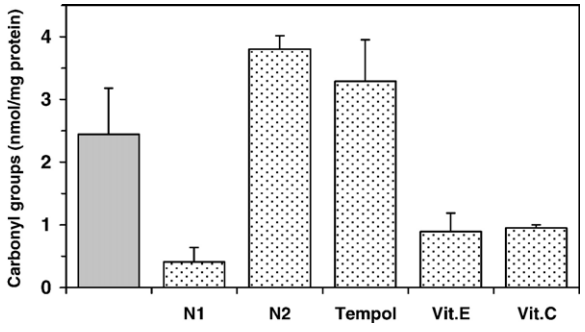

Fig. 6. Quantification of collagen-bound carbonyl groups resulting from exposure to UV-A. Acid-soluble type III collagen (2 mg/ml) from calf skin was exposed to 40 min UV-A (730 kJ/m^2) in the absence (dark bar) or presence (spotted bars) of antioxidants (100 μM) and the increment in carbonyl groups was determined spectrophotometrically (see Materials and methods for details).

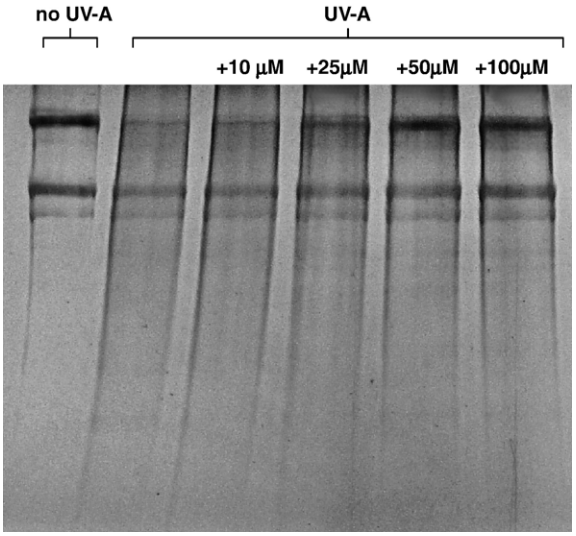

Fig. 7. Effects of different concentrations of nitroxide 1 on UV-A-induced collagen damage. Acid-soluble type III collagen (2 mg/ml) from calf skin was exposed to 40 min UV-A (730 kJ/m^2) in the absence or presence of increasing concentrations of nitroxide 1 and protein integrity was examined by SDS-PAGE analysis with Coomassie Brilliant Blue stain.

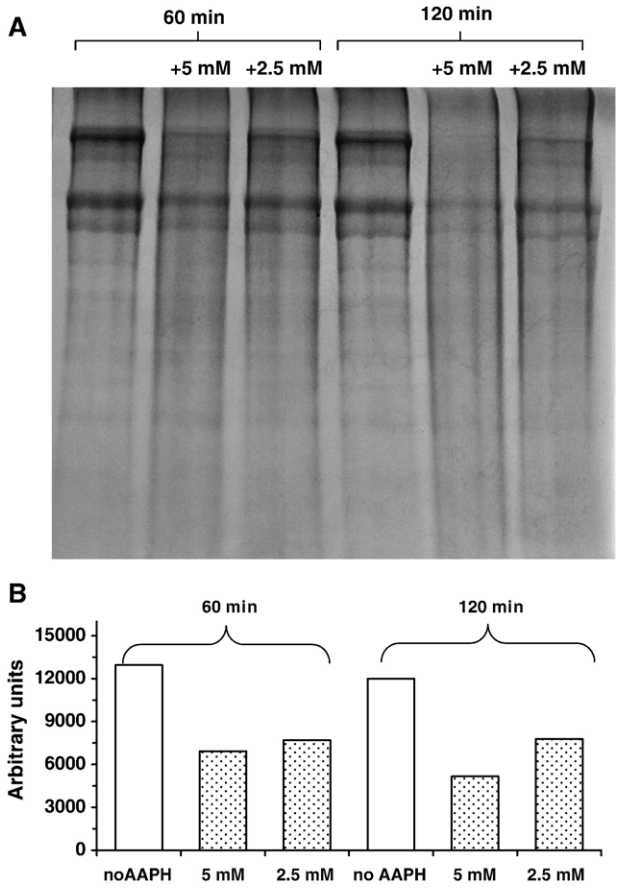

Fig. 8. Collagen damage as a result of AAPH exposure. Acid-soluble type III collagen (2 mg/ml) from calf skin was exposed to increasing incubation times with different concentrations of AAPH and protein integrity was examined by SDS-PAGE analysis with Coomassie Brilliant Blue stain (A). (B) Densitometry of gel's γ bands showing a decrease in intact α-chains following AAPH exposure.

already effective at inhibiting collagen fragmentation, which is almost totally inhibited at 100 μM.

