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Enzyme-linked immunosorbent assay for 4-hydroxynonenal–histidine conjugates

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Abstract
Highly reactive aldehyde 4-hydroxynonenal (HNE) is the final product of lipid peroxidation, known as a second messenger of free radicals and a signaling molecule. It forms protein conjugates involved in pathology of various diseases. To determine cellular HNE–protein conjugates we developed indirect ELISA based on well-known, monoclonal antibody against HNE–histidine (HNE–His) adducts. The method was calibrated using HNE–albumin conjugates as standards ($R^2 = 0.999$) and validated on human osteosarcoma cell cultures (HOS). The ELISA showed good sensitivity (8.1 pmol HNE–His/mg of protein), precision (±8% intra-assay and ±12% inter-assay) and spiking recovery (±9%). The assay revealed 60-fold increase of cellular HNE–His adducts upon copper-induced lipid peroxidation of HOS. The ELISA matched HNE-immunocytochemistry of HNE-treated HOS cells and quantified the increase of cellular HNE–His conjugates in parallel to the decrease of free HNE in culture medium. The ELISA was developed as ELISA Stress for severe lipid peroxidation and ELISA Fine for studies on HNE physiology.

Keywords: *Oxidative stress, protein modification, 4-hydroxynonenal, indirect ELISA*

Abbreviations: *BSA, bovine serum albumin; DAB, 3,3′-diaminobenzidine tetrahydrochloride; ddH_2O, double-distilled water; DMEM, Dulbecco's Modified Eagle's Medium; ELISA, enzyme-linked immunosorbent assay; FCS, fetal calf serum; His, histidine; HNE, 4-hydroxynonenal; HPLC, high performance liquid chromatography; HRP, horseradish peroxidase; MDA, malondialdehyde; PBS, phosphate-buffered saline; PMSF, phenylmethylsulphonylfluoride; ROS, reactive oxygen species; TBARS, thiobarbituric acid reactive substances; TMB, 3,3,5,5-tetramethylbenzidine*

Introduction

During the lipid peroxidation of polyunsaturated fatty acids, α,β-unsaturated aldehydes are generated and involved in the onset and progression of many diseases such as atherosclerosis and related cardiovascular diseases, neurodegeneration, cancer, fibroproliferative disorders, etc. [1–3]. Among these aldehydes, 4-hydroxynonenal (HNE) is one of the most important lipid-derived compounds, generated through the oxidation of ω-6 polyunsaturated fatty acids [4,5]. It is a highly cytotoxic compound, which alters several cellular functions such as membrane integrity and function, mitochondrial respiration, synthesis of DNA, RNA and proteins, etc. [4]. HNE is also a potent mutagenic and carcinogenic agent [6,7]. Despite these effects, HNE is found to be a physiological constituent of various tissues from animal and human origin, while its physiological roles are not yet understood [1,4,8,9]. Relatively high steady state concentrations of HNE in cell membranes were determined as the primary site of its origin (5–10 μM), [10] from where it can diffuse acting as a

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"second messenger" of free radicals [11]. HNE also modulates the expression of several genes, including *c-myc*, *c-fos*, *procollagen type I*, *aldose reductase*, *c-myb*, transforming growth factor *β1* gene, etc. [11,12]. Today it is accepted that HNE acts as a signaling molecule, modulating biological processes such as neutrophil chemotaxis, signal transduction, cell proliferation, differentiation, necrosis and apoptosis [1,2,13–15].

Different methods can be used to quantify the extent of lipid peroxidation. The most used and the simplest method, which does not require expensive equipment, is the assay for malondialdehyde (MDA) using thiobarbituric acid (TBARS). This method is, however, unspecific, and chromatographic (HPLC) analysis with fluorescence detection is necessary to increase the specificity of the method. HPLC analysis will also increase the cost of the assay, but it can be used to measure MDA in plasma and tissue samples as well as in cell cultures [16]. On the other hand, the determination of HNE is very complicated and time consuming. The HPLC method for the determination of free HNE may be used for metabolic studies, where high concentrations of HNE are applied to cell cultures, but the sensitivity of this method does not allow precise HNE determination below 1 μM, which is a usual HNE range in plasma and tissue samples [17]. More complicated methods using derivatization protocols were used for HNE measurements below this concentration, but sample purification does not allow the processing of a high number of samples [18]. Advanced methods for free HNE as well as protein-bound HNE quantification by GC-MS are even more expensive, time consuming and not practical for routine use [19].

Immunoassays take advantage of HNE's high reactivity with proteins to determine amount of HNE–protein conjugates. HNE reacts with amino acid side chains (mostly cysteine, lysine, arginine, proline and histidine), resulting in the generation of antigenic epitopes. Thus, polyclonal and monoclonal antibodies were developed to prove the presence of HNE-modified proteins, especially in tissue sections by immunohistochemistry [20–22]. The advantage of immunohistochemistry is to demonstrate the distribution of HNE–protein conjugates in tissues and distinguish HNE-positive from HNE-negative cells, but the quantity of the HNE–protein conjugates remains uncertain and can be estimated only semi-quantitatively. Another way to demonstrate the amounts of HNE–protein conjugates is to use Dot-blot or Western-blot methods, which are also semi-quantitative immunochemical methods. If used with a scanning system, these methods are better for HNE–protein conjugate quantification, but still, they are not precise, standardized methods and offer only approximate quantification [21].

We present here the ELISA assay for HNE–protein conjugate determination in cell cultures, which allows the determination of HNE–histidine (HNE–His) conjugates expressed as nmol HNE–His/mg of protein. The antibody we used was developed for HNE–His conjugates [23] and is already well known and often used in immuno-cyto and -histochemistry of various human and animal cell and tissue samples [15,22,24–32].

