Macromolecular carbonyls in human stratum corneum: a biomarker for environmental oxidant exposure?

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Abstract The presence of stratum corneum carbonyls may serve as an intrinsic dosimeter for environmental oxidative damage to skin. To investigate the accumulation of carbonyls in human stratum corneum, skin was tape-stripped, then tapes were sequentially incubated with 2,4-dinitrophenyl hydrazine (DNPH), rat anti-DNP, mouse anti-rat IgG conjugated with alkaline phosphatase, *p*-nitrophenyl phosphate and absorbance (405 nm) measured and carbonyls estimated. Stratum corneum exposed in vitro to oxidants: hypochlorous acid (1, 10, 100 mM), ozone (0, 1, 5, 10 ppm for 2 h) or UV light (280–400 nm; 0, 4, 88, or 24 J/cm^2) contained increased carbonyls. Furthermore, stratum corneum carbonyls were elevated in tanned compared with untanned sites: dorsal hand (0.43 ± 0.06 nmol/cm^2) vs. lower arm (0.32 ± 0.04, mean ± S.E.M., $n = 11$; $P < 0.003$) and lower back (0.26 ± 0.02) vs. buttock (0.21 ± 0.02; $n = 6$, $P < 0.01$) indicating in vivo oxidative damage.

Key words: Skin; Ultraviolet light; Ozone; Oxidative stress; Carbonyl

1. Introduction

Skin is directly and frequently exposed to a pro-oxidative environment. Sources of environmental oxidants include oxygen, solar UV irradiation and ozone found in polluted air. Thiele et al. [1–3] examining the susceptibility of skin exposed to ozone found that the skin surface is readily oxidizable. When hairless mice were exposed to 10 ppm ozone for 2 h, it increased skin malondialdehyde (MDA), and depleted topically applied vitamin E, but not the vitamin E inherent in the whole skin [1]. When the upper layers were examined, ozone (10 ppm for 2 h) was found to damage the upper epidermis, decreasing both vitamins C and E and increasing MDA concentrations [2]. Notably, antioxidants in the lower layers of skin were unaffected. Therefore, assessment of the antioxidant content of the uppermost skin layer, the stratum corneum, was evaluated [3]. Sequential tape-strippings of mouse stratum corneum demonstrated that ozone exposures as low as 1 ppm for 2 h on 6 consecutive days depleted vitamin E and ozone increased MDA formation. Thus, skin is especially susceptible to oxidative stress and the outermost layer, the stratum corneum, serves as a barrier to oxidative insults.

The introduction of carbonyl groups into amino acid residues of proteins is a hallmark of oxidative modification and is often used as a definitive method for assessing oxidative protein damage [4]. However, carbonyl groups may also be introduced into proteins by mechanisms that do not involve oxidation of amino acid residues. For example, α,β-unsaturated aldehydes produced during the peroxidation of polyunsaturated fatty acids react with protein thiol groups to form stable covalent thiol ether adducts carrying a carbonyl group [5], as do aldehydes present in cigarette smoke [6]. Increased carbonyls have been detected in several disease states such as rheumatoid arthritis [7], ischemia-reperfusion injury to heart muscle [8,9] and skeletal muscle damage due to exhaustive exercise [10]. Furthermore, we have found that carbonyls are an useful marker of LDL oxidation because unlike lipid peroxidation products, carbonyls are relatively stable [11–14].

Exposed skin may react with oxidizing species in the environment, and even aldehydes in polluted air (e.g. cigarette smoke [15]) to form carbonyl groups. Carbonyl formation most likely involves the production of hydroperoxides, which can further react to yield active oxygen species, such as HO•, with some amino acids being converted to carbonyl derivatives [16]. The direct oxidation of amino acid residues, however, may not be the only mechanism for the generation of carbonyl derivatives. Lipid radical-propagated chain reactions begin with oxidation of polyunsaturated fatty acids to lipid hydroperoxides [17], which can serve as sources for production of carbonyls.

The objective of this study was to evaluate whether carbonyls could be detected in human skin and whether their formation in skin varied in response to oxidative stress. For this purpose, we obtained stratum corneum, the uppermost layer of skin, by tape-stripping skin of human volunteers. An ELISA technique was developed to detect the carbonyls in the tissue adherent to the tapes because it is not possible to obtain large amounts of protein needed for the more conventional spectrophotometric carbonyl assays. We present here a non-invasive method for detection of carbonyl formation in human skin using a tape-stripping-based technique.

2. Materials and methods

2.1. Tape-strippings

Human stratum corneum was obtained in accordance with approvals obtained from the UC Berkeley Committee for the Protection of Human Subjects; all subjects gave written, informed consent. Samples of human stratum corneum were obtained by tape-stripping the skin with 5×5 cm pieces of cellophane tape. In preliminary studies, various tapes were tested for applicability and interference with non-specific binding. Scotch Superstrength Mailing Tape (3M, St. Paul, MN) was found to be suitable.

