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UV irradiation-induced methionine oxidation in human skin keratins: Mass spectrometry-based non-invasive proteomic analysis

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ABSTRACT

Ultraviolet (UV) radiation is the major environmental factor that causes oxidative skin damage. Keratins are the main constituents of human skin and have been identified as oxidative target proteins. We have recently developed a mass spectrometry (MS)-based non-invasive proteomic methodology to screen oxidative modifications in human skin keratins. Using this methodology, UV effects on methionine (Met) oxidation in human skin keratins were investigated. The initial screening revealed that Met^{259}, Met^{262}, and Met^{296} in K1 keratin were the most susceptible oxidation sites upon UVA (or UVB) irradiation of human tape-stripped skin. Subsequent liquid chromatography/electrospray ionization-MS and tandem MS analyses confirmed amino acid sequences and oxidation sites of tryptic peptides D^{290}VDGAYMTK^{298} (P1) and N^{258}MQDMVEDYR^{267} (P2). The relative oxidation levels of P1 and P2 increased in a time-dependent manner upon UVA irradiation. Butylated hydroxytoluene was the most effective antioxidant for artifactual oxidation of Met residues. The relative oxidation levels of P1 and P2 after UVA irradiation for 48 h corresponded to treatment with 100 mM hydrogen peroxide for 15 min. In addition, Met^{259} was oxidized by only UVA irradiation. The Met sites identified in conjunction with the current proteomic methodology can be used to evaluate skin damage under various conditions of oxidative stress.

Biological significance: We demonstrated that the relative Met oxidation levels in keratins directly reflected UV-induced damages to human tape-stripped skin. Human skin proteins isolated by tape stripping were analyzed by MS-based non-invasive proteomic methodology. Met^{259}, Met^{262}, and Met^{296} in K1 keratin were the most susceptible oxidation sites upon UV irradiation. Met^{259} was oxidized by only UVA irradiation. Quantitative LC/ESI-SRM/MS analyses confirmed a time-dependent increase in the relative oxidation of target peptides (P1 and P2) containing these Met residues, upon UVA irradiation of isolated human skin. The relative oxidation levels of P1 and P2 along with the current proteomic methodology could be applied to the assessment of oxidative stress levels in skin after exposure to sunlight.

1. Introduction

As the major barrier of the human body, the skin is continuously exposed to external stimuli such as ultraviolet (UV) radiation, air pollutants, and industrial chemicals. These agents can induce the generation of reactive oxygen species (ROS) [1–3], which mediate oxidative stress. Oxidative stress causes damage to important cellular components such as proteins, DNA, and membrane lipids, and can lead to cell death [4]. Oxidative stress is also thought to be a major contributor to skin disorders such as skin cancer, photoaging, and atopic dermatitis [5–7]. UV radiation is the most common external stimulus that induces oxidative skin damage. UVA (320–400 nm) represents 95% of the UV radiation reaching the Earth's surface. It penetrates the skin dermis more deeply than UVB and mainly produces ROS through interactions with photosensitizers such as vitamin B, porphyrin, and aromatic amino acids [8,9]. By contrast, UVB (290–320 nm) causes direct damage to DNA, proteins, and lipids in addition to generating ROS [9]. UVA- and UVB-induced oxidative effects on skin include the depletion of antioxidants (*e.g.* vitamin E) and the modulation of enzymatic antioxidant defense systems (*e.g.* Met sulfoxide reductases (MSRs), superoxide dismutase, and catalase) [10]. Therefore, antioxidant levels and antioxidant enzyme activities in the dermis, epidermis, and stratum corneum (SC) have been investigated as markers of UV-induced photo-oxidation

Abbreviations: ACN, acetonitrile; Ang, angiotensin; AscA, ascorbic acid; BHT, butylated hydroxytoluene; DHB, 2,5-dihydroxybenzoic acid; DNPH, 2,4-dinitrophenylhydrazine; DTT, dithiothreitol; EIC, extracted ion chromatogram; ESI, electrospray ionization; FA, formic acid; IAA, iodoacetamide; LC, liquid chromatography; MALDI/TOF, matrix-assisted laser desorption ionization/time of flight; Met, methionine; MetO, methionine sulfoxide; $[M + H]^+$, protonated molecule; $[M + 2H]^{2+}$, doubly protonated molecule; MS, mass spectrometry; MS/MS, tandem mass spectrometry; MSR, methionine sulfoxide reductase; 8-oxo-dGuo, 8-hydroxydeoxyguanosine; PMF, peptide mass fingerprinting; RCM, reduction and carboxymethylation; ROS, reactive oxygen species; SC, stratum corneum; SDS, sodium dodecyl sulfate; SEM, standard error of the mean; SRM, selected reaction monitoring; TEMPO, 2,2,6,6-tetramethylpiperidin-1-oxyl; TFA, trifluoroacetic acid; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; UV, ultraviolet.