Materials and methods

Materials

The materials were as follows: acetic acid (Kemika, Croatia), acetonitrile (Merck, Germany), BSA (Sigma, USA), $CaCl_2$ (Kemika), $CuSO_4$ (Kemika), DAB (DAKO, Denmark), DMEM (Sigma), fat-free dry milk (BioRad, USA), FCS (Sigma), flat ELISA plate (high binding, Costar), H_2O_2 (Kemika), H_2SO_4 (Kemika), HNE–dimethylacetal (Alexis, Switzerland), KH_2PO_4 (Kemika), $MgCl_2$ (Kemika), $MgSO_4$ (Kemika), microwell plate, 96-well (TPP, Switzerland), NaCl (Kemika), NaN_3 (Kemika), $NaHCO_3$ (Kemika), NaH_2PO_4 (Kemika), Na_2HPO_4 (Kemika), PMSF (Sigma), sodium deoxycholate (Sigma), SDS (Sigma), TMB (Sigma), Triton X-100 (Sigma), trypsin (Sigma) Tween 20 (Merck), genuine monoclonal mouse anti-HNE-His antibody (produced and provided by Georg Waeg, Karl-Franzen's University in Graz, Institute of Molecular Biosciences, Austria), anti-mouse HRP-linked IgG (DAKO). All the reagents used were prepared in double-distilled water. Phosphate-buffered saline, pH 7.4, contained 8.08 mM, Na_2HPO_4; 17.7 mM, KH_2PO_4; 136.7 mM, NaCl; 170 μM, $CaCl_2$; 123 μM, $MgCl_2$; 2.68 mM KCl. Krebs-Heneseleit buffer, pH 7.4, contained 25 mM, $NaHCO_3$; 118 mM, NaCl; 4.7 mM, KCl; 1.2 mM, $MgSO_4$; 1.2 mM, NaH_2PO_4 and 1.2 mM, $CaCl_2$.

Determination of free HNE by HPLC method

HNE standards were prepared by serial dilution from HNE stock solution stored at −20°C. Serum-free cell culture samples were stored at −80°C prior to analysis. After thawing, the samples were mixed and analyzed by HPLC as already described [33]. The samples (20 μl) were injected into the HPLC system, which consisted of a Beckman System Gold Solvent module 128 (Beckman, Germany), a UV Detector (Beckman) and a Midas Spark Holland autosampler (Spark Holland, Netherlands). The mobile phase consisted of 42% (v/v) acetonitrile (Merck). The flow was set to 1 ml/min and the absorbance at 223 nm. The samples were analyzed on a Beckman Ultrasphere ODS, 5 μm, 4.6 × 150 mm column (Beckman).

Human osteosarcoma cell cultures

The human osteosarcoma (HOS) cell line HOS was obtained from the American Type Culture Collection (ATCC). Cells were maintained in DMEM with 10% (v/v) FCS in an incubator (Heraeus, Germany) at 37°C, with a humid air atmosphere containing 5% CO_2. The cells were detached from semiconfluent cultures with a 0.25% (w/v) trypsin solution for 5 min. Viable cells (upon trypan blue exclusion) were counted on a Bürker-Türk haemocytometer and used for experiments.

Treatment of HOS cells with HNE for ELISA sensitivity testing

Cells were seeded in serum free DMEM in sterile glass tubes at 2×10^5 cells/ml. HNE was added to final concentrations in the range of 0–100 μM, i.e. 0–50 nmol/10^5 cells. After 1 h of incubation the cells were washed twice with 1 ml of PBS. For the ELISA experiment, cell pellets were frozen immediately and kept at −20°C until analysis.

In parallel, the cells were also seeded and treated with HNE in the same way for cell viability evaluation. After an hour of incubation, cells were centrifuged for 5 min at 1100 rpm (Beckman), resuspended in trypan blue and the number of damaged, blue cells was determined as a percentage of total cells for triplicates of cultures.

Treatment of HOS cells with HNE for immunocytochemistry

The cells were seeded in a 1% FCS-supplemented DMEM in 96-well microwell plates at 5×10^4 cells/well and incubated overnight. The medium was replaced with fresh 250 μl serum free DMEM containing HNE as in the treatment for the ELISA experiment: in the range of 0–100 μM, i.e. 0–50 nmol/10^5 cells. After a 1 h incubation the cells were washed twice with PBS and were stored in 10%-buffered formalin until analysis [29].

HNE elimination and binding in HOS cell cultures

Cells were washed twice with sterile Krebs-Heneseleit buffer and placed as 10^6 cells/ml buffer into sterile glass tubes. HNE was added into cell samples to reach a final concentration of 20 μM (2 nmol/10^5 cells) and the cells were incubated in an incubator (Heraeus, Germany) at 37°C, with a humid air atmosphere containing 5% CO_2. At different time points (15, 30, 60, 90 and 120 min), samples were taken out of the incubator and centrifuged. The supernatants were mixed with an equal volume of acetonitrile/acetic acid (24:1 v:v), centrifuged and the supernatants were further stored at −80°C for HPLC analysis of free HNE. Cell pellets were washed twice with PBS and treated with acetonitrile/acetic acid as described above. The samples were centrifuged and the supernatants were also stored at −80°C for the determination of free HNE.

For the HNE-binding studies, a corresponding set of HNE-treated HOS cell samples was prepared. Samples were centrifuged, the supernatants were discarded and the cell pellets were washed twice with PBS to be stored at −80°C for the ELISA.

Cell viability was determined after HNE-treatment by trypan blue exclusion assay.

Induction of HNE in HOS cell cultures

The method for HNE induction during lipid peroxidation of cells was used according to a similar protocol for the induction of MDA in cell cultures [34]. HOS cells (1×10^6) were incubated in Krebs-Heneseleit buffer in the presence of 50 μM $CuSO_4$ in a final volume of 1 ml in an incubator at 37°C for 3 h. Cells were then centrifuged, washed once with Krebs-Heneseleit buffer, immediately frozen and kept at −20°C until analysis. Cell pellets were used to test the presence of HNE–protein conjugates in cell cultures.

For the viability study, a corresponding set of $CuSO_4$-treated HOS cell samples was prepared. Samples were centrifuged, the supernatants were discarded and the cell pellets were dispersed in trypan blue. The number of trypan-positive, i.e. damaged cells was counted and expressed in percentage of the total cell count.