To improve the reproducibility of the tape-stripping technique, a standardized protocol was used: tapes were cut exactly (5×5 cm), then smoothly adhered onto the skin, equally flattened three times,

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and gently removed using moderate and even traction. The resultant stratum corneum layers adhering on the tapes appeared by light microscopy to be of uniform thickness. Each tape had approximately 2 mg of adherent tissue.

Due to the possibility of surface contamination and to remove surface lipids, the first (uppermost) tape-stripping was discarded; subsequent tape-strippings were used for analyses.

2.2. ELISA assay of carbonyls

Stratum corneum samples on tape strips were either analyzed immediately, or were oxidized in vitro (as described below), then an ELISA procedure was performed in situ on the tissue adherent to the tape.

To prevent non-specific reactions, tapes with adherent skin were agitated for 30 min at room temperature in blocking buffer (2.5% blotting grade blocker non-fat dry milk; Bio-Rad Laboratories, Hercules, CA), rinsed three times with phosphate buffered saline (PBS)-Tween (quickly twice, then for 2 min). To identify carbonyl, tapes were incubated with 0.2 mM 2,4-dinitrophenyl hydrazine (DNPH) in PBS to form a carbonyl specific conjugate, then were washed as described above. In the absence of stratum corneum, there was little or no reaction of the tape to DNPH.

DNPH conjugates were identified by incubation with a rat antibody to synthetic DNP (Zymed Laboratories Inc., South San Francisco, CA). Tapes were placed individually (tissue side down) onto 3 ml antibody solution in 90 mm Petri dishes (Becton Dickinson labware, Lincoln Park, NJ). The dishes were kept in the dark for 2 h, then each tape was rinsed in PBS-Tween, and placed (tissue side down) onto 3 ml secondary antibody solution (mouse anti-rat IgG conjugated with alkaline phosphatase, Boehringer Mannheim, Indianapolis, IN). After 2 h in the dark, the tapes were rinsed in PBS-Tween, then placed (tissue side down) onto 2 ml substrate solution (*p*-nitrophenyl phosphate, Sigma, St. Louis, MO). After 30 min in the dark, 200 μl of the substrate solution was placed in a well of a 96-well tissue culture plate (Nunclon, from Delta, Denmark). Absorbances were read at 405 nm using a Bio-Rad microplate reader, model 3550. The nmol/cm^2 skin was estimated from the tape size (25 cm^2) and a standard curve (carbonyls = (absorbance−0.067)/0.056, $r^2 = 0.9898$, $n = 3$ per six dilutions) comparing the absorbance with a dilution of the second antibody, reaction with substrate for 1 h, and the assumption that a single carbonyl reacted with one DNPH and this was recognized by one primary and one secondary antibody. This is only an estimate of the carbonyl concentrations; the relative differences between treatments or between exposed and non-exposed sites do not depend on the absolute values shown.

2.3. Modification by hypochlorite

The concentration of hypochlorite/hypochlorous acid present in the diluted commercial NaOCl solution was determined spectrophotometrically using published extinction coefficients ($\varepsilon_{290} = 350$ M^{-1} cm^{-1}) [18]. Hypochlorous acid ($pK_a = 7.5$ [19]) is present as both OCl^- and HOCl; therefore, 'HOCl' is used to describe the mixture of OCl^- and HOCl. Tapes were incubated with 0, 1, 10, or 100 mM HOCl overnight with shaking at room temperature, washed twice with PBS, and then were analyzed for carbonyl contents.

2.4. Ozone exposure

Ozone was produced from oxygen by electric discharge (Sander ozonizer model IV, Eltze, Germany). The ozone was then mixed with filtered (ozone-free) ambient air and allowed to flow into an exposure chamber at a constant rate (200 l/min). The concentration in the exposure chamber was adjusted to 1, 5, and 10 ppm and continuously monitored with an ozone detector (Dasibi model 1003-AH, Glendale, CA). Tapes were exposed for 2 h.

2.5. UV irradiation

Skin tapes were irradiated using an Oriel 1000 W Solar Simulator xenon arc lamp (Oriel, Town, CT) fitted with an Oriel model 81051, UVC WG-335 filter. This lamp simulates the full solar UV spectrum (UVA and UVB, 280–400 nm). The output was 1.85 mW/cm^2, as measured with the IL 443 radiometer, and 5.16 mW/cm^2, as measured with the UVX-36 radiometer. For comparison, the irradiances of sunlight measured on a sunny day (15 July at noon) on the campus of the University of California at Berkeley (latitude 38°N) were 0.5 mW/cm^2 (IL 443 radiometer), and 2.2 mW/cm^2 (UVX-36 radiometer). The solar UV simulator set-up included an in-built UVB/UVA dichroic mirror that allows wavelengths of 280–400 nm to pass and greatly reduces the VIS and IR output of the lamp. Tape-stripped stratum corneum was exposed to 0, 4, 8, or 24 J/cm^2.

