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Exposure of HL-60 human leukemic cells to 4-hydroxynonenal promotes the formation of adduct(s) with α-enolase devoid of plasminogen binding activity.

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ABSTRACT

4-Hydroxynonenal (HNE), the major product of lipoperoxidation, easily reacts with proteins through adduct formation between its three main functional groups and lysyl, histidyl, and cysteinyl residues of proteins. HNE is considered to be an ultimate mediator of toxic effects elicited by oxidative stress. It can be detected in several patho-physiological conditions, in which it affects cellular processes by addition to functional proteins. We demonstrated by mass spectrometry and confirmed by immunoblotting experiments the formation of HNE-α-enolase adduct(s) in HL-60 human leukemic cells. α-Enolase is a multifunctional protein that acts as a glycolytic enzyme, transcription factor (c-myc binding protein, MBP-1) and plasminogen receptor. HNE did not affect α-enolase enzymatic activity, expression, intracellular localization and did not change the expression and localization of MBP-1 either. Confocal and electronic microscopy results confirmed the plasmamembrane, cytosolic and nuclear localization of α-enolase in HL-60 cells and demonstrated that HNE was colocalized with α-enolase at the surface of cells, early after its addition. HNE caused a dose-and time-dependent reduction of the binding of plasminogen to α-enolase. As a consequence, HNE reduced adhesion of HL-60 cells to endothelial cells (HUVECs). These results could suggest a new role for HNE in the control of tumour growth and invasion.
Keywords: 4-hydroxynonenal, HNE-adducts, HL-60 cells, α-enolase, plasminogen binding, cell adhesion.

INTRODUCTION

Reactive intermediates produced under oxidative stressful conditions cause the oxidation of polyunsaturated fatty acids in membrane lipid bilayers, leading eventually to the formation of aldehydes [1]. Among many different aldehydes, which can be formed during lipid peroxidation, the most intensively studied has been the 4-hydroxyalkenal class, in particular 4-hydroxynonenal (HNE). HNE has three main functional groups: the aldehyde group, the C=C double bond and the hydroxyl group, which can participate, alone or in sequence, in chemical reactions with other molecules [2]. HNE is a highly electrophilic molecule that easily reacts with low molecular weight compounds, such as glutathione, with proteins and, at higher concentration, with DNA. It has been demonstrated that HNE modifies proteins, either by forming simple Michael adducts with lysyl, histidyl, and cysteinyl residues [3], or through Schiff base formation with lysyl residues, leading to pyrrole formation [4]. In addition, HNE modification can result in cross-linking of two lysyl residues through reversibly formed Schiff base Michael adducts [5]. HNE has been detected in vivo in several pathological conditions, which entail increased lipid peroxidation, including inflammation, atherosclerosis, chronic degenerative diseases of the nervous system, and chronic liver diseases [6]. However, under physiological conditions, HNE can be found at low concentrations in human tissues and plasma [7,8], where it participates in the control of biological processes, such as signal transduction, cell proliferation and differentiation [8]. Addition to and modification of functional and/or signalling proteins most likely are among the main mechanisms by which HNE can modulate physiological and pathological processes. HNE-protein adducts have been found in mammalian organisms [7-13], but no data have been provided for leukemic cells.

In leukemic cell models, HNE, at doses similar to those detected in normal cells, inhibits proliferation and induces differentiation of HL-60, MEL and K562 cells [14-16]. Recently, HNE has been identified as a novel agent for the eradication of human leukaemia stem cells [17]. Since HNE is a normal constituent of human plasma in physiological and pathological conditions [18], the identification of HNE adducts may contribute to clarify the consequences of the systemic oxidative stress response on leukemic cells.