As mentioned in Introduction, collagen was also exposed to a non-photoinduced source of oxidative stress. AAPH was used for this purpose whereby the first radical species generated are carbon-centred (alkyl) radicals (Eq. (1)) which subsequently react with molecular oxygen at an almost diffusion controlled rate to give a constant flux of peroxyl radicals (Eq. (2)) [51], an important propagating species of the free radical reactions occurring in proteins (Eq. (3)) [46].

$$\underset{\text{AAPH}}{\bar{Cl}[(H_2\overset{+}{N})_2CC(CH_3)_2N=]_2} \longrightarrow \underset{2AP^{\bullet}}{2\bar{Cl}(H_2\overset{+}{N})_2C\dot{C}(CH_3)_2} + N_2 \quad (1)$$

$$AP^{\bullet} + O_2 \longrightarrow APOO^{\bullet} \quad (2)$$

$$APOO^{\bullet} + \text{protein} \longrightarrow \text{protein carbon-centred radical}^{\bullet} + APOOH \quad (3)$$

When both collagen and radical generator (AAPH) were incubated in solution, variable degrees of collagen fragmentation reflecting radical damage to the protein were detected, depending on the concentration of AAPH and length of incubation. As shown in Fig. 8, incubation with 2.5 mM AAPH for both 60 min and 120 min lead to significant protein fragmentation as detected by SDS-PAGE, which was even more evident using 5 mM AAPH and for twice the amount of time. Hence, this latter condition was used to study the effects of the different antioxidants at equimolar concentrations (200 μM) on AAPH-induced collagen damage. Fig. 9A shows that the only antioxidants which were effective at reducing protein fragmentation were the nitroxide compounds, with Tempol being the least effective out of the three. The natural vitamins appeared to be ineffective against this damage. The corresponding densitometric analysis (Fig. 9B) shows that inhibition of protein fragmentation decreases in the order: nitroxide 1 > nitroxide 2 > Tempol > vitamin C > vitamin E.

AAPH-induced radical damage to collagen was also quantified through the increment in protein carbonyl residues present in the protein. Fig. 10 shows that the increase in carbonyl residues mediated by AAPH-derived radicals is almost totally inhibited by the presence of the nitroxide compounds contrary to what happens in the presence of the vitamins, thus confirming the data observed using SDS-PAGE (Fig. 9A). It is

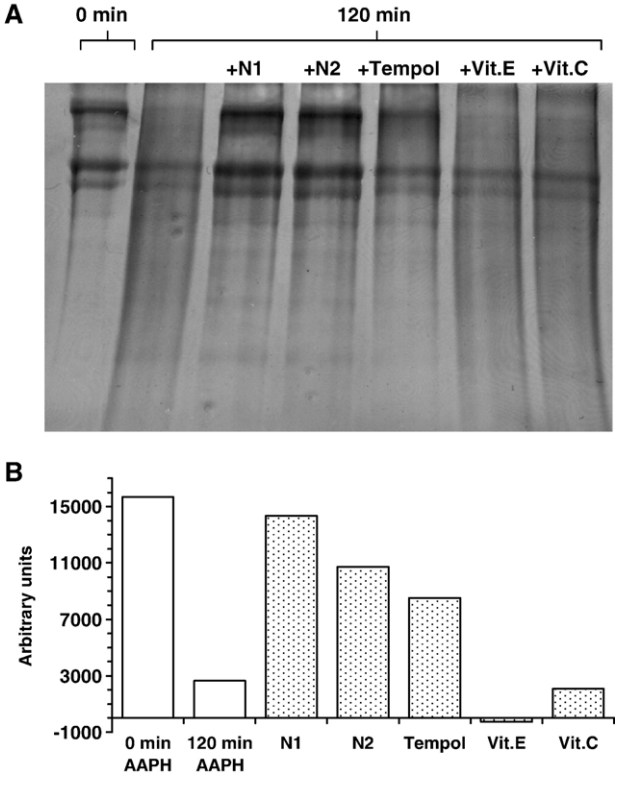

Fig. 9. Effects of antioxidant compounds on AAPH-induced collagen damage. Acid-soluble type III collagen (2 mg/ml) from calf skin was incubated for 120 min with 5 mM AAPH in the absence or presence of antioxidants (200 μM) and protein integrity was examined by SDS-PAGE analysis with Coomassie Brilliant Blue stain (A). (B) Densitometry of gel's γ bands showing decrease in intact α-chains after AAPH exposure which is inhibited to different extents by nitroxides 1 and 2 and Tempol.

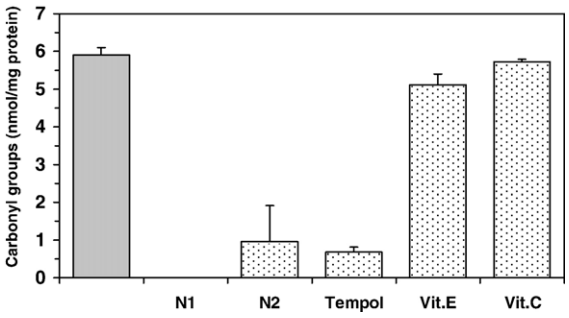

Fig. 10. Quantification of collagen-bound carbonyl groups resulting from exposure to AAPH. Acid-soluble type III collagen (2 mg/ml) from calf skin was incubated for 120 min with 5 mM AAPH in the absence (dark bar) or presence (spotted bars) of antioxidants (200 μM) and the increment in carbonyl groups was determined spectrophotometrically (see Materials and methods for details).

also noteworthy that exposure of collagen to AAPH-derived radicals generates approximately twice more protein carbonyl residues (Fig. 10) than exposure to 730 kJ/m^2 UV-A (Fig. 6) as deduced by the values reported in the respective graphs. Although twice the amount of vitamins was used in this system compared to the concentration used for UV-A exposure, they were unable to protect against carbonyl groups' formation. In fact, when collagen was exposed to UV-A, these two vitamins were capable of inhibiting their formation to a reasonable extent (Fig. 6) although they were ineffective at inhibiting protein fragmentation (Fig. 5A). This suggests that carbonyl group formation in AAPH-exposed collagen arises mainly from the oxidative cleavage of the protein backbone rather than from the direct oxidation of amino acid residues [46,47,52] which in turn depends on the different radical species generated upon UV-A exposure and from AAPH. Instead, the encouraging results obtained in the presence of nitroxide 2 and Tempol using the non-photoinduced system of oxidative stress mediated by AAPH, confirm that their inefficacy at protecting collagen exposed to UV-A (Figs. 5 and 6) was most likely due to photosensitization reactions as mentioned earlier. Fig. 11 shows that the protection conferred by the most effective nitroxide, i.e. nitroxide 1, against AAPH-induced collagen damage is again dose-dependent, where highest protection was achieved using 200 μM.

The results concerning the AAPH system described above assumed that peroxyl radicals were being generated. In fact, the AAPH system produces peroxyl radicals in aerated solutions but only at relatively low concentrations of added substrate. Considering that collagen is a polymer, and assuming a MW of about 100 for an amino acid, the concentration per monomer at the amount used in this study (2 mg/ml) is about 570 μM (lower concentrations, ≤1 mg, could not be used as bands were too faint after SDS-PAGE and carbonyl levels hardly detectable) while the concentration of oxygen at 50 °C is c.a. 175 μM O_2. Hence it may be assumed that peroxyl radicals under the experimental conditions of this study would hardly be formed. Therefore to check whether APOO$^\bullet$ radicals were actually being generated (Eq. (2)) and that they were the damaging species, i.e. that oxygen competes efficiently with collagen for AP$^\bullet$ radicals, control experiments were carried out also in deareated solution (therefore only in the presence of AP$^\bullet$ radicals) and in the presence or absence of 200 μM nitroxide 1, Tempol and Tempo (the non-hydroxy derivative of Tempol). Interestingly, as can be observed from the SDS-PAGE gel in Fig. 12, collagen fragmentation was considerable even in the presence of only AP$^\bullet$ radicals, i.e. alkyl radicals, and that all three nitroxides inhibited this fragmentation similarly. Measurement of carbonyl groups (not shown) was also in agreement with the above findings and in all three cases the nitroxides totally inhibited their formation. Hence, it is likely that the damage observed using AAPH under the aerated experimental con-

