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Method

Measurement of HNE-protein adducts in human plasma and serum by ELISA—Comparison of two primary antibodies

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ABSTRACT

There is increasing evidence that non-enzymatic post-translational protein modifications might play key roles in various diseases. These protein modifications can be caused by free radicals generated during oxidative stress or by their products generated during lipid peroxidation. 4-Hydroxynonenal (HNE), a major biomarker of oxidative stress and lipid peroxidation, has been recognized as important molecule in pathology as well as in physiology of living organisms. Therefore, its detection and quantification can be considered as valuable tool for evaluating various pathophysiological conditions.

The HNE-protein adduct ELISA is a method to detect HNE bound to proteins, which is considered as the most likely form of HNE occurrence in living systems. Since the earlier described ELISA has been validated for cell lysates and the antibody used for detection of HNE-protein adducts is non-commercial, the aim of this work was to adapt the ELISA to a commercial antibody and to apply it in the analysis of human plasma samples.

After modification and validation of the protocol for both antibodies, samples of two groups were analyzed: apparently healthy obese ($n=62$) and non-obese controls ($n=15$). Although the detected absolute values of HNE–protein adducts were different, depending on the antibody used, both ELISA methods showed significantly higher values of HNE–protein adducts in the obese group.

Introduction

In recent decades evidence has arisen that not only genetic but also epigenetic, mostly stress-related, mechanisms are involved in pathophysiology of aging and age-associated disorders. Among these mechanisms are non-enzymatic post-translational protein modifications which may alter structural and biological properties of proteins in living organisms. These alterations might be recognized as key events in certain diseases like degenerative diseases associated with protein storage. Such modifications of proteins are often influenced by their environment, their structural features, by protein folding states, as well as by free radicals generated in their vicinity. Abundant generation of reactive oxygen species (ROS) causes oxidative stress, which represents an imbalance between production of ROS and their elimination through antioxidative defense mechanisms [1]. ROS can attack all cellular macromolecules of which lipids are particularly susceptible. Since cholesterol esters, phospholipids, and triglycerides all contain polyunsaturated fatty acids (PUFAs) they are subject to free radical attack. Lipid peroxidation is marked by the breakdown of PUFAs yielding oxidation products such as reactive aldehydes of 3–9 carbons length of which 4-hydroxynonenal (HNE), malondialdehyde (MDA) and acrolein (ACR) are the most studied ones. These bioactive compounds can react with all major biomolecules of the cell, thus changing their structure and function and consequently influencing cellular physiology and pathophysiology.

Compared to free radicals, aldehydic products such as MDA and HNE are relatively stable and are able to roam freely and attack molecules, e.g. DNA, proteins, lipids far from the site of their origin. Amongst others, these aldehydic fragments may modify proteins and alter protein function, but are also considered as cytotoxic second messengers of oxidative stress which

Abbreviations: ACR, Acrolein; BSA, Bovine serum albumin; c-Ab, Commercial antibody; Cys, Cysteine; DEPC, Diethyl pyrocarbonate; ELISA, Enzyme-linked immunosorbent assay; HCl, Hydrochloric acid; His, Histidine; HNE, 4-Hydroxy-*trans*-2-nonenal; HPLC, High performance liquid chromatography; HRP, Horseradish peroxidase; KLH, Keyhole limpet hemocyanin; LOD, limit of detection; LOQ, Limit of quantification; Lys, Lysine; MDA, Malondialdehyde; nc-Ab, Non-commercial antibody; PQL, Practical quantitation limit; PUFA, Polyunsaturated fatty acid; ROS, Reactive oxygen species

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makes them highly utilized biomarkers in biological research [2,3]. Therefore, developing new methods for detection and quantification of these compounds in all kinds of biological samples, as well as improving the current ones, is of the utmost importance in the field of research related to oxidative stress.

4-Hydroxynonenal (HNE), a well known biomarker of oxidative stress and lipid peroxidation, is derived from ω-6 PUFAs such as arachidonic acid and linoleic acid and has been recognized as important molecule in pathology, as well as in physiology of living organisms [3–5]. Intracellular HNE reacts rapidly with thiol groups of glutathione and cysteine, with ε-amino groups of lysine, and with histidine residues of proteins [6,7]. Increased concentrations of HNE–modified proteins have been detected in various diseases, among them cancer, atherosclerosis, neurodegenerative disorders, ischemia, inflammation, diabetes, autoimmune diseases, bone diseases and aging [8–13].

As already mentioned, HNE can bind to proteins, forming relatively stable adducts, which are considered as the most likely form of its occurrence in living systems. We have previously reported that these HNE–protein adducts can be measured and quantified by an ELISA method using a mouse monoclonal antibody that specifically recognizes the HNE-histidine epitope [14]. Taking into consideration that ELISA methods require only micrograms of protein, they are suitable for clinical trials where only small amounts of material is available. Because it is becoming of increasing interest to evaluate the level of HNE-protein adducts in different human pathologies as well as in healthy human subjects, our aim was to establish a method that can be widely applied. Up to now, the only reliable and convenient method was the ELISA established for cell lysates by Borovic et al. [14]. To allow other researchers that are not able to use the non-commercial antibody to measure protein adducts of HNE by the ELISA used there, we selected one of the few available monoclonal antibodies against adducts of HNE. By comparing these two antibodies, it allows to compare results obtained with the commercial antibody in the future with those obtained with the non-commercial antibody.