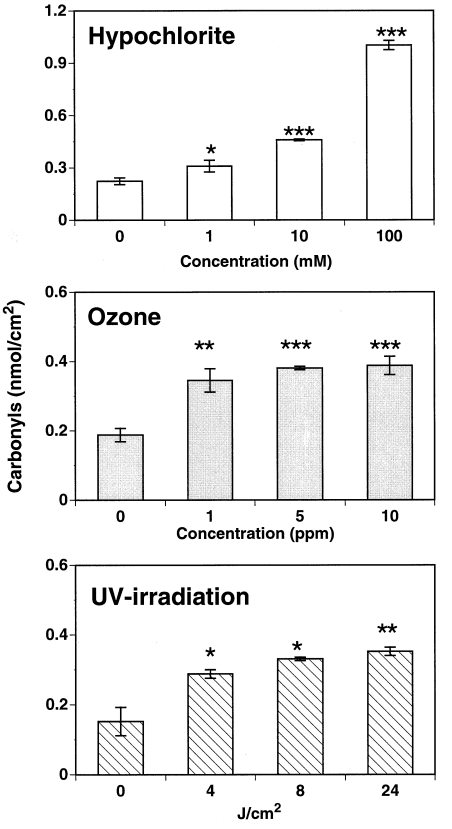

Fig. 1. Carbonyl formation in human stratum corneum attached to tapes by three different known pro-oxidative systems. Top: Incubation overnight with hypochlorite (0–100 mM). Middle: Exposure for 2 h to ozone (0–10 ppm). Bottom: Exposure to solar-simulated UV light (280–400 nm; 0–24 J/cm^2) $^*P < 0.05$, $^{**}P < 0.001$, $^{***}P < 0.0001$; all results are given as means ± S.E.M., $n = 3$.

2.6. Statistical analysis

Statistical analysis was carried out using Super Anova (Abacus Concepts, Berkeley, CA). Data were analyzed by one-way analysis of variance (ANOVA) and least square means analysis. For analysis of intraindividual differences between exposed and non-exposed skin sites ANOVA with repeated measure design was used. The $P < 0.05$ level was selected as the point of minimal acceptable statistical significance. All data in text and figures are expressed as mean ± standard error (S.E.M.).

3. Results

3.1. In vitro exposure of human stratum corneum to oxidants

To test whether macromolecular carbonyl formation could be detected in human skin, stratum corneum was obtained by tape-stripping and then was exposed to known oxidants. Since hypochlorite is known to cause carbonyl formation [12], this was the first agent tested; high concentrations were used to induce maximal carbonyl formation. Incubation of human stratum corneum (adherent to tapes, $n = 3$ per group) with sodium hypochlorite resulted in dose-dependent increases of carbonyls (Fig. 1, upper panel). As compared with control levels (0 mM sodium hypochlorite; 0.22 ± 0.02 nmol/cm^2 skin surface), incubation with 10 mM hypochlorite doubled

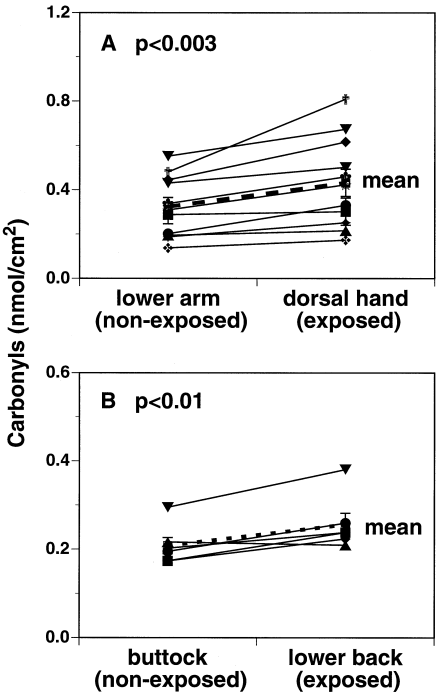

Fig. 2. Differences in carbonyl levels between sun-exposed (tanned) and non-exposed (pale) skin sites; values for individual subjects are shown. A: Comparison between lower (inner, non-exposed) arm and dorsal hand ($n=11$, $P<0.003$). B: Comparison between lower (non-exposed) buttock and lower back ($n=6$, $P<0.01$).

carbonyl concentrations (0.46 ± 0.01; $P<0.001$), while 100 mM hypochlorite increased them more than four-fold (1.00 ± 0.03; $P<0.001$).

Exposure of human stratum corneum (adherent to tapes; $n=6$ per treatment) to ozone also resulted in increases of carbonyls (Fig. 1, center panel). As compared with control levels (0 ppm ozone; 0.19 ± 0.02 nmol/cm^2 skin surface), exposure for 2 h to 10 ppm increased carbonyls to 0.39 ± 0.03 ($P<0.001$).

Exposure of stratum corneum (adherent to tapes; $n=4$) to solar-simulated UV light also resulted in dose-dependent increases of carbonyls (Fig. 1, lower panel). As compared with control levels (0 J/cm^2; 0.15 ± 0.04 nmol/cm^2), exposure to 4 ($P<0.02$), 8 ($P<0.003$), or 24 J/cm^2 ($P<0.001$) doubled the carbonyls to approximately 0.3 nmol/cm^2.

3.2. Intraindividual comparison between carbonyl levels in tanned (exposed) skin versus pale (non-exposed) skin

In 11 individuals, stratum corneum carbonyl levels measured in tanned skin from the dorsal hand were compared with those in pale skin from the lower arm. In 10 of 11 cases, the carbonyl contents of stratum corneum obtained from the tanned sites were higher than those of stratum corneum obtained from pale sites. The carbonyl content of tanned skin was higher (0.43 ± 0.06 nmol/cm^2) compared with pale sites (0.32 ± 0.04; $P<0.003$; Fig. 2A). It should be noted that in this portion of the study a larger number of subjects were investigated and the few subjects studied in the validation section above had lower skin carbonyl contents in the unoxidized state.

In six of the individuals, stratum corneum carbonyls from the tanned lower back skin and the pale lower buttock were also obtained. Again, in five of six cases, the carbonyl content of stratum corneum obtained from the tanned sites (0.26 ± 0.02 nmol/cm^2) was higher than that obtained from pale sites (0.21 ± 0.02, $P<0.01$; Fig. 2B). In addition to the described intraindividual differences, a high interindividual range of stratum corneum carbonyl levels at both pale and tanned skin sites was observed.

4. Discussion

Higher levels of carbonyls were found in stratum corneum isolated from human skin exposed to the environment (tanned) compared with areas that were relatively protected (pale). The ELISA technique used to detect carbonyl formation in human stratum corneum is non-invasive; it utilizes stratum corneum adherent to tape, and unlike other ELISA techniques for measuring carbonyls [20], it is not necessary to extract protein from the tissue. Importantly, we demonstrate that when human stratum corneum adherent to tapes was exposed to known oxidants, a measurable increase in carbonyl content was found; thereby suggesting that the increased carbonyls found in sun-exposed skin resulted from oxidative damage.

Fig. 1 demonstrates that the absolute levels of carbonyls formed on the stratum corneum varied depending upon the in vitro oxidation conditions. Carbonyl derivatives could arise through interaction of lysine, cysteine, or histidine residues with lipid peroxidation products such as malondialdehyde [21] and 4-hydroxynonenal [22] via Michael additions and Schiff base adducts [23]. During hypochlorous acid modification of stratum corneum, formation of reversible lysine chlorohydrins may also take place. These chlorohydrins can decompose to carbonyls and other products [24]. It is possible that reactions such as condensation and crosslinking, as well as direct attack on amino groups by hydroperoxides or other oxidizing species, result in the loss of amino groups, some of which are converted to non-carbonyl species. Thus, processes which generate lipid peroxidation (ozone and UV light exposure, or aldehydes themselves) might, therefore, yield different amounts of carbonyls than would hypochlorite exposure of the stratum corneum. Furthermore, the hypochlorite could penetrate the stratum corneum during the prolonged incubation and thereby produce higher carbonyl levels.

Routine in vivo exposure of skin to high hypochlorite concentrations is not very likely, but UV light exposure is quite common. The detection of carbonyls in tanned compared with untanned skin suggests that oxidation of skin components occurs in vivo as a result of sun exposure. Melanin in the skin and stratum corneum may act as a photoprotective agent at long UV and visible wavelengths, but could act as a photosensitizer in the short UV range [25]. Since melanin is present in variable amounts in the stratum corneum [26], its wavelength-dependent potential to quench or to generate free radicals and to absorb UV light may modulate α-tocopherol depletion, lipid peroxidation, and carbonyl formation during and after solar exposure. We have recently determined the vitamin E content of human stratum corneum [27] and found that the surface stratum corneum layers are virtually depleted of vitamin E as compared with deeper layers [28].

Although we did not specifically examine formation of carbonyls as a result of aldehyde exposure, this is an important area for subsequent investigations. Aldehydes in cigarette

smoke could readily cause carbonyl formation in the skin of the face and hands.

Taken together these findings suggest that the outermost barrier of skin is a barrier to oxidative stress and is relatively ravaged by environmental factors. Hence, stratum corneum carbonyls may serve as intrinsic dosimeters for this environmental oxidative skin damage.

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