In the present work, proteomic analysis of proteins extracted from HL-60 cells revealed that the first target of HNE in 10 µM HNE-treated cells was α-enolase, located on the surface of cells. α-enolase was discovered to act as a glycolytic enzyme (2-phospho-D-glycerate hydrolyase, EC4.2.1.11), which catalyzes the dehydration of 2-phospho-D-glycerate (PGA) to phosphoenolpyruvate (PEP) [19]. For a long time, it had been considered as a “dull” enzyme, highly conserved during the phylogensis. However, unlike the gene coding glyceraldehyde-3-phosphate dehydrogenase (GAPDH), the gene that codes α-enolase is not a housekeeping gene, because its expression varies according to the pathophysiological, metabolic and developmental conditions of cells [20]. Indeed, it has been uniformly reported that the expression of the α-enolase-specific mRNA increases to a very high level in exponentially growing cells, but remains almost at an undetectable level in the stationary/resting/quiescent phase [21]. Moreover, α-enolase levels have been found to be significantly altered in blast cells from all types of acute myeloid leukaemia, compared with normal mononuclear blood cells [22]. In recent years, it has become evident that α-enolase is a multifunctional protein [23]. It has been shown that the α-enolase mRNA can be alternatively translated, to produce a protein which corresponds to the C-terminal fragment of α-enolase. The two proteins are highly homologous and are recognized by the same antibody in immunoblots as two bands of different molecular masses: 47 K for α-enolase and 37 K for MBP-1 [24]. The 37-K protein (Myc Binding Protein -1, MBP-1) does not show enolase activity, but functions as a transcriptional
repressor of the c-myc gene, through the binding to the minor groove of the P2 c-myc promoter, thus preventing the formation of the transcription initiation complex [25,26]. In addition to its enzymatic and transcriptional roles, α-enolase, expressed on the surface of a variety of eukaryotic cells, functions as a strong plasminogen receptor [20, 27]. Plasminogen receptors include: α-enolase (47 K), annexin 2 (36 K) and histone H2B (17 K) [28]. These proteins mediate the response to plasminogen binding, including fibrinolysis, inflammatory cell recruitment [29], tumour cell invasion [30] and recruitment of monocytes to inflammation sites [31]. In this work, we studied, by mass spectrometry and immuno-blotting, the formation of HNE-α-enolase adducts in HL-60 human leukemic cells exposed to low HNE concentrations, close to those detected in vivo under pro-oxidant conditions. Moreover, the consequences of α-enolase-HNE adduct formation on α-enolase expression and enzymatic and plasminogen-binding activities have been examined.

EXPERIMENTAL

Cells and culture conditions

HL-60 cells were cultured in standard conditions, in RPMI 1640 medium supplemented with 2 mM glutamine, antibiotics and 10% foetal calf serum (FCS) (Biochrom AG Seromed, Berlin, Germany). Human umbilical vein endothelial cells (HUVECs) were isolated and cultured as described elsewhere [32]. HUVECs were utilized between two and five passages.

HNE treatments

Before treatment, cells were washed and resuspended in serum-free RPMI 1640 medium. HNE (Calbiochem, La Jolla, CA, USA) was added to the cell suspension (200,000 cell/ml) at the final concentration of 10 µM. After 15 min or 2 h from the start of the treatment, 10 x 10^6 cells were washed twice in cold PBS and total proteins were extracted. For the assay of α-enolase activity and plasminogen binding, cells were resuspended (200,000 cell/ml) and treated with 1, 5, 10, or 20 µM HNE. For plasminogen binding and cell adhesion assays, repeated HNE treatments were performed. Briefly, 1 µM HNE was added to the cell suspension (200,000 cells/ml) in RPMI 1640 medium supplemented with 10% FCS. The aldehyde was added at regular intervals (45 min) up to 10 treatments (the overall time of exposure to the aldehyde was 7.5 hours). No washing steps were performed. This experimental procedure has been adopted because HNE vanishes from the culture medium within 45 minutes [33] and must be repeatedly added, in order to prolong the HNE effects [34, 35]. Protein extracts from control and test cell cultures were prepared as described below.

Preparation of total and nuclear protein extracts

Total cell extracts were prepared by suspension of 10 x 10^6 cells in lysis buffer, containing 0.050 M Tris/HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% Nonidet, 1mM sodium orthovanadate, 1 mM PMSF and 0.05% aprotinin. Debris were discarded by centrifugation at 13,000 g at 4°C. Protein concentration was measured in triplicate, using a commercial assay (Bio-Rad Laboratories, Segrate, Italy). The nuclear fraction was isolated from cells (10 x 10^6), as previously described [36]. The postnuclear supernatant was harvested and utilized for the analysis of the α-enolase content. Nuclear proteins were purified from debris by high speed centrifugation after incubation for 15 minutes at 4°C in 20 mM HEPES buffer, pH 7.9, containing 0.4 M NaCl, 1 mM EDTA, 1mM sodium orthovanadate, 1 mM PMSF and 0.05% aprotinin. Protein concentration was measured as reported above.
Antibodies

The sensitivity and specificity of various anti-HNE antibodies was evaluated in preliminary experiments, with extracts of cells exposed to HNE, separated by SDS-PAGE and blotted onto nitrocellulose. A monoclonal anti-HNE antibody, produced in-house by Prof. Koji Uchida, proved to be more specific than a number of antibodies from commercial sources and was used for all subsequent experiments. The sources of the other antibodies used in this study are provided as online supplemental data.

