

Epidermal *trans*-urocanic acid and the UV-A-induced photoaging of the skin

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ABSTRACT The premature photoaging of the skin is mediated by the sensitization of reactive oxygen species after absorption of ultraviolet radiation by endogenous chromophores. Yet identification of UV-A-absorbing chromophores in the skin that quantitatively account for the action spectra of the physiological responses of photoaging has remained elusive. This paper reports that the *in vitro* action spectrum for singlet oxygen generation after excitation of *trans*-urocanic acid mimics the *in vivo* UV-A action spectrum for the photosagging of mouse skin. The data presented provide evidence suggesting that the UV-A excitation of *trans*-urocanic acid initiates chemical processes that result in the photoaging of skin.

Most of the visible signs of aging result from chronic exposure of the skin to ultraviolet radiation (1–2). Unlike chronologically aged skin that results from a general atrophy and a gradual decline in the production of the dermal matrix (3), UV-A (320–400 nm) photoaged skin is characterized by a gross increase in the elastic fibers (elastin, fibrillin, and desmosine) of the skin replacing the collagenated dermal matrix (elastosis) (4–5), an increase in glycosaminoglycans (4–5), collagen cross-linking, epidermal thickening (4–6), and an increase in the number of dermal cysts (7). The deep lines, leathery appearance, and the sagging of the skin surface typically associated with “old age” are thought to result from UV-induced photodamage to the skin and to occur over the course of a lifetime (3). Although obvious differences between photoaged and chronologically aged skin exist, the anatomic basis of the visible signs of photoaging is not understood fully (4, 8). Absorption of UV-A must induce photobiologic effects within the skin that lead to the visible and histological differences of photoaged skin, and, although the mechanisms by which UV-A-induced photodamage occur have not been completely determined, reactive oxygen species are postulated to play a role (9). The natural shift toward a more prooxidant state in chronologically aged skin then could be exacerbated by absorption of UV-A radiation by endogenous chromophores like NADH/NADPH (10–14), tryptophan (15), and riboflavin (9), which then sensitize the formation of reactive oxygen species. Solar radiation has been shown both to reduce the antioxidant population in the skin (16) and to sensitize the production of reactive oxygen species such as singlet oxygen, hydrogen peroxide, and the superoxide anion (9–19), increasing the potential for reactions like the oxidation of lipids and proteins (17) that influence the degree of cross-linking between collagen and other proteins (9) within the skin.

The first step toward identifying the chromophore(s) responsible for physiological changes, such as those seen in photoaged skin, is to match the action spectrum for the physiological change with the absorption spectrum of the chromophore (20). Once

such a comparison is made, the pathways that lead to the physiological change can be unraveled. For example, the epidermal chromophore *trans*-urocanic acid (*t*-UA) has received considerable attention in the last 15 years, following the discovery that its broad and structureless absorption spectrum from 260 nm to 320 nm mimicked that of the immune suppression action spectrum of contact hypersensitivity (21). Now, a direct relationship between UV absorption by *t*-UA and UV-induced immunomodulation has been postulated (21), and the mechanism by which *t*-UA mediates these physiological events is a topic of current research (22–29). A similar quantitative comparison between photoaging action spectra and the absorption spectra of endogenous chromophores in the UV-A has remained elusive.

In this report, results from pulsed laser photoacoustic spectroscopy (30, 31) are presented. This technique allows one to study the energetics and dynamics of nonradiative processes. These processes can be photophysical (e.g., internal conversion, intersystem crossing) or photochemical (e.g., isomerization, electron transfer) in nature. The underlying principle of the technique is that a nonradiative decay generates a thermal acoustic wave in the sample, which is detected by a piezoelectric transducer. The experiment measures dynamic processes that occur within a certain time window. This time window is determined by the sample cuvette design and the frequency response of the detection transducer. The sample cuvette is calibrated so that the observed signal can be quantified in terms of the fraction of the photon energy that is released by the sample as heat on the timescale of the transducer response. In addition, kinetic information can be extracted from the differences in the temporal properties of the waveforms for the sample and reference compound. In the present study, excitation in the UV-A is achieved by using either a picosecond or femtosecond laser system.