Cell lysates

Cell pellets were lysed with 400 μl of lysis buffer: 0.6055 g, TRIS; 0.8766 g, NaCl in 100 ml, ddH_2O; pH 7.5 with 1% (v/v) Triton X-100, 2% (w/v) sodium deoxycholate and 2% (w/v) SDS; and immediately before use, PMSF was added to reach final concentration of 1 mM/10^6 cells. All experiments were thus standardized so that 20 μl of cell lysate corresponded to 5×10^4 cells—the volume of sample, which was used in ELISA assay.

After addition of lysis buffer to the cell pellets, samples were left for 60 min at 4°C and vortexed every 10 min. Samples were immediately analyzed by the ELISA.

The stability of cell lysate samples was additionally tested with respect to lysis duration of 30, 60 and 90 min and by repeated freeze–thaw cycles. Repeated freeze–thaw cycles were carried out in the following manner: for each freeze–thaw cycle samples were taken from the freezer, left at room temperature for about 10 min, vortexed and frozen again.

HNE-histidine antibody

The same monoclonal antibody was used for both immunocytochemistry and the ELISA [22,29].

The monoclonal antibody was obtained from the culture medium of the clone derived from a fusion of Sp2–Ag8 myeloma cells with B-cells of a BALBc mouse immunized with HNE-modified keyhole limpet hemocyanine. The antibody is specific for the HNE-His epitope in HNE-protein (peptide) conjugates [23].

Immunocytochemistry

The immunostaining of HNE-His conjugates was performed on HOS cells as described before [29]. The primary antibody against HNE-His conjugates was added and the possible endogenous peroxidase activity of samples was blocked with 1.5% (v/v) H_2O_2, 0.1% (w/v) NaN_3 and 2% (w/v) BSA. For the immunocytochemical detection of HNE-adducts the immunoperoxidase technique was used, with secondary rabbit-anti-mouse antibody applying DAB as chromogen. The immunostaining intensity was evaluated by the use of SFORM image analysis software (Vamstec, Croatia). The staining intensity was analyzed for ten cell areas per culture by optical densitometry. The significance was calculated according to the Student's *t*-test, with respect to control cells.

ELISA design

The amounts of HNE–protein conjugates determined by the ELISA are expressed as nmol HNE–His/mg of proteins. Because the very broad range of concentrations could be expected *in vitro* and *in vivo* in different types of cells under physiological and pathological conditions, it is impossible to analyze all samples with one set of standards. The procedure was, therefore, developed for two assays, denoted as ELISA Stress to determine amounts of HNE–His conjugates in severe oxidative stress, which generates cytotoxic amounts of HNE–His adducts, and ELISA Fine, which could be useful for HNE physiology studies.

ELISA standards

BSA was dissolved in ddH_2O for 24 h by magnetic stirring. HNE was prepared from HNE–dimethylacetal by addition of 1 mM HCl and an incubation of 1 h at RT. The HNE concentration was determined spectrophotometrically (DU-70 Spectrophotometer, Beckman) by measuring the HNE maximal absorbance at 223 nm ($\epsilon = 13750\,M^{-1}\,cm^{-1}$) [4]. HNE was diluted to the desired concentrations and mixed with BSA solutions for 24 h at 4°C. Aliquots of standards were then stored at −20°C.

ELISA procedure

Carbonate binding buffer, pH 9.6, (200 μl) was added into the wells followed by 20 μl of standards or samples. The plate was incubated overnight at 4°C and then washed once with 400 μl H_2O. The blocking solution consisting of 5% (w/v) fat-free dry milk (400 μl) was added and the plate incubated for 3 h at room temperature and then washed once with 400 μl washing buffer (0.5 ml Tween 20/l of PBS). The anti-HNE-His antibody (200 μl) was added and incubated for 2 h at room temperature and then washed seven times with 400 μl washing buffer. The peroxidase-blocking solution containing 0.1% NaN_3 and 1.5% H_2O_2 (400 μl) was added and incubated for 30 min at room temperature and then washed seven times with 400 μl washing buffer. The secondary antibody (200 μl) was added and incubated for 1 h at room temperature and then washed seven times with 400 μl washing buffer. Two hundred microliter of TMB substrate (0.1 mg/ml) was added and the reaction was stopped after 5–10 min (ELISA Stress) or 30–60 min (ELISA Fine) by adding 2 M 50 μl H_2SO_4. The absorbance was read at 450 nm with the reference filter set to 620 nm.

Accuracy of the ELISA for spiked cell lysate samples

The accuracy of the ELISA for spiked cell lysate samples was done by addition of different doses of HNE to the control, HNE-free cell lysates, which were afterwards processed as the other test samples. The accuracy was estimated by comparison of the expected, calculated values of the HNE–His adducts and those determined by the ELISA using the concentrations of HNE that match both ELISA Fine and ELISA Stress.

Statistical analysis

The results obtained in the experiments performed are expressed as nmol of HNE-His/mg of proteins or nmol of HNE/10^5 cells. All assays were carried out in triplicates. The comparison of the mean values was done using Student's *t*-test considering values of $p < 0.05$ as significantly different.

Results

Standard curves

The standard curves of both ELISAs are shown in Figure 1. The linear range of the standard curve gave a correlation of 0.999 for both assays. The ELISA Stress detection range was 0–1.714 nmol HNE-His/mg of protein, and the ELISA Fine range was 0–0.143 nmol HNE-His/mg of protein. In both assays zero standards were very good, i.e. 0.031 absorbance units for Stress and 0.075 for ELISA Fine. Although the background values originating from the unspecific binding of secondary antibody are minimal, a parallel "background" ELISA plate was run without the secondary