Western blot analysis

Proteins extraction and separation was performed as previously described [35] Densitometric analysis of bands was performed by using a dedicated software (Multi-Analyset, version 1.1, Bio-Rad Laboratories, Segrate, Italy).

Two-dimensional gel electrophoresis (2-DE)

Protein cell lysates were separated by two dimensional gel electrophoresis (2-DE). Both control and test lysates were analyzed in two replicas, one to be silver-stained, the other to be transferred to PVDF filters for immunoblotting. Samples containing 40-50 µg each of cell proteins were subjected to isoelectrofocusing (IEF) in immobilized, linear pH gradients, using 11-cm-long, 0.1-mm-thick, pre-cast Immobiline DryStrips, pH 3-10L (GE Healthcare), equilibrated in rehydration buffer (300 µl of 8 M urea, 2% CHAPS, 2% IPG Buffer (GE Healthcare), 2.8 mg/ml dithiothreitol, bromophenol blue as tracking dye), in a thermostatted Multiphor II flatbed electrophoresis unit (GE Healthcare). Samples were cup-loaded onto DryStrips halfway between the anode and the cathode. For preparative purposes, 100 µg of cell proteins were dissolved in rehydration buffer and sample loading occurred during rehydration. IEF was conducted for 30 min at 500 V, then 6 h at 3000 V at 15°C. Proteins in DryStrips were reduced and alkylated in 10 ml of 6 M urea, 0.05 M Tris/HCl, pH 8.8, 2% SDS, 30% glycerol, bromphenol blue (traces), 1% dithiothreitol for 15 min at RT, followed by 15 min in the same buffer, with 2.5% iodoacetamide in the place of dithiothreitol. Thereafter, each Immobiline DryStrip was layered onto an 8-18% total acrylamide vertical gradient gel in 0.375 M Tris/HCl, pH 8.6, 0.1% SDS, mounted in a Hoefer SE600 vertical electrophoresis unit. SDS-PAGE was conducted in 0.025 M Tris base, 0.2 M glycine, 0.1% SDS, at the constant current of 10 mAmp per gel, for 16 h at 15°C. Low molecular weight protein standards were from Bio-Rad. Analytical gels to be silver-stained were fixed in 30% (vol/vol) ethanol, 5% (vol/vol) acetic acid.

Electrophoretic transfer and immunodetection of proteins separated in 2-D gels

Proteins separated by 2-DE were transferred onto PVDF membranes (Immobilon P, Millipore) by semi-dry electrophoretic transfer in 0.025 M Tris, 0.01 M glycine. Membranes were blocked overnight at 4°C, rinsed 3 times in PBS, 0.2% Tween 20 and incubated with a 1:2000 dilution of a murine monoclonal anti-HNE-histidine primary antibody (prepared by K. U.) in PBS-Tween 20 with 3% BSA, for 1 h at RT. After two rinses in PBS-Tween, membranes were incubated with a 1:50,000 dilution of HRP-conjugated, secondary anti-mouse IgG antibodies for 1 h at RT. Immunoreactive spots were detected by chemiluminescence (ECL PLus, GE Healthcare).

Silver staining of SDS-PAGE gels

Analytical gels were stained, using the PlusOne Silver Staining kit (GE Healthcare), as previously described [38]. Preparative gels to be used for identification of protein spots by Mass Spectrometry were stained using a modified, MS-compatible protocol. Briefly, gels were fixed overnight in 50%
(vol/vol) methanol, 5% (vol/vol) acetic acid. After rinsing in 50% methanol for 10 min, and in milli-Q (Millipore)-purified water for another 10 min, the gels were sensitized with 0.2% sodium thiosulfate for 1 min, and then rinsed quickly twice in milli-Q-purified water. The gels were then incubated in 0.1% silver nitrate for 20 min at 4°C and rinsed in milli-Q-purified water twice more. Staining was developed in 0.04% formalin, 2% sodium carbonate, and blocked in 5% acetic acid. Gels were stored in 1% acetic acid at 4 °C until peaking of protein spots for MS analysis.