In this report, we focus on the UV-A absorption of *t*-UA between 310 nm and 351 nm and the potential role of *t*-UA in the photoaging of the skin. Examination of Fig. 1 shows that this region corresponds to the tail of the absorption spectrum. Irradiation throughout this spectral region has been shown recently to induce photoisomerization of *t*-UA to its *cis* isomer (*c*-UA) (32–34). Excitation of a pH 7.2 solution of *t*-UA at 310 nm results in photoisomerization in which the quantum yield for the conversion of *t*-UA to *c*-UA at this wavelength is ≈ 0.49 (35, 36). Previous pulsed laser photoacoustic spectroscopic results revealed that essentially all of the incident photon energy is released as heat on the subnanosecond time-scale at this excitation wavelength (37). This result is consistent with time-resolved absorption studies that indicate that the photoisomerization reaction takes place within the singlet manifold of electronic states on a picosecond timescale (38). Intersystem crossing from the initially populated excited singlet state to the triplet-state manifold does not compete with isomerization to *c*-UA after irradiation at 310 nm (37, 38). In the present report, pulsed laser photoacoustic spectroscopy (37) is used to study the UV-A portion of the *t*-UA

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Abbreviation: *t*-UA, *trans*-urocanic acid.

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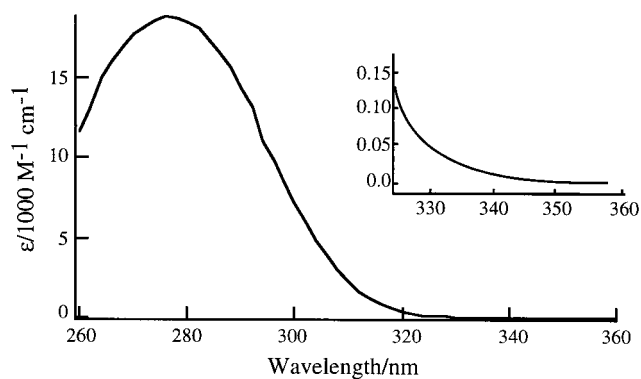


FIG. 1. The absorption spectrum of naturally occurring *trans*-urocanic acid, pH 7.2 (solid line). The broad and structureless spectrum masks the complicated wavelength-dependent photochemistry exhibited by the chromophore. Wavelength-dependent isomerization between *t*-UA and photoinduced *cis*-urocanic acid results from the presence of weakly coupled distinct electronic states between 266 and 400 nm (35–37). In addition, as shown in this report, there is a weak absorption band of *t*-UA from 320 to 360 nm. This region of the spectrum is enlarged in the inset.

absorption spectrum from 310 nm to 351 nm. We find that the photoacoustic signal in this region of the spectrum is wavelength-dependent. Starting at 320 nm, the amount of energy retained by the molecule increases with increasing excitation wavelength. The energy retained reaches a maximum at ≈ 340 nm and then decreases with increasing excitation wavelength. Because of the low extinction coefficient for *t*-UA for wavelengths >370 nm, the photoacoustic technique could not be used to determine whether energy is retained by *t*-UA after excitation in this region. Recent studies establish that photoisomerization occurs after excitation of *t*-UA in the UV-A (320–400 nm) (32–34). The data obtained in the present study reveal that the energy retained after excitation at 340 nm is almost three times the energy difference between the two isomers of UA [$110 \text{ kJ}\cdot\text{mol}^{-1}$ vs. $40 \text{ kJ}\cdot\text{mol}^{-1}$ (37)]. Consequently, we can conclude that photoisomerization is not the sole photochemical process that is initiated by UV-A exposure. To account for this observed energy storage, a long-lived intermediate must be formed. The absence of any time delays between the sample and reference photoacoustic waves indicate that this intermediate is formed on the subnanosecond timescale and that its lifetime is longer than the instrument response time, hundreds of nanoseconds (37). Therefore, its decay kinetics do not contribute to the photoacoustic signal. The absence of any photochemistry except isomerization contributing to the photoacoustic signal requires that this intermediate be a long-lived excited state of the molecule, and so it is reasonable to conclude that this state is an excited triplet state of *t*-UA, *vide infra*. Because excitation at 310 nm accesses an electronic state that only leads to isomerization (37–39), it is also reasonable to conclude that excitation between 320 nm and 351 nm populates two distinct but overlapping excited electronic states: the same state that leads to isomerization at 310 nm and an electronic state that either directly or by intersystem crossing populates the long-lived excited triplet state. As a result, the wavelength-dependent photoacoustic measurements determine the action spectrum for triplet state formation for excitation of *t*-UA in the UV-A. *t*-UA does not exhibit any phosphorescence in room temperature solutions. As a result, conventional optical spectroscopic techniques, e.g., collection of a phosphorescence excitation spectrum, cannot be used to determine the line shape of action spectrum for triplet formation. Fig. 2A shows this action spectrum as determined from these photoacoustic measurements. This action spectrum is in excellent agreement with the action spectrum for photoinduced sagging of mouse skin (Fig. 2B; ref. 40).