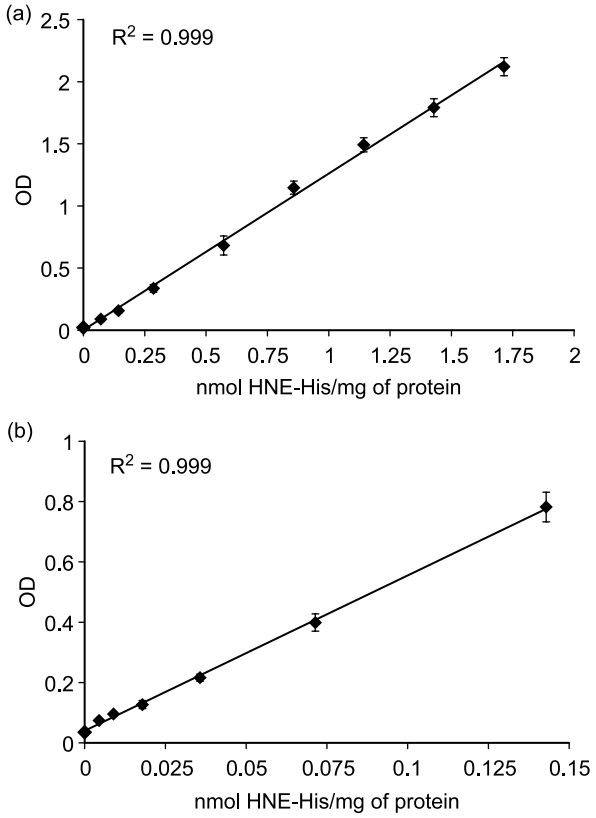

Figure 1. Standard curves for HNE-His ELISA. (a) ELISA Stress standard curve; and (b) ELISA Fine standard curve. Results were expressed as mean values ± SD ($n = 3$).

antibody and the values obtained, for ELISA Stress 0.010, and for ELISA Fine 0.040 absorbance units, were subtracted from measured values.

Analysis of HNE binding in cell cultures

Although background values were negligible in standards, the unspecific binding of secondary antibody in cell samples gave elevated background values. It was, therefore, important to run a "background" plate without the secondary antibody to increase the assay specificity. These values were then subtracted from the measured values (0.024 for ELISA Stress and 0.093 absorbance units for ELISA Fine).

To determine the applicability of the method in experimental conditions, HOS cells were treated with different HNE concentrations. For easier interpretation and comparison, HNE concentrations used to treat the cells were calculated as nmol of HNE/10^5 cells. Results are presented in Figure 2. ELISA Fine (Figure 2b) was able to determine HNE-His conjugates when the cells were treated with less than 3 nmol HNE/10^5 cells. Results showed a linear correlation between cellular HNE–His adducts and concentrations of exogenous free HNE used to treat the cells ($R^2 = 0.99$). When the cells were treated with 0.5 nmol/10^5 cells, an increase in HNE–His conjugates was observed in cell cultures ($p < 0.05$). Lower concentrations of HNE did not generate measurable amounts of HNE-His conjugate. Cell viability was not affected during this period of time (Figure 2b). If the cells were treated with concentrations of HNE higher than 3 nmol HNE/10^5 cells, it was necessary to use the ELISA Stress assay (Figure 2a), which gave a logarithmic correlation ($R^2 = 0.99$) between the treatment concentration and HNE–His conjugate when cells were treated with 3–20 nmol/10^5 cells. If HOS cells were treated with higher amounts of HNE there was no further increase in HNE–His conjugate creation and saturation was observed. Cell viability gradually declined with increased HNE concentration (Figure 2a, $p < 0.05$ for concentrations above 4 nmol HNE/10^5 cells).

The same HNE-treatment was applied to cells attached to the surface of a multiwell plate to compare the ELISA sensitivity with determination of HNE–His conjugate by immunocytochemistry; the results are presented in Figures 2c,d and 3. A similar HNE-concentration dependence of the HNE–His adducts was also detected by immunocytochemistry, while the ELISA was more effective in the detection of HNE–His conjugates when low concentrations of HNE were added to the cells (below 3 nmol HNE/10^5 cells).

HNE–His adducts could not be seen by immunocytochemistry in control HOS cell cultures or in those treated with < 1 nmol HNE/10^5 cells. The dose of 1–3 nmol HNE/10^5 cells resulted in slight to moderate immunopositivity of HOS cells (significant raise of OD for 2–4-fold, $p < 0.05$), but the typical morphology of these mesenchymal cells, polygonal with two or three prominent extensions, was not changed. The doses of 1–3 nmol HNE/10^5 cells correspond to the values of 0.062–0.177 nmol HNE-His/mg of protein as determined by the ELISA.

Treatment with 4 or 5 nmol HNE/10^5 cells, which corresponds to 0.321 or 0.531 nmol HNE-His/mg of protein, caused not only strong intensity of the HNE–His immunopositivity (OD raise of 5-fold, $p < 0.001$) but also the altered morphology of the cells. The cells were becoming oval, losing cytoplasmatic extensions and polygonal shape. These signs of cytotoxicity of HNE were even more obvious if the cells were treated with 10 nmol HNE/10^5 cells, which corresponds to 1.262 nmol HNE–His/mg of protein. The cells were mostly picnotic, darkly stained (OD raise of 8-fold) due to strong immunopositivity and mostly aggregated in case of colonies, while individual cells or polygonal cells could hardly be noticed.

If the cells were exposed to 20 nmol HNE/10^5 cells or higher doses of the aldehyde, corresponding to the 1.814 nmol HNE–His/mg of protein and more, only picnotic, darkly stained cells were found (giving a maximal OD raise of 8–9-fold), together with the cellular debris remaining after necrotic cells