**Identification of HNE-modified proteins by Mass Spectrometry**

Silver-stained 2-DE gels of the proteome of HL-60 cells, under control conditions and after exposure to HNE, were aligned with the chemiluminescent detection maps of the corresponding immunoblots with anti-HNE antibodies, in order to locate the proteins detected by anti-HNE antibodies within the 2-D separation patterns. Separation and immunodetection patterns obtained under test conditions and control conditions were compared with each other, in order to identify the proteins selectively affected by HNE. Protein spots of interest were excised from the gels with a scalpel, placed in Eppendorf tubes, washed twice with 50 µl of Milli-Q-purified water and destained with 0.050 M NH₄HCO₃ in 50% (vol/vol) aqueous acetonitrile. Destained spots were dehydrated by submersion into acetonitrile, dried under vacuum and incubated in 20 µl of 0.05 M NH₄HCO₃, containing 12 ng/µl of trypsin, on ice. After 45 min of digestion, the supernatants were removed and incubated overnight at 37°C. The tryptic digests were extracted in 40 µl of 50% acetonitrile, 2.5% formic acid and concentrated to a tenth of the original volume in vacuum centrifuge. Samples were desalted and concentrated for analysis by Matrix-Assisted Laser Desorption Ionization-Time of Flight/Mass Spectrometry (MALDI-TOF/MS), using Poros Oligo R3 microcolumns [39]. The columns were washed with 20 µl of 0.1% TFA. Retained peptides were eluted onto the MALDI target with 0.5 µl of a matrix solution containing 10 mg of alpha-cyano-4-hydroxycinnamic acid (4-HCCA) in 1 ml of 50% aqueous acetonitrile. Measurements were carried out using a PerSeptive Biosystems Voyager DE-PRO instrumentation (Framingham, MA, USA), equipped with a N2 laser (337-nm, 3-ns pulse width). Mass spectra acquisitions were performed in positive reflectron mode, by accumulating 200 laser pulses. The accelerating voltage was 20 kV. External mass calibration was performed with low-mass peptide standards (PerSeptive Biosystems). Peptide assignment were done using the GPMAW software (http://www.gpmaw.com). MS data were searched against the NCBIInr protein sequence databases, using the Mascot server (http://www.matrixscience.com).

**Immunoprecipitation**

Total protein extracts (100-1000 µg) were incubated with 2 µg of primary antibody, under shaking, overnight at 4°C. Thereafter, 35 µl of protein A-Sepharose (Sigma Aldrich, Segrate, Italy) was added to the samples for 2 h at 4°C. The pellets were collected by centrifugation and supernatants were discarded. The pellets were washed extensively in 2 mM Tris/HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% Nonidet, 1mM sodium orthovanadate, 1 mM PMSF and 0.05% aprotinin, after which they were added to 35 µl of 0.2 M Tris/HCl buffer, pH 6.8, containing 2% SDS, 30% glycerol, 16% 2-mercaptoethanol, and were boiled for 2 min. The supernatants were collected and subjected to SDS-PAGE, followed by western blot analysis.

**Confocal microscopy**

5x10⁴ cells were smeared onto a non-charged slide, fixed for 15 min in 4% paraformaldehyde, washed twice, permeabilized with 1% Triton X100 for 30 min at RT and washed with PBS. Thereafter, the slides were incubated with 1% BSA in PBS for 30 min at RT, after which they were incubated overnight with primary antibodies (1:100 in 1% BSA dissolved in PBS) at 4°C. The slides were then washed, incubated for 1 h at RT with secondary fluorochrome-conjugated
antibodies (diluted 1:100 in 0.1% BSA in PBS), washed again and mounted on coverslips. The slides were examined under a Leica TCS SP2 confocal microscope, equipped with a water immersion objective HCX Apo 0.8.

Electron microscopy

HL-60 cells were fixed and samples were infiltrated in 2:1 (vol:vol) ethanol/London Resin White (LRW) for 1 h, 1:2 (vol:vol) ethanol/resin for 2 h and 100% LRW overnight at 4°C (all other steps are carried out at RT) [40]. Samples were embedded in gelatine capsules [40] and processed for ultramicrotomy [41]. Single antibody immunogold localization was performed as previously described [42].