Given the agreement between the two action spectra shown in Fig. 2, we undertook *in vitro* experiments to elucidate the

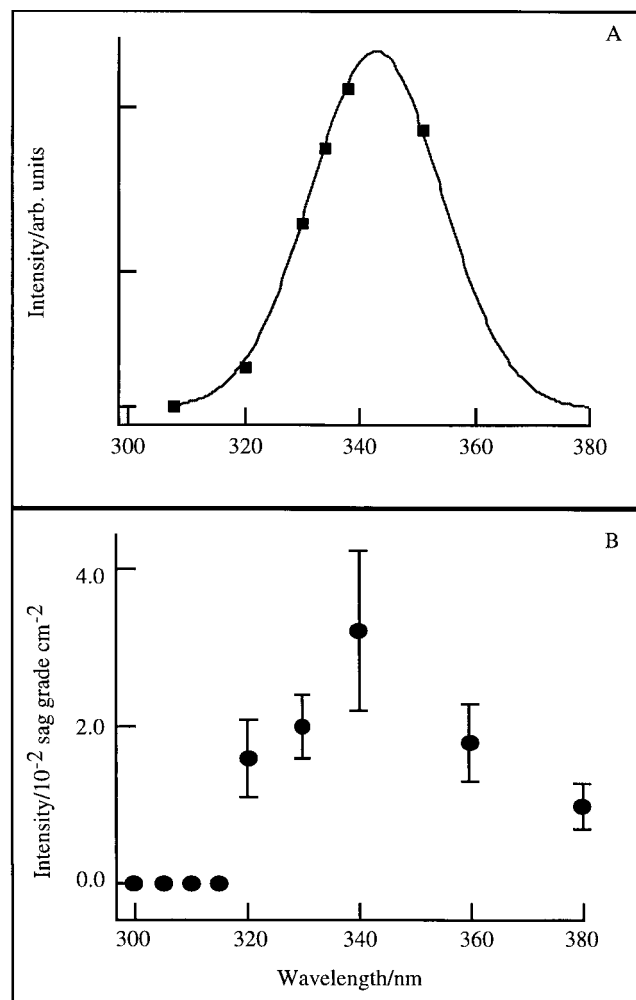


FIG. 2. (A) The line shape of action spectrum for triplet formation (and singlet oxygen generation) for *trans*-urocanic acid in a deoxygenated pH 7.2 solution (solid line). The line-shape was determined by fitting a Gaussian line-shape function to the photoacoustic data collected from 320 to 360 nm (data points). Each irradiation wavelength was generated by using a home-built temperature-tuned optical parametric amplifier pumped by a home-built Nd:YLF (1 kHz, $1.054 \mu\text{m}$) regenerative amplifier. The 10^{-3} M *t*-UA samples had an optical density of between 0.05 and 0.1, and, at each irradiation wavelength, the *t*-UA optical density was matched to within 3% of the optical density of the standard bromocresol purple. (B) The measured *in vivo* action spectrum for the photosagging of mouse skin taken from the results of Bissett and coworkers (reprinted with permission from ref. 40). The *in vivo* action spectrum mimics the action spectrum shown in A.