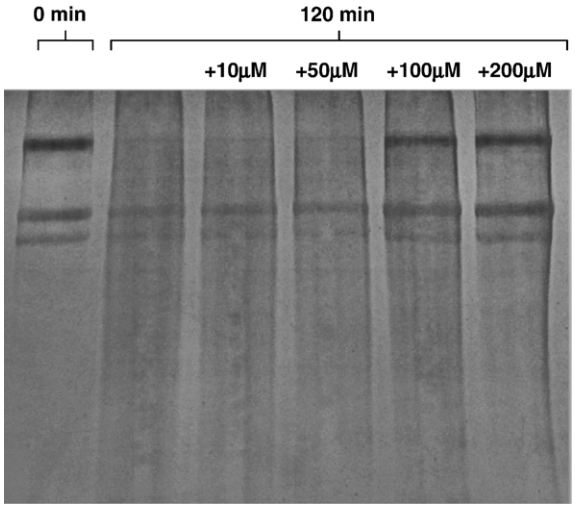

Fig. 11. Effects of different concentrations of nitroxide 1 on AAPH-induced collagen damage. Acid-soluble type III collagen (2 mg/ml) from calf skin was incubated for 120 min at 50 °C with 5 mM AAPH in the absence or presence of increasing concentrations of nitroxide 1 and protein integrity was examined by SDS-PAGE analysis with Coomassie Brilliant Blue stain.

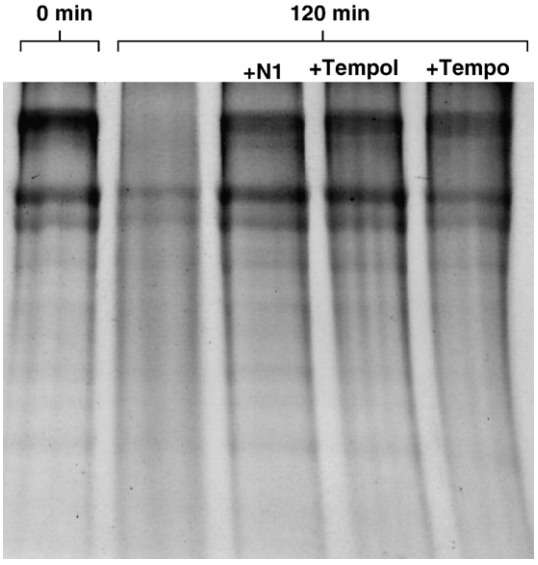

Fig. 12. Effects of nitroxide compounds on AAPH-induced collagen damage under anoxic conditions. Acid-soluble type III collagen (2 mg/ml) from calf skin was incubated under anoxic conditions for 120 min at 50 °C with 5 mM AAPH in the absence or presence of nitroxides (200 μM) and protein integrity was examined by SDS-PAGE analysis with Coomassie Brilliant Blue stain.

ditions of this study is most likely due to alkyl radicals. However it is unclear why carbonyl groups were detected even in anoxic conditions in the absence of nitroxides. These might arise from attack of the alkyl radicals on the collagen molecule which could in some way generate ketonic or aldehydic carbonyl groups during the degradation process of the target molecule.

4. Discussion

Many researchers have reported that collagen, the major protein skin constituent, is damaged by active oxygen species (hydroxyl, superoxide anion, peroxynitrite, singlet oxygen) [53–58] either photo- or non photo-induced, resulting in both fragmentation and a greater susceptibility to enzymatic degradation and that this contributes to cutaneous photodamage and ageing. Hence it seems reasonable to expect that antioxidants should be capable, within certain limits, of protecting against this oxidative injury. Indeed, the cutaneous levels of antioxidant enzymes SOD and CAT and vitamins C, E, and glutathione present in skin are altered, for example, by chronic UV exposure [59,60], which makes the addition of exogenous antioxidants in topical formulations attractive as a strategy for sun-protection and ageing in general [14,16]. Interestingly, however, there are very few reports in the literature on the effects of antioxidants on collagen. To date, only the protective effects of flavonoids [54,56], caffeoyl derivatives extracted from *Echinacea* [61], and β-carotene on oxidative degradation of collagen have been studied [62].

The results reported above reveal that nitroxides could also be explored for their potential application in preventing acute sun damage and photo/chronological ageing since the data demonstrate their efficacy at inhibiting oxidative damage of collagen. Nitroxide radicals are an important class of antioxidants which are increasingly gaining interest for topical applications against radiation-induced alopecia [63], for dermatological research [64] and for the development of the EPR skin imaging technique [65]. They are among the most stable free radicals known that are largely exploited as biophysical probes, spin labels and contrast agents for many biophysical/medical studies [66]. However, because of their versatile ability to neutralize free radicals chiefly via radical–radical coupling, or by donating/accepting electrons [27,30], they have also been used as antioxidants in biological systems [28,29,67] as well as in other fields, such as rubbers [68], polymers [69], paints [70] and the paper industry [71]. Their well-known capacity to react via radical–radical coupling at the nitroxide function with carbon-centred radicals, deriving either from decomposition of AAPH (Eq. (1)) or from the protein (Eq. (3)) as an effect of AAPH or UV-A exposure, at an almost diffusion controlled rate, to give non-radical species (alkylated hydroxylamines), explains why they inhibited collagen oxidation (Fig. 13). In addition, the aromatic indolinonic nitroxides 1 and 2, and more recently aliphatic nitroxides, have been shown to be capable of reacting also with oxygen-centred radicals, via radical–radical coupling to give quinoneimine N-oxides for the former group [72], and via electron-transfer to give oxoammonium cations for the latter [73,74] (Fig. 13) which are additional features accounting for their antioxidant activity in the system

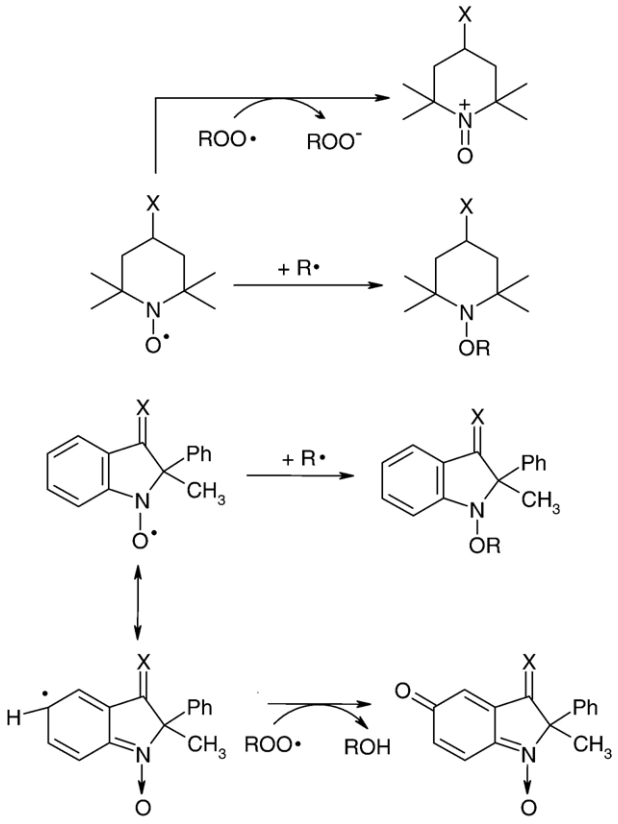

Fig. 13. Scheme showing the scavenging of alkyl radicals ($R^{\bullet}$) and peroxyl radicals ($ROO^{\bullet}$) by aromatic indolinonic and aliphatic piperidine nitroxides.

here investigated. In this context, it is worth mentioning that if part of the damage to collagen in the aerated AAPH system is due to peroxyl radicals then the differences in protection observed between N1 and Tempol (Fig. 9) could be due to the formation of the oxoammonium cation in the case of Tempol, which is known to be a highly oxidizing species [75].

Moreover, the data obtained in this study suggest that precautions should be taken when considering which nitroxides to use for possible topical applications where extensive UV-exposure is foreseen. In fact, nitroxide 1 protected collagen against UV-A oxidation contrarily to what was observed with the structurally similar aromatic nitroxide 2 and the aliphatic nitroxide Tempol, whereas these two latter nitroxides were efficient antioxidants in the absence of UV-exposure. The nitroxide Tempol has previously been shown to protect against UV radiation in a transgenic murine fibroblast culture model of cutaneous photoaging [76] and in human dermal fibroblasts [77]. The fact that no protection against protein oxidation was observed when Tempol was co-illuminated with collagen could most probably be due to the different experimental systems investigated: cell cultures vs. pure, native protein as well as to the different UV sources and intensities employed in these studies.