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[11]. Several other biomarkers have also been used to assess the extent of oxidative stress in the skin. Carbonylated proteins have been detected by labeling with 2,4-dinitrophenylhydrazine (DNPH) as markers of ROS-mediated protein oxidation in various positions and under different skin conditions [10,12]. The level of 8-hydroxydeoxyguanosine (8-oxo-dGuo) was measured to evaluate oxidative DNA damage in atopic dermatitis [13]. In our recent study, the relative methionine (Met) oxidation of keratin-derived target peptides was successfully employed to estimate the oxidation levels of human skin after treatment with hydrogen peroxide (H_2O_2) [14]. Protein carbonyls and 8-oxo-dGuo are common biomarkers of oxidative stress [15,16]. In comparison, the use of Met oxidation as an oxidative stress marker has been somewhat limited, possibly due to the existence of MSRs that can repair the lesion. However, the level of Met sulfoxide is still considered to be a useful marker of *in vivo* oxidative damage because Met residues are highly susceptible to oxidation by almost all forms of ROS, including hypochlorite, nitric oxide, and H_2O_2 [17,18]. In addition, Met sulfoxide is a more general and sensitive indicator of the overall exposure of a protein to oxidative, glycoxidative, and lipoxidative damage than advanced glycoxidation or lipoxidation end products [19].

Keratins are the main constituents of the skin. Basic keratin K1 and acidic keratin K10 are mainly expressed in the SC and assembled as heterodimers [20]. Modifications of human skin keratin have been reported as potential biomarkers of dermal exposure to sulfur mustard (a chemical warfare agent), naphthalene (jet fuel), and organophosphates (agricultural chemicals) [21–23]. Keratins have also been identified as the major oxidative target proteins in human skin [24]. Keratinocytes that produce the keratins are constantly shed and replaced by cells from the lower layers of the epidermis, creating a new epidermis every 28–35 days [25]. Therefore, keratins could provide up-to-date information on the oxidative status of the skin. However, there have been few studies using keratins as markers of skin condition or damage because of the difficulties associated with their insolubility and handling. Our laboratory has recently developed a mass spectrometry (MS)-based proteomic methodology to screen oxidative modifications in human skin keratins [14]. Human skin proteins were obtained non-invasively by tape stripping and solubilized in sodium dodecyl sulfate (SDS) buffer, followed by purification and digestion using the filter-aided sample preparation method. The tryptic peptides were then analyzed by matrix-assisted laser desorption ionization/time-of-flight (MALDI/TOF)-MS.

In this study, we aimed to investigate the effects of UV radiation on Met oxidation in human skin keratins, and to assess the potential use of relative Met oxidation levels as quantitative biomarkers of UV-induced oxidative skin damage. By using the developed methodology, the most susceptible Met sites were determined by UVA or UVB irradiation of human tape-stripped skin. Liquid chromatography/electrospray ionization (LC/ESI)-MS and tandem MS (MS/MS) were used to confirm the identified oxidation sites. Various antioxidant systems were tested to avoid artificial Met oxidation during sample preparation. The relative Met oxidation levels were then determined by LC/ESI-selected reaction monitoring (SRM)/MS based on calibration curves generated using synthetic target peptides. Finally, UV-induced Met oxidation levels in human skin keratins were compared with those mediated by H_2O_2.

2. Materials and methods

2.1. Materials

Adhesive skin tape was purchased from Asahi Biomed Co. Ltd. (Tokyo, Japan) and a mini cordless grinder was purchased from Funakoshi Co., Ltd. (Tokyo, Japan). A homogenizing pestle was purchased from Scientific Specialties, Inc. (Omaha, NE). PL-S 9 W UV-A/2P 1CT and PL-S 9 W/12/2P 1CT was purchased from Philips (Aachen, Germany). Dermalight 80 was purchased from Dr. K. Hönle GmbH (Munich, Germany). HPLC-grade acetonitrile (ACN), urea, 2-mercaptoethanol, hydrochloric acid (HCl), disodium hydrogenphosphate 12·H_2O, sodium dihydrogenphosphate dihydrate, sodium chloride (NaCl), SDS, sodium periodate ($NaIO_4$), H_2O_2, ammonium bicarbonate (NH_4HCO_3), dithiothreitol (DTT), iodoacetamide (IAA), formic acid (FA), phosphoric acid, L-Met, and L-tyrosine were purchased from Nacalai Tesque, Inc. (Kyoto, Japan). Human angiotensin (Ang) II was obtained from Calbiochem (a brand of EMD, San Diego, CA). Ang I, 2-amino-2-hydroxymethyl-1,3-propanediol (Tris), butylated hydroxytoluene (BHT), and trifluoroacetic acid (TFA) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). ACTH (18–39) was purchased from Bachem (Bubendorf, Switzerland). L-Ascorbic acid (L-AscA), deferoxamine mesylate, and 2,2,6,6-tetramethylpiperidin-1-oxyl (TEMPO) were purchased from Sigma-Aldrich, Inc. (St. Louis, MO). Sequencing-grade modified trypsin was purchased from Promega (Madison, WI). 2,5-Dihydroxybenzoic acid (DHB) was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Chelex-100 chelating ion-exchange resin (100–200 mesh size) was purchased from BIO-RAD Laboratories (Hercules, CA). Amicon Ultra centrifugal filters (0.5 mL, 30 K) and ZipTip C18 were purchased from Millipore (Billerica, MA). Ultrapure water was obtained from a Milli-Q Integral 10 (EMD Millipore, Billerica, MA) equipped with a 0.22 μm membrane cartridge. Peptide standards, DVDGAYMTK (Asp-Val-Asp-Gly-Ala-Tyr-Met-Thr-Lys, P1), DVDGAY*MTK (Asp-Val-Asp-Gly-Ala-Tyr-MetO-Thr-Lys, $P1_{OX}$), NMQDMVEDYR (Asn-Met-Gln-Asp-Met-Val-Glu-Asp-Tyr-Lys, P2), N*MQDMVEDYR (Asn-MetO-Gln-Asp-Met-Val-Glu-Asp-Tyr-Lys, $P2_{OX-1}$), and NMQD*MVEDYR (Asn-Met-Gln-Asp-MetO-Val-Glu-Asp-Tyr-Lys, $P2_{OX-2}$) were purchased from Toray Research Center, Inc. (Tokyo, Japan) (* represents an oxidized amino acid residue, +16 Da).

2.2. MALDI/TOF-MS conditions

MALDI/TOF-MS experiments were carried out on an AXIMA Performance MALDI/TOF mass spectrometer (Shimadzu Co., Kyoto, Japan) equipped with a nitrogen laser (337 nm) at the Biomedical Research Core (School of Medicine, Tohoku University, Japan). All spectra presented herein were acquired in the positive ion mode with an accelerating voltage of 20 kV and in reflectron mode with an average of 1000 laser shots. TOF-MS experiments were performed in the mass range of m/z 500–4000. Calibration was conducted using three external calibrants as follows: Ang II at m/z 1046.5345, Ang I at m/z 1296.6775, and ACTH (18–39) at m/z 2465.1911. Prior to MALDI/TOF-MS analysis, the peptide solutions were desalted and eluted with ZipTip C18 cartridges. In brief, the ZipTips were conditioned with ACN (10 μL × 5) and equilibrated with 0.1% aqueous TFA (10 μL × 5). The peptide solutions in 5% aqueous ACN containing 0.1% TFA were subsequently loaded onto the ZipTip and washed with 0.1% aqueous TFA (10 μL × 5). Finally, the samples were eluted with 50% aqueous ACN containing 0.1% TFA (10 μL × 2) and diluted twice with 50% aqueous ACN containing 0.1% TFA to give a final volume of 40 μL. Aliquots (5 μL) of samples in 50% aqueous ACN containing 0.1% TFA were mixed with 5 μL of matrix solution (150 mM DHB in 25% aqueous ACN containing 1% phosphoric acid). Aliquots (1 μL) of the mixture were loaded on the MALDI sample plate and allowed to dry at room temperature.

Shimadzu Biotech Launchpad Ver.2.9.3.20110624 (Kratos Analytical Ltd., New York, NY) was used for data processing and MASCOT database search engine (Matrix Science Inc., Boston, MA) was used for peptide mass fingerprinting (PMF) analysis. Search parameters were as follows: Database, Swiss Prot; enzyme, trypsin; missed cleavage, allow up to 2; taxonomy, *Homo sapiens* (human); mass values, $[M + H]^+$, monoisotopic; fixed modifications, carbamidomethyl (C); variable modification, oxidation (M); and peptide tolerance, ±0.3 Da. Peaks with signal-to-noise ratio greater than 4 were used for database searching.

2.3. LC/ESI-MS, MS/MS and SRM/MS conditions

Chromatography for LC/MS analyses were carried out using two different systems. For all systems, 0.1% (*v*/*v*) FA in water and 0.1% (*v*/*v*) FA