photochemical event that mediates this physiological response. Specifically, pulsed laser photoacoustic data after UV-A excitation of *t*-UA were collected both in deoxygenated and oxygen-saturated solutions. In the presence of oxygen, the identical line shape as that reported in Fig. 2A is observed. However, when compared with the deoxygenated sample, the oxygen-saturated solution retained significantly less heat. These data showed that oxygen quenches the long-lived state of *t*-UA generated from UV-A irradiation. Quenching reactions of triplet states of organic molecules by O_2 are common, and energy transfer from an excited triplet state to O_2 leads to the formation of $\text{O}_2(^1\Delta_g)$ (41). We have confirmed that $\text{O}_2(^1\Delta_g)$ is generated after the UV-A excitation of *t*-UA at 351 nm by measuring the subsequent $^1\Delta_g \rightarrow ^3\Sigma_g$ emission of O_2 . This result confirms the above assignment that the long-lived excited state of *t*-UA that is formed on UV-A irradiation is an excited triplet state of the molecule. From the similarity of the

action spectra shown in Fig. 2, we propose that the generation of $O_2(^1\Delta_g)$ that results from the UV-A excitation of *t*-UA initiates chemical processes that lead to the physiological responses characteristic of UV-A photoaged skin. It needs to be noted that, once a photostationary state is achieved, the concentration of *c*-UA exceeds that of *t*-UA in the skin. For example, the photostationary state for photoexcitation at 313 nm is 34% *t*-UA (36). Thus, the reactions of *c*-UA also could contribute to the action spectra of urocanic acid *in vivo*. Whether *c*-UA generates $O_2(^1\Delta_g)$ after UV-A excitation is not known. Work is currently in progress to quantify the photo-reactions of *c*-UA *in vitro*.

It is important to emphasize that we are not comparing the *in vivo* action spectrum of the photosagging of skin with the overall absorption spectrum of *t*-UA. Rather, the *in vivo* action spectrum is being compared with a weak transition that contributes to the photochemically complicated, yet structureless absorption spectrum of *trans*-urocanic acid. At first glance, the *t*-UA absorption spectrum would appear to be completely unrelated to any physiological response in the UV-A. However, the present work shows that one must consider the possible role of weak transitions of endogenous absorbing chromophores in initiating physiological responses. This includes chromophores like NADH/NADPH, tryptophan, and riboflavin that have been postulated to play a role in photoaging (18, 19, 42). These chromophores have been implicated in photoaging, but a quantitative comparison between their absorption spectra and photoaging action spectra has not been made. Additional action spectra (skin fold thickening, glycosaminoglycan production, collagen damage, cellularity increase, and elastosis in mice models) also reflect a UV-A component that have a similar line shape as the triplet state action spectrum of *t*-UA (40). It would not be surprising if the generation of $O_2(^1\Delta_g)$ by energy transfer from the excited triplet state of *t*-UA in the UV-A also contributes to these physiological responses.

$O_2(^1\Delta_g)$ is not a "selective" reactant, and it initiates a wide range of physiological responses. Recent reports of the UV-A irradiation of fibroblasts indicate that singlet oxygen is both an early intermediate in the signaling pathway of interstitial collagenase induction, preceding the synthesis of the proinflammatory cytokines interleukin 1 and interleukin 6 (43), and can activate JNKs (stress-activated kinases), which can affect gene expression (44). Correlation between UV-A and singlet oxygen also have been discussed for the induced synthesis of mRNA heme oxygenase-1 (45, 46), collagenase (43, 47, 48), and intercellular adhesion molecule-1 (49). Whether *t*-UA is a major contributing source of the singlet oxygen that causes these responses requires more research.

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