On the other hand, the existing HNE–protein adduct ELISA was originally developed and validated for cell lysates and since the primary antibody used is non-commercial, we wanted to accomplish two goals: (a) to validate and if necessary modify the current protocol to be applicable for testing plasma/serum samples and (b) to compare the original non-commercial antibody (nc-Ab) with a commercial antibody (c-Ab), thus developing an ELISA method that differs only in the primary antibody used and that could be applied in all research laboratories related to lipid peroxidation and oxidative stress research. The here presented method is applicable to serum and plasma samples, when using a non-commercial as well as a commercially available antibody.

Materials and methods

Chemicals of best analytical grade available were purchased from Sigma-Aldrich (Taufkirchen, Germany) or Carl Roth (Karlsruhe, Germany), unless otherwise stated. Distilled water was used throughout for preparation of all solutions.

Primary antibodies used throughout were: commercial Ab (c-Ab): mouse monoclonal anti-4-Hydroxynonenal antibody (clone HNEJ-2; Abcam, Cambridge, UK); and non-commercial Ab (nc-Ab): genuine monoclonal mouse anti-HNE-His antibody in cell culture supernatant (clone HNE 1g4; produced and provided by Dr. Georg Waeg, Karl-Franzen's University in Graz, Institute of Molecular Biosciences, Austria). Secondary antibody was EnVision+ System-HRP Labeled Polymer anti-mouse (Dako, Denmark).

HNE–BSA standard preparation

HNE-dimethylacetal (Enzo Life Sciences) solution was dried under nitrogen flow and dissolved in 1 mM HCl at room temperature (RT) overnight. The concentration of this solution was determined by measuring the absorbance at 223 nm (ε=13,750 M^{-1} cm^{-1} [15]) with water as blank (Shimadzu, UV160-A). Afterwards, the solution was diluted to 1 mM with water.

For preparation of HNE–BSA standards, fatty acid free BSA was diluted in PBS to a protein concentration of 10 mg/ml. The zero standards consisted of fatty acid free BSA (no HNE). The highest standard concentration was prepared by adding different amounts of 1 mM HNE to fatty acid free BSA giving concentrations of 250 pmol/mg (ELISA with nc-Ab) and 5000 pmol/mg (ELISA with c-Ab). Prepared stock solutions were incubated for 3 h at 37 °C or overnight at 4 °C, to achieve complete binding of HNE to BSA, and stored at −20 °C until analysis.

ELISA procedure

This ELISA protocol was adapted from Borovic et al. [14]. Standards were prepared from stock solutions (stored at −20 °C) in concentrations ranging from 0 to 250 pmol/mg (ELISA with nc-Ab) or from 0 to 5000 pmol/mg (ELISA with c-Ab), respectively, by mixing varying proportions of HNE-BSA and fatty acid free BSA while the total protein concentration remained 10 mg/ml. Standards and diluted plasma/serum samples (10 mg/ml), respectively, were diluted 10-fold in 0.05 M carbonate binding buffer (pH 9.6; 0.015 M sodium carbonate, 0.035 M sodium bicarbonate). 100 µl of this mixture were transferred into wells of an ELISA plate (Nunc Immuno Maxisorp, Thermo Scientific) for triplicate analysis. Proteins were adsorbed to wells of the plate overnight at 4 °C. The following day, the plate was washed once with PBS (300 µl). Freshly prepared blocking solution (5% fat free dry milk in carbonate binding buffer) was transferred into wells and the plate was blocked for 2.5 h at RT followed by one washing step (0.1% Tween 20 in PBS). Primary antibody solution in 1% BSA in PBS (nc-Ab 1:100; c-Ab 1:500) was incubated for 2 h at 37 °C. To eliminate sample background values one well of each sample was incubated with 1% BSA in PBS (without primary antibody). After washing the plate seven times, it was incubated for 30 min with peroxidase blocking solution (3% H_2O_2 in PBS) at RT. Then it was again washed seven times. 100 µl of the goat anti-mouse secondary antibody solution in 1% BSA in PBS (1:100; Dako) were transferred into the wells and incubated for 1 h at RT, followed by a washing step (seven times). Freshly prepared TMB substrate solution (0.05 mg/ml) was transferred into the wells and after 30 min at 37 °C the reaction was stopped by adding 50 µl of stopping solution (2 M sulfuric acid). Absorption was read at 450 nm with the reference filter set to 620 nm. All analyses were performed in triplicate or quadruplicate and the amounts of HNE–protein adducts measured by the ELISA are expressed as pmol HNE/mg of proteins.

Analysis of samples

Sixty-two serum samples of morbidly obese candidates for surgical intervention were obtained from the Department of Surgery, University Hospital Mannheim (Germany) on the day before laparoscopic Roux-en-Y gastric bypass surgery. Oxidative stress markers HNE and MDA were compared with those in Na-heparin plasma obtained from 15 healthy controls which were kind gifts from Dr. Ina Bergheim from the University of Hohenheim (Germany). The study was approved by the Ethics Committees of the Institutions and informed consent was obtained from all participants. Blood samples were collected into appropriate tubes and serum or plasma, respectively, was separated from