Numerous studies have shown that topical and systemic supplementation of vitamins C and E, the major antioxidant vitamins present in skin, are valuable agents in the prophylaxis and treatment of photoaging, skin cancer and numerous skin disorders [15,16]. These vitamins are related in part through

their ability to act as antioxidants, principally via electron/hydrogen donation thereby quenching free radicals, such as peroxyl radicals in the case of vitamin E [33], and superoxide radical in the case of vitamin C [78]. However, their benefits to skin health are not only related to their antioxidant activity but also to other mechanisms of action which modulate skin disorders. For example, vitamin C is a cofactor in the hydroxylation reactions of collagen production [79], while vitamin E has anti-inflammatory properties [80]. The results obtained in this study indicate that with regards to collagen, both vitamins were capable of inhibiting carbonyl group formation in UV-A exposed collagen but not when it was exposed to AAPH-derived radicals, and that both were unable to prevent collagen fragmentation in both systems employed of oxidative stress. As already mentioned in the results, the different protective behaviour of these two vitamins may be explained considering the different radical species that could be generated upon UV-A exposure and from AAPH. In fact, a wide range of different radicals can be formed on reaction of a protein with an attacking radical and this is due to the varied nature of the amino acid side chains which offer a multitude of possible sites of attack, in addition to attack on the backbone [46,47,52]. The nature of the radicals formed on proteins therefore depends on the nature and reactivity of the attacking radical. For example, electrophilic radicals (e.g. hydroxyl, alkoxyl radicals) preferentially oxidise electron-rich sites, whereas nucleophilic species (such as carbon-centred radicals) attack electron-deficient sites [46]. Since UV-A generates several ROS which include singlet oxygen, superoxide radical and hydrogen peroxide [40], while AAPH mainly generates alkyl and peroxyl radicals [51], protein oxidative modifications are expected to proceed differently and hence antioxidant responses will also be diverse.

Another possibility which should be considered when trying to find plausible explanations for antioxidant behaviour is the compatibility in terms of hydrophobicity/hydrophilicity of the antioxidant in question with the target molecule. In fact, it has previously been reported that vitamin E was ineffective at protecting bovine serum albumin (BSA) against oxidation from AAPH-derived radicals [52] and this was explained considering that vitamin E is a hydrophobic antioxidant while BSA is a water-soluble protein, hence the extent of radical attack by the hydrophilic radicals from AAPH on BSA can be affected by the localization of the antioxidant. If the antioxidant is not within the vicinities of the attacking radical or the target molecule, its efficacy as antioxidant will be greatly reduced. This is the most plausible explanation for the apparent inefficacy of vitamin E here observed, to inhibit AAPH-induced damage to collagen which is also a water-soluble protein at acidic pH. However in our system, vitamin C which is a hydrophilic antioxidant, also had no effect. Thus on an equimolar basis, these vitamins were not as effective antioxidants in our system as the nitroxides due either to their lower capacity to scavenge collagen radical species generated by exposure to a photo- and non photo-induced source of oxidative stress, or because of incompatibility reasons in terms of localization of the antioxidant with the target protein and radicals generated. Since the nitroxides appear to be efficient in inhibiting collagen damage, it is possible that besides their versatile radical scavenging abilities, they might also localize better within the collagen molecule compared to the vitamins here tested.

This study also demonstrates for the first time the direct degradation of collagen by alkyl radicals and possibly by peroxyl radicals, the latter being important oxidant species. Alkyl radicals are the precursors of peroxyl radicals and since both are well known to be generated during lipid peroxidation processes from polyunsaturated fatty acids which constitute not only the outermost layer of cells in the form of phospholipids, but also the epidermal lipids which make up the skin barrier system of the stratum corneum, it was of interest to study their effect on collagen. In fact, peroxyl radicals generated from unsaturated lipids during photo- or non photo-oxidative stress are well known to react with surrounding molecules leading to membrane damage and loss in barrier function and with any amino acid residues of proteins in the vicinity as well, such as lysine and arginine, leading to protein oxidative modifications including fragmentation [81]. Hence a scenario could be envisaged in skin whereby peroxyl radicals generated during lipid peroxidation of skin barrier lipids or of cell membranes could attack nearby collagen molecules and upon degradation, collagen responsible for intrinsic tension of skin, loses its water binding capacity leading to skin wrinkling and ageing.

In conclusion, the results of this study support the view that appropriately tailored nitroxides could be used as alternative agents for reducing oxidative skin damage promoted by exogenous or endogenous factors, where effective local concentrations of these antioxidants could be achieved by topical application. Additional favourable features are their very low potential to cause acute or subacute skin toxicity [82], their low immunogenicity and their inefficacy as mutagens [83].

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