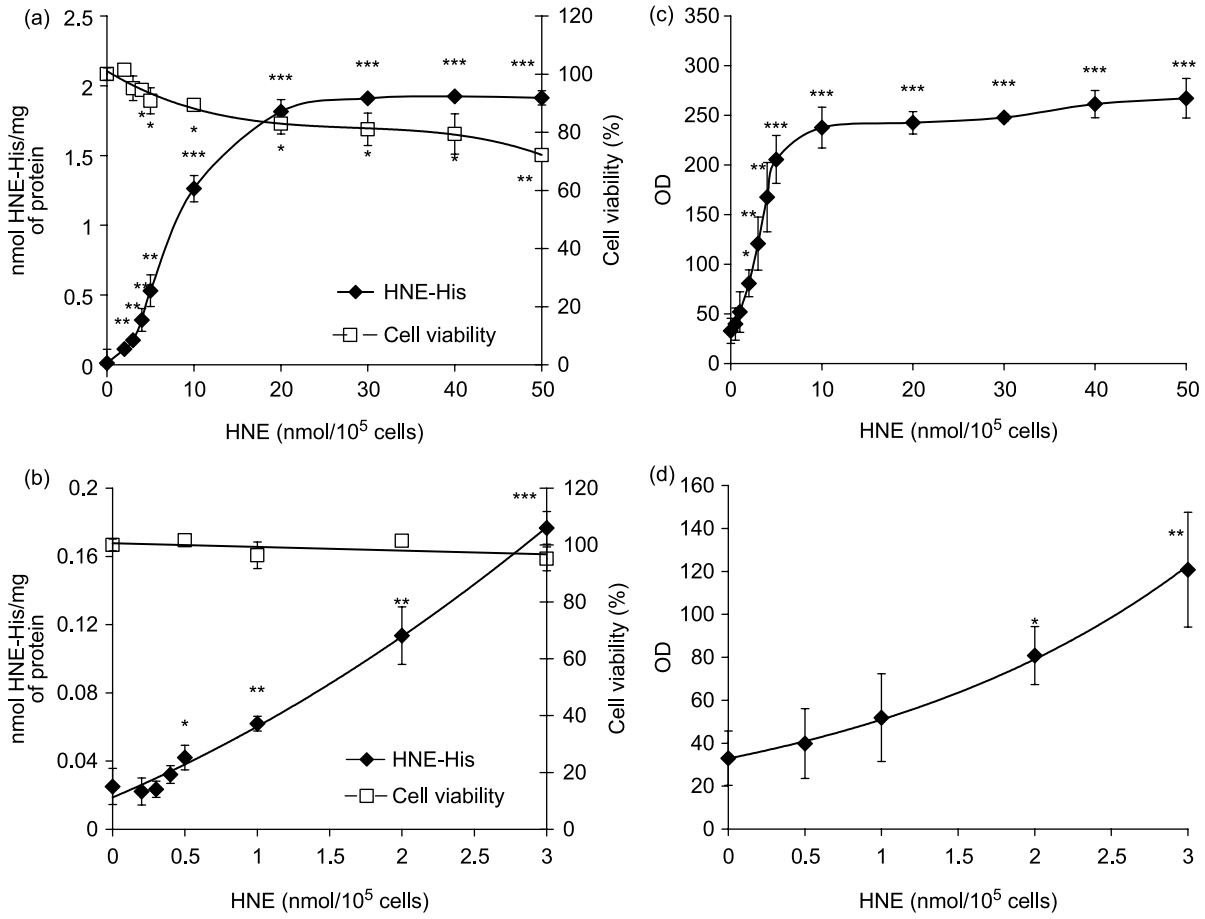

Figure 2. Comparison of cell viability (a, b), the amount of HNE-His conjugates (a, b) measured by ELISA and immunocytochemistry quantification by image analysis (c, d) in HOS cell cultures. Cells were treated with HNE concentrations from 0 to 50 nmol/10^5 cells for 1 h. An increase in HNE-His conjugates was observed by both ELISA and immunocytochemistry followed by a gradual decrease of cell viability. Results were expressed as mean values ± SD ($n = 3$). The comparison of mean values was done using Student's t-test (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).

(mostly pronounced as randomly spread small, dark particles in case of 50 nmol HNE/10^5 cells). On the remaining picnotic and strongly immunopositive cells typical membrane "blebs" were often noticed as a consequence of lipid peroxidation and cell destruction.

Induction of HNE in cell cultures

To determine whether it is possible to induce HNE–His conjugates in cell cultures, HOS cells were treated with 50 μM $CuSO_4$. Results are presented at Figure 4.

In the control HOS cell cultures the amounts of HNE-His adducts was in the range of 0.012 nmol HNE–His/mg of protein. Treatment with 50 μM $CuSO_4$ for 3 h induced 0.73 nmol HNE–His/mg of protein, which is equivalent to cell treatment with 6.2 nmol HNE/10^5 cells for 1 h. This increase corresponded to a 60-fold increase of HNE–His conjugates above control values. The cell viability was significantly lower in the presence of $CuSO_4$ ($p < 0.001$).

Analysis of HNE elimination and binding kinetics

HOS cells treated with 2 nmol HNE/10^5 cells retained their viability during 120 min of incubation (Figure 5c). The concentration of free HNE was analyzed by HPLC (Figure 5a). When HNE was added to the cell cultures, the concentration of the aldehyde gradually decreased during 120 min, reaching $t_{/2}$ after 54 min. After 24 h of incubation, all HNE was removed from cell cultures (data not presented). The concentration of free HNE in the cells remained below the limit of detection (50 pmol), while the concentration of HNE in buffer alone was stable during the 120 min of experiment. The elimination of free HNE from cell culture medium was associated with the gradual increase in HNE–His conjugates in the cells determined by the ELISA as presented in Figure 5b.