α-enolase activity assay

An α-enolase activity assay was performed according to Witkowska et al. [43]. Briefly, cell lysates (20-40 µg) were incubated at 37°C in 50 mM imidazole/HCl buffer, pH 6.8, containing 0.4 M KCl, 2.0 mM MgSO4. The reaction was started by adding 2 µM 2-phospho-D-glycerate (Sigma, Segrate, Italy), and the optical density at 240 nm was recorded every 15 sec for 10 min. Enzyme activity was expressed as units per mg of protein.

Plasminogen binding

Ligand blot assay was performed according to Mundodi et al. [44]. Total protein extracts were subjected to electrophoresis, blotting and blockage, as previously described. The membranes were then incubated overnight at 4 °C with human plasminogen (35 µg/ml) dissolved in 2% non fat dry milk in TBS (10 mM Tris-HCl, pH 7.4; 150 mM NaCl), 0.1% Tween 20 (Sigma, Segrate, Italy). After incubation with plasminogen, the membranes were washed extensively in TBS (10 mM Tris-HCl, pH 7.4; 150 mM NaCl), 0.1% Tween 20 and then incubated for 1 h at RT with an anti-human plasminogen antibody (2 µg/ml) (R&D Systems), followed by a secondary anti-mouse HRP-conjugated antibody. The bands were revealed by using an ECL Kit by GE Healthcare.

Fluorescent labeling of HL-60 cells

Commercial fluorescent cell linker kit PKH67 (Sigma Chemical Co., St Louis, MO) was used for membrane labeling of HL-60 cells. The whole procedure was performed at 25°C as previously described [45].

Adhesion assay

HUVECs were grown to confluence in 24-well plates, washed, and let stay for one day in M199 medium plus 10% FCS without bovine brain extract. HNE-treated or untreated HL-60 cells (15 min or 8 h), were labeled as described above, and plated (5x10^5 cells/wells) in a final volume of 0.25 ml of M199 medium on untreated HUVECs and left in place for 1 h at 37°C in 5% CO2. After incubation, non-adherent HL-60 cells were removed by washing 3 times with 1 ml of M199 medium. In order to evaluate the effect of α-enolase on HL-60 cell adhesion to HUVECs, HL-60 cells were treated with 50 µg/ml of an anti-α-enolase monoclonal goat antibody for 15 min. The ability of this mAb to block HL-60 cell adhesion in a selective way was previously tested in our laboratory (data not shown), and the mAb were used at the concentration that demonstrated saturation in binding assays and maximal inhibitory effects in adhesion assays. After incubation, HL-60 cells where seeded on HUVECs, and left in place for 1 h at 37°C in 5% CO2.
The center of each well was analyzed by fluorescence image analysis [45]. Adherent cells were counted, using Image Pro Plus Software for micro-imaging (Media Cybernetics, version 5.0). Single experimental points were assayed in quadruplicate, and the standard deviation of the four replicates was below 10% in all cases.

Statistical analysis

Data are expressed as means ± SD. Statistical analysis was performed with GraphPad Prism 3.0 software. Paired t-test was used to determine significant differences between means, \( p \leq 0.05 \) being considered significant.

RESULTS

Identification of HNE adducts with \( \alpha \)-enolase

In preliminary experiments, in which HL-60 cell lysates obtained under basal conditions and after exposure to HNE were separated by SDS-PAGE, electrophoretically transferred to nitrocellulose membranes and probed with a murine monoclonal anti-HNE antibody (prepared by K.U.), protein adducts with HNE were detected already after 15 min of exposure to the aldehyde. Therefore, protein extracts obtained after 15 min of treatment with HNE were prepared for separation by 2-DE and analysis. Fig.1 shows the separations of total cell proteins of control (panel A) and HNE-treated HL-60 cells (panel C), and the respective immunoblots, obtained by electrophoretic transfer of the respective gel replicas onto PVDF and probing with a murine monoclonal anti-HNE antibody (panels B and D). The spots marked as number 1 and number 2 in the gels shown in panels A and C were not further analyzed, as they were present both in control and test samples. Spot number 3, obtained from a preparative-scale replica of the gel shown in panel C, prepared with 100 µg of total proteins from HL-60 cells treated with HNE for 15 min at 37 °C, was subjected to in-gel tryptic digestion, as described under Experimental Procedures, and analyzed by MALDI-TOF/MS. The peptide masses obtained from the MALDI-TOF/MS spectrum (Fig. 1, panel E) of the tryptic digest of spot number 3 were aligned with the NCBI nr database, using the Mascot server. Nineteen peptides were identified, whose experimentally determined masses matched the theoretical masses of tryptic peptides of human enolase-1 (code: gi/4503571) (human \( \alpha \)-enolase, 2-phospho-D-glycerate hydro-lyase, non-neural enolase (EC 4.2.1.11), having nominal mass (Mr) 47,481. We also performed immunoprecipitations of protein extracts obtained from HL-60 cells under basal conditions and after 15 min of exposure to HNE. Immunoprecipitation with an anti-\( \alpha \)-enolase antibody and western blotting with an anti-HNE antibody (Fig. 2, panel A) confirmed the formation of HNE adducts with \( \alpha \)-enolase. The same result was obtained by immunoprecipitation with an anti-HNE antibody and western blotting with an anti-\( \alpha \)-enolase antibody (Fig. 2, panel B).

Subcellular localization of HNE adducts with \( \alpha \)-enolase

In order to investigate the subcellular localization of \( \alpha \)-enolase and HNE after HNE treatment, confocal microscopy was performed after 15 and 120 min of treatment. No traces of HNE were detected with the anti-HNE monoclonal antibody in control cells (Fig 3, panel A). In cells treated with HNE for 15 min, HNE was localized prevalently at the plasma membrane level (Fig.3, panel B). In cells treated for 120 min, HNE also formed a halo underneath the cytoplasmic aspect of the plasmamembrane and some fluorescence signals were detectable in the nucleus too (Fig. 3, panel C). \( \alpha \)-enolase was localized at plasma membrane level, in the cytosol, and, to a lesser extent, in the nucleus.
nucleus, both in control and in treated cells, and this distribution did not change after HNE treatment (Fig. 3, panels D, E and F). Panels G, H and I of Fig. 3 show the colocalization of HNE and α-enolase. After 15 min of treatment, most of HNE and α-enolase were colocalized at the plasma membrane level. After 120 min, only a fraction of HNE was colocalized with α-enolase at the plasmamembrane level and the remainder was apparent as a green fluorescent signal within cells. The localization of α-enolase in HL-60 cells was confirmed by electron microscopy experiments (Fig. 4, upper side) that demonstrated the presence of α-enolase on the surface of plasma membrane and in the cytosolic and nuclear compartments. After treatment with HNE, the latter was detected on the surface of the plasmamembrane after 15 min and in the cytosol and nucleus after 2 hours (Fig. 4, lower side).

α-enolase expression and activity

In order to determine the biological consequences of HNE-α-enolase adduct formation, we tested the α-enolase expression and activity in control cells and in cells treated with 10 µM HNE. No changes of either expression or activity were detected (results are reported as supplementary data).

Nuclear localization of α-enolase and MBP-1

Because the main effect displayed by HNE in human leukemic cells is the inhibition of c-myc expression [37], which can be regulated by MBP-1, we tested the amount and nuclear localization of the 37-K product of the ENO1 gene in HNE-treated cells. The 37-K protein was expressed in very small amounts in the total protein extract of HL-60 cells, even in overexposed films (Fig. 6, panel A). The expression of the 37-K protein did not change after HNE treatment in the total protein extracts. When the nuclear fraction was isolated (Fig. 5, panel B) only the 47-K protein was apparent in the nuclear fraction, with no significant differences between control and treated cells.

Plasminogen binding activity of α-enolase

In order to determine whether the binding of plasminogen to α-enolase was affected by HNE, HL-60 cells were treated with increasing concentrations of the aldehyde (from 1 to 20 µM) and cell proteins were extracted after 15 min of treatment. The results obtained (Fig. 6, panel A) showed that HNE caused a dose-dependent decrease of the binding of plasminogen to α-enolase, already evident after treatment with 5 µM HNE. The reduction of plasminogen binding was apparent already after 7.5 min and maximal after 15 min of treatment (Fig. 6, panel B). The effect progressively vanished, as the time of treatment increased, conceivably as a consequence of HNE decay and of α-enolase neosynthesis. However, when the cells were maintained in presence of 1 µM HNE for 7.30 h, through 10 repeated treatments at intervals of 45 min, the HNE inhibitory effect on plasminogen binding was evident until the end of the treatment (Fig. 6, panel C).

HL-60 cell adhesion to a HUVEC monolayer

HL-60 cells have been widely used in studies characterizing leukocyte-endothelial interactions [46]. