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Proteomic approach for the analysis of acrylamide–hemoglobin adducts Perspectives for biological monitoring

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ABSTRACT

The formation of adducts between acrylamide and hemoglobin *in vitro* was investigated by using mass spectrometric methodologies to identify the amino acid residues sensitive to alkylation. Liquid chromatography–electrospray ionisation mass spectrometry analysis of either intact or trypsin-digested α- and β-globin chains isolated from hemolysate samples incubated *in vitro* with acrylamide at different molecular ratios allowed us to identify Cys93 of β-globin as the most reactive site in hemoglobin, according to a Michael-type addition reaction between acrylamide and the sulphydryl group of cysteine. The only other reactive sites were Cys104 of α-globin and the N-terminal amino groups of both chains. The method developed, based on electrospray ionisation quadrupole time-of-flight tandem mass spectrometry analysis of intact globin chains was able to specifically detect low levels of adducts. In this way, rapid identification of alkylated portion of Hb was achieved to be potentially used as a biomarker for high-sensitivity biological monitoring.

1. Introduction

Acrylamide (AA, CH_2=CHCONH$_2$, CAS Registry Number 79-06-1) is an important industrial chemical widely used in the production of polyacrylamide and as an intermediate reagent in a variety of reactions of chemical synthesis. AA is used in research laboratories for preparing the polyacrylamide gel electrophoresis support for the separation of biological macromolecules which is also the basis technique for proteomic analysis. The polyacrylamide polymers contain varying amounts of residual monomer AA [1]. AA is also present in cigarette smoke.

High levels of AA have been found in heated food as the product of the Maillard reaction between amino acids (mainly Asn, but also Gln and Met) and reducing sugars (D-fructose, D-galactose, lactose, glucose) [2,3]. Human exposure may occur by the ingestion of AA-containing foods [4], inhalation or skin contact. AA is neurotoxic, clastogenic, carcinogenic and is a toxicant for the reproductive tract of animals. AA is classified by the International Agency for research on Cancer (IARC) as "probable human carcinogen" 2A group [5].

AA is a mutagen towards either mammalian somatic cells or germ cells, inducing high frequency of dominant lethal mutations, heritable chromosomal translocations, and specific locus mutations in postmeiotic sperm and spermatogonial stem cells of male mice [6]. Glycidamide (GA), the main metabolite formed by the epoxidation of AA operated by cytochrome P450 (CYP2E1 isoform [7]), is believed to be responsible for the carcinogenicity of AA [8–12].

The neurotoxic effects of monomer AA are mental status changes, ataxia and skeletal muscle weakness related to injury to nerve terminals and cerebellar Purkinje cells. Although the molecular mechanism of the synaptic dysfunction due to AA is unknown, the membrane fusion processes which are at the basis of neurotransmission are highly sensitive to inhibition by alkylation of protein thiols [13] and/or alteration of the redox state of certain cysteine sulphydryl groups on proteins. It has been recently hypothesized [14,15] that electrophilic neurotoxicants selectively adduct thiol groups located in the catalytic triad of NO-receptors and that the resulting loss of fine gain control impairs neurotransmission.

Both AA and GA are protein alkylating agents; they can bind to human hemoglobin (Hb) to form covalent adducts which were detected in the blood of animals and humans exposed to AA [16–22]. Since the 1990s, Hb adducts have been considered valuable markers of human exposure to chemicals and constitute a good measure of the internal dose [23–29]. Human biomonitoring of

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occupational and dietary exposure to AA through Hb adduct determination has been carried out with various analytical methods. The standard method is based on the *N*-alkyl Edman degradation procedure to release the N-terminal Val–AA adduct which is then derivatised and determined by gas chromatography–mass spectrometry [7–12,16–22] or liquid chromatography–mass spectrometry (LC/MS) analysis [30,31] of the resulting derivatised thiohydantoin. Previously a method based on determination of *S*-(2-carboxyethyl)cysteine in hydrolyzed hemoglobin was used [32].

Paulsson et al. [33] have carried out three case studies of occupational exposure to AA. In the two individuals in which AA–Hb levels were present in fairly high concentrations (0.3 and 23 nmol/g Hb), the adducts remained measurable for several months after exposure had ceased, rendering AA–Hb adducts potentially useful for occupational surveillance and intervention.

Similarly to other α-, β-unsaturated chemicals, AA reacts through Michael addition of nucleophiles across its vinyl group which is characterized by a polarized double bond. Protein adduction is dependent not only upon the chemical nature of the electrophilic agent, but also upon the microenvironment surrounding the nucleophilic group. As a consequence, the reactivity of free sulfhydryl (or other nucleophilic groups) on proteins can be different and, consequently, soft electrophilic chemicals such as AA may specifically react only with the most reactive thiol groups [34,35].

However, the structural nature of AA–Hb adducts has been never investigated. In this work, the characterization of interaction between AA and Hb was accomplished by mass spectrometry analysis of either whole globins or protein tryptic digests.

2. Experimental

2.1. Materials

Acrylamide (electrophoresis-grade, 99%) was purchased from Bio-Rad (Hercules, CA, USA). 98% enriched [2,3,3′-$^2H_3$]acrylamide (AA-d_3) was obtained from Cambridge Isotope Labs. (Woburn, MA, USA). Acetonitrile (ACN), trifluoroacetic acid (TFA), and all other chemical and solvents used were of analytical grade. HPLC-grade solvents and reagents were from Carlo Erba (Milan, Italy).

2.2. In vitro alkylation of hemoglobin with AA

Blood samples from healthy, non-smoking subjects (not occupationally exposed to AA) collected in EDTA tubes were centrifuged for 10 min at 3000 rpm to separate red blood cells from plasma. Using a glass Pasteur pipette, the plasma was removed and transferred to a different tube. Red blood cells were washed three times with an equal volume of isotonic (0.9%) NaCl solution and centrifuged at 3000 rpm for 15 min at room temperature. After removing the last saline wash, tubes containing the red blood cells were added with 2 volumes of water purified with a Milli-Q system (Millipore, Milan, Italy) at 4 °C to obtain cell membrane lysis. Soluble Hb was separated from cell membranes by centrifugation at 10 000 rpm for 30 min at 4 °C and transferred to new tubes [36] after dilution with 2 volumes of water. Hb concentration was adjusted to 15.0 g/dl. Hb was incubated *in vitro* with aqueous solutions of AA at various molecular AA:Hb ratios in a final volume of 1 ml at 37 °C for 18 h at pH 7.4. In these samples, the concentration of AA is 0.775 mM (AA:Hb ratio 1:1) and 7.75 mM (AA:Hb ratio 10:1). An Hb sample not incubated with AA was processed for assessing chromatographic retention times and for mass value reference.

2.3. HPLC purification of α- and β-globin chains

Alkylated globin chains were fractionated by HPLC using a Hewlett-Packard 1100 System (Agilent, Palo Alto, CA, USA) using a reversed-phase Jupiter C4 column (250 mm × 2.0 mm, 5 μm, 300 Å; Phenomenex, St. Torrance, CA, USA) at 0.2 ml/min flow rate. Sample elution was achieved using a gradient from 5 to 70% of solvent B in 60 min. Solvent A was a mixture H_2O/ACN/TFA 80/20/0.1 (v/v/v); solvent B was a mixture H_2O/ACN/TFA 40/60/0.1 (v/v/v). A volume of 150 μl was injected for each chromatographic run. The UV detector was set at 280 nm.

2.4. Nano-electrospray-tandem mass spectrometry (nano-ESI-MS/MS) analysis of AA-modified globins

ESI-MS analysis for the intact globins was performed with a Micromass quadrupole time-of-flight (Q-TOF) Ultima hybrid orthogonal mass spectrometer (Waters, Manchester, UK).

Before nano-ESI-MS analysis, HPLC fractions of globin chains were resuspended in 50 μl of 50/50/0.1 H_2O/ACN/TFA (v/v/v). HPLC-purified globin samples (10 ml, 25–50 pmol) were injected into the ion source at a flow rate of 1 μl/min operating in positive ion mode to run nano-ESI-MS and MS/MS experiments; the spectra were scanned from *m*/*z* 1600 to 600 at 10 s/scan. Mass-scale calibration was carried out by using the multiple charged ions of a separate introduction of myoglobin. In nano-ESI-MS/MS experiments Argon was used as collision gas. The following instrumental conditions were used for MS analysis: number of microscans = 3, length of microscans = 200 ms, capillary temperature = 160 °C, spray voltage = 1.9 kV, capillary voltage = 35 V, tube lens offset = −14 V. The mass spectrometer was tuned using the static nanospray calibrated with a 5-μM solution of angiotensin I (M_r 1296.5). Data were processed using the MassLynx software provided by Waters.

2.5. Tryptic digestion of modified globin chains

The HPLC-purified fractions containing the alkylated globins were freeze-dried before tryptic hydrolysis, which was carried out using sequencing grade trypsin (Sigma) in 0.4% ammonium bicarbonate (Fluka, Milan, Italy) pH 8.5, with a 1:100 E:S ratio at 37 °C for 8 h. The reaction was stopped by the addition of 1 μl of 10% (v/v) formic acid solution.

2.6. LC/ESI-MS and LC/ESI-MS/MS analysis of tryptic peptide mixture

Peptides from tryptic hydrolysis of α- and β-globin chains were analysed by liquid chromatography–ion trap mass spectrometry (LC/ESI-IT-MS). LC was performed using a Hewlett-Packard 1100 System with a reversed-phase Phenomenex C18 column (250 mm × 2.0 mm, 5 μm, 300 Å) with a flow rate of 0.2 ml/min. Solvent A was 0.1% TFA (v/v) in water; solvent B was 0.1% TFA in acetonitrile. After a 5-min hold at 5% solvent B, elution of peptides was performed by a linear gradient raging from 5 to 70% of solvent B in 90 min. The column effluent was monitored at 220 nm.

The LC/ESI-MS analysis was carried out using a LCQ DECA (Thermo, San José, CA, USA) ion trap mass spectrometer, equipped with an electrospray ion source. The ES mass spectra were scanned from *m*/*z* 300–2000 at a scan cycle of 5 s/scan. HPLC flow was directly connected to the electrospray ion source with a heated capillary (280 °C). Data were acquired and processed using the Xcalibur program (version 1.1, Thermo). The cone voltage was 40 V. Data from the tryptic peptide mixtures were first acquired in MS^1 mode in order to define signals to each peptide of interest; these signals were subsequently used as precursor ions for MS/MS experiments.