Validation of the ELISA

The lower limit of detection of the ELISA determined by analyzing 20 zero standards and adding two SD was

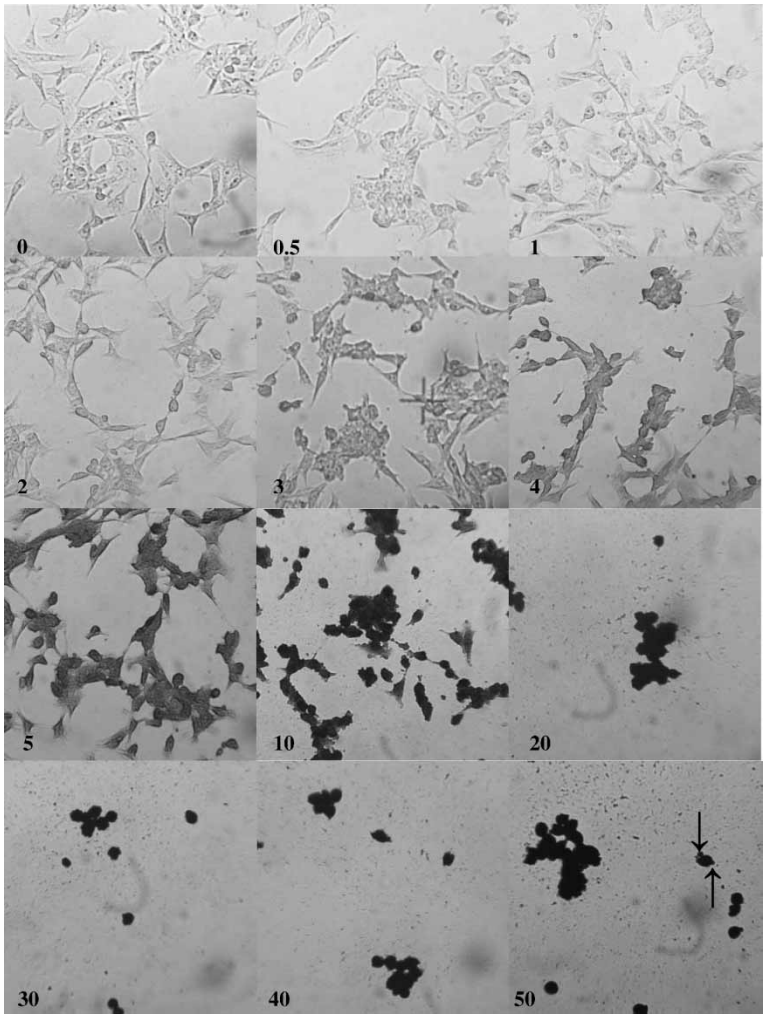

Figure 3. HNE-protein adducts determined by immunocytochemistry in HOS cell cultures after treatment with HNE (300x). Concentrations of HNE used to treat the cells were expressed in nmol/10^5 cells and indicated on the photographs. The rise of HNE-His immunopositivity corresponded to the doses of the aldehyde used and was associated with the change of cellular morphology. In the case of 20 nmol HNE/10^5 cells and above the remaining cells were picnotic and strongly immunopositive showing typical membrane "blebs" as a consequence of lipid peroxidation and cell destruction (indicated by arrows).

8.1 pmol HNE–His/mg of protein. To analyze the precision of the ELISA, the coefficients of variation (%CV) of intra and inter-assay were determined. The intra-assay %CV of samples analyzed in triplicate was 8.3 and 7.3%, while that of inter-assay was 11.2 and 12.0% for ELISA Stress and ELISA Fine, respectively.

The recovery percentage of HNE-spiked cell lysate samples was in the range of ±9% for all samples analyzed as shown in Table I. Observed tendency of the slight increase of the HNE-His values in the spiked samples was not significant (for all, $p > 0.05$). Because this is the most important validation test, we believe that both ELISA Fine and ELISA Stress could be used to determine amounts of HNE–His adducts in biological samples similar to the HOS cell lysates we used.

The stability of cell lysate samples was additionally tested with respect to lysis duration and repeated freeze–thaw cycles (Table II). Shorten lysis of the cells for 30 instead of 60 min as well as extended lysis of the cells for 90 resulted in a 10–20% decrease of HNE-His adducts ($p < 0.05$ for all except ELISA Fine 30 min). Thus, the suggested 60-min protocol was validated as optimal.

Stability of HNE-His adducts in the cell lysates was remarkable in case of freeze–thaw treatment. Namely, after the first freeze–thaw cycle identical results to control samples were obtained ($p > 0.1$). After the second freeze–thaw cycle a slight decrease in HNE–His values obtained was observed for the ELISA Fine (7–20%, $p < 0.05$), but not for the ELISA Stress ($p > 0.1$).

Discussion

Proteins are essential macromolecules, whose unique biological functions can be disrupted during oxidative

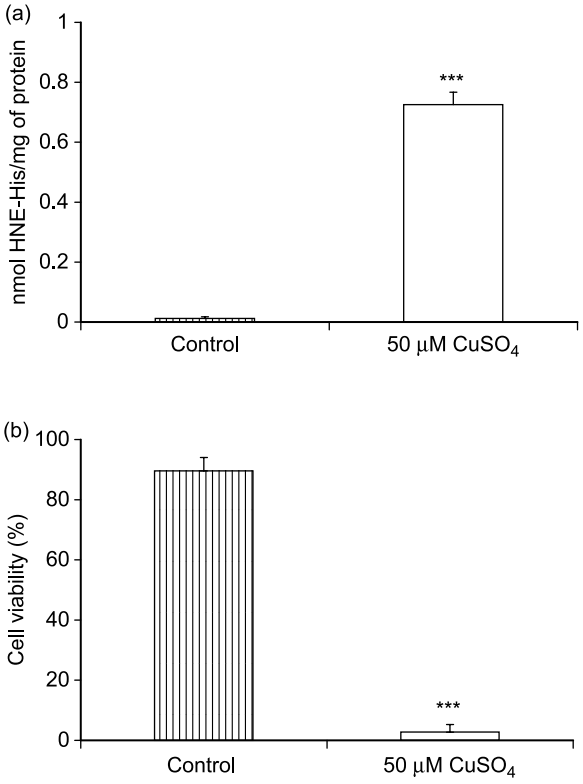

Figure 4. HNE-His conjugates measured by ELISA (a) and cell viability measured by trypan blue exclusion (b) in HOS cell cultures after treatment with $CuSO_4$. Results were expressed as mean values ± SD ($n = 3$). The comparison of mean values was done using Student's t-test considering values of $p < 0.05$ as significantly different (***$p < 0.001$).

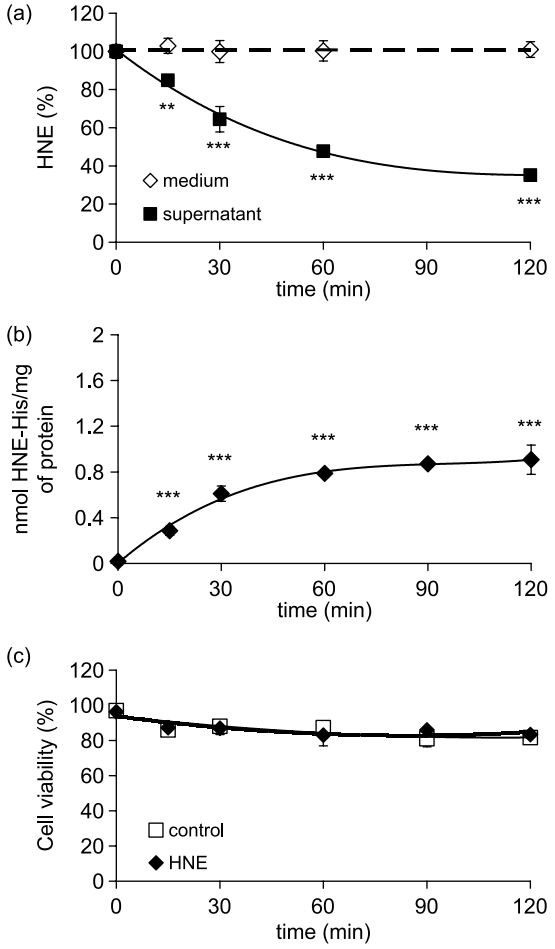

Figure 5. HNE elimination, binding kinetics and cell viability determined in HOS cell cultures. Cells were treated with 2 nmol HNE/10^5 cells. Free HNE was measured by HPLC in cell free media and cell culture supernatants (a), while HNE-His conjugates were measured by ELISA in control and HNE-treated HOS cells (b). The viability of control and HNE treated cells was counted as a percentage of viable cells upon trypan blue exclusion (c). Results were expressed as mean values ± SD ($n = 3$). The comparison of mean values was done using Student's t-test considering values of $p < 0.05$ as significantly different (**$p < 0.01$; ***$p < 0.001$).

stress [35]. Oxidative modifications have been shown to inhibit a wide array of enzymatic activities, [35,36] while oxidative modification of low-density lipoproteins (LDL) has been found in atherosclerotic plaques indicating that the oxidation of LDL plays an important role in the pathology of atherosclerosis and cardiovascular disorders [37,38]. The presence of oxidized proteins has been demonstrated in numerous other diseases as well as in aging [39] and their incomplete removal and accumulation is thought to be a cause of the functional breakdown of affected proteins and cells [40,41].

Products of protein oxidation are relatively stable and could therefore be detected as potential markers of oxidative stress. The most commonly measured products of protein oxidation in biological samples are protein carbonyls [42]. Sensitive ELISA methods for protein carbonyl measurements are available [43,44]. The introduction of carbonyls through adduction of a lipid peroxidation product such as HNE [45] will give a positive result in a carbonyl assay. However, the amount of HNE-induced increase in protein carbonyls will be overestimated as these assays measure any carbonyl in a protein, regardless of the source. The presence of HNE modified Lys, His and Cys amino acid residues in proteins can be detected with specific antibodies, thus increasing specificity of the HNE determination [45,46]. The antibody we used is highly specific to the HNE–His epitope in HNE–protein conjugates. HNE–Lys and HNE–Cys give 5 and 4% cross-reactivity with the antibody [23]. The antibody

Table I. Accuracy of the ELISA for spiked cell lysate samples.

Expected values⋆	Measured values⋆	Recovery (%)
0.453†	0.478	106
0.241†	0.261	109
0.149‡	0.159	106
0.084‡	0.079	104

⋆values are expressed as nmol HNE-His/mg of protein.†values analysed by ELISA Stress.‡values analysed by ELISA Fine.

Table II. Accuracy of the ELISA in dependence of lysis duration or freeze-thaw treatment of the cell lysates.

Shortened—30 min lysis⋆	Extended—90 min lysis⋆	One freeze-thaw treatment⋆	Two freeze–thaw treatments⋆
87.53 ± 3.70†	92.79 ± 2.46†	100.50 ± 10.69†	96.27 ± 4.14†
100.77 ± 29.53‡	79.58 ± 9.34‡	102.20 ± 7.24‡	80.21 ± 8.95‡

⋆ values are expressed as percent of the respective control values, i.e the values used for ELISA as described in Materials and Methods.† values analysed by ELISA Stress.‡ values analysed by ELISA Fine.

was previously verified for cross-reactivity with several other aldehydes and it was found to have about 0.1% cross-reactivity with MDA–BSA and other possibly related aldehyde-albumin conjugates [23]. The use of anti-HNE antibodies is widely applied both in immunohistochemistry and immunoblotting analyses and revealed the involvement of HNE in several pathophysiological conditions such as Alzheimer's disease and other CNS and degenerative diseases, traumatic injuries, immune disorders, liver diseases, cancer, etc. [22,25,47–50]. Immunohistochemsitry of HNE–protein adducts in various diseases together with biological effects of the aldehyde make HNE one of the most attractive bioactive markers of oxidative stress [1–3,51]. Although, essential evidence of the presence of HNE in various tissues is given by the immunohistochemistry, in particular the morphological distribution of HNE-protein adducts, this approach does not allow quantitative measurement of these derivatives in biological samples. The use of HNE–His ELISA can, therefore, be considered as a complementary, quantitative method to the immunohistochemistry of the HNE-protein adducts. Recently, the novel ELISA was developed for a 1,4-dihydroxynonane-mercapturic acid, which is a urinary metabolite of HNE [52]. This assay represents a novel approach to study a particular HNE metabolite as a marker of lipid peroxidation, but it does not detect HNE or its protein adducts. Therefore, the HNE-His ELISA may also be used as a complementary method to the 1,4-dihydroxynonane-mercapturic acid ELISA and to the protein carbonyl ELISA.

The use of a well-known monoclonal antibody directed to HNE–His conjugate that was proven in immunohistochemical studies of human and animal samples is an advantage of the ELISA method we used. Met and Cys are the two amino acid residues that are the most susceptible to oxidative attack, but the modification of these residues may not always damage protein functions [53] as they can serve as internal scavengers which protect critical amino acid residues from oxidative attack [54]. Similarly, HNE is highly reactive with thiol groups of proteins, [4] and the reaction of HNE with peptide glutathione (GSH) is a well-known HNE-detoxification pathway [55,56]. The antibody used here was found to be highly selective for HNE–His and its cross-reactivity with HNE-Lys or Cys was negligible [23,57].

The analytical range of the ELISA assay was designed by taking into account possible pathophysiological concentrations of HNE that could be found *in vitro* and *in vivo*. To test the assay's applicability, the model of human osteosarcoma HOS cell line was chosen because it is a reliable model of human malignant cells of mesenchymal (bone) origin on which the effects of HNE were recently well described [58]. Therefore, it is of high importance that excellent correlation was observed between results obtained by immunocytochemistry and ELISA of HNE-treated HOS cells. The ELISA appeared to be superior to the immunocytochemistry of HOS cells not only because it gave a possibility to quantify HNE-protein conjugates, but also as it had a broader limits of detection than immunocytochemistry.

The amount of HNE-His conjugates and free HNE detected in the culture medium will depend upon experimental conditions, in particular if low concentrations of the aldehyde are used or if the medium contains serum. However, not all HNE molecules will react with amino acids and proteins (released by cells) in medium. If present in medium, proteins will decrease interaction of HNE with intracellular proteins [29] and attenuate cytotoxicity of the aldehyde. Previous studies led to interpretation of HNE as a growth regulating substance that acts interfering with serum growth factors and cytokines [2,12,14,28]. It is also possible that under most circumstances *in vivo* HNE is also distributed rather in the form of adduct with proteins than as free HNE.

In spite of the ELISA's high sensitivity, when concentrations of HNE used for the treatment of HOS cells were lower than 0.5 nmol/10^5 cells, the amount of HNE–His conjugates was undetectable. Low concentrations of HNE were thus unable to influence cell viability. We suppose that such low amounts of HNE, at least for HOS cells cultured under serum-free conditions, could not modify cellular proteins, but are either metabolized, removed by glutathione or utilized in dynamic processes of cell signaling and growth regulation [2,8,11,58].

On the other hand, a linear correlation between protein-bound HNE and concentrations of HNE used to treat the cells was present when HOS cells were treated with 0.5–3 nmol HNE/10^5 cells. The logarithmic correlation between the HNE concentration used for cell treatment and HNE–His conjugates

in the cells was present when HOS cells were treated with 3–20 nmol/10^5 cells. We previously found that supraphysiological concentration of HNE (10 μM; or 10 nmol/10^5 cells) causes growth and differentiation inhibition and induces apoptosis in HOS cell cultures, while lower concentrations were able to only slightly stimulate apoptosis [58]. Higher concentrations of HNE, from 20 nmol of HNE/10^5 cells and higher, are known to cause a breakdown of cellular detoxifying mechanisms and are cytotoxic [4]. High doses of the aldehyde would thus certainly generate maximal amounts of HNE-His conjugates, but with possible artifacts due to the acute necrosis associated with oxidative damage of the cellular proteins. We found that the 2 nmol HNE/10^5 cells concentration of HNE used for the elimination and binding study did not influence cell viability during short-term treatments. Although this concentration was non-toxic, 10^5 cells did not easily metabolize 2 nmol of HNE as they needed one day of incubation to entirely metabolize the free aldehyde (data not shown).

HNE-His conjugates were formed in HOS cells reaching saturation at 0.9 nmol/mg of protein. When taking into account the initial concentration of HNE and the measured concentration of HNE-His conjugates, it appears that approximately 4.5% of HNE was bound as HNE-protein conjugates and that 35% was not metabolized during a 2 h incubation. In other metabolic studies, lower amounts of HNE were used causing a faster and more complete HNE elimination, mostly by glutathione conjugation [59,60]. Our findings are in accordance with other studies showing HNE binding to cellular proteins between 1 and 8.5% [61].

It should be said also that the stability of thus obtained HNE-His adducts is very good, at least if the cell lysates are prepared according to the protocol described. Namely, shorten or extended lysis of the cells caused slight reduction of the HNE-His adducts values. These could be explained as a consequence of insufficient cell lysis during the 30-min treatment, while after 90 min due to the detergents present in the lysis buffer destruction of the macromolecules and consequential reduction of HNE-His epitopes could occur. Thus, the chosen protocol of 60-min lysis was additionally validated. As in case of extended lysis, we assume that repeated freeze–thaw treatments reduced HNE-His values due to denaturation of the macromolecules and consequential reduction of HNE-His epitopes. In fact, in the cell lysates HNE-His adducts were found as very stable to the freeze–thaw treatment and we believe that this is at least in part due to the reliable lysis protocol used, i.e. optimal dispersion of the macromolecules in the lysates. In favor of this are findings of the most important validation test that has proven accuracy of the ELISA for spiked cell lysate samples.

Complementary to the treatment of the cells with exogenous HNE, it is convenient to induce the production of HNE-protein conjugates *in vitro* and *in vivo* by various LPO inducers, such as cadmium [62], copper and iron [63]. We treated HOS cell cultures with copper, and obtained a 60-fold increase of HNE-His conjugates above control values. This proved the applicability of our model for HNE-His conjugates analysis with this ELISA.

Hence, according to the amounts of HNE-His adducts determined, the ELISA was developed eventually into two assays; HNE-His ELISA Stress for values from 0 to 1.714 nmol HNE-His/mg and HNE-His ELISA Fine for values from 0 to 0.143 nmol HNE-His/mg of proteins. From the obtained results, we conclude that the ELISA Stress should cover the oxidative stress-induced increase in HNE-His (protein) conjugates, which will affect normal cell function and viability, while ELISA Fine should cover concentrations that will not influence cell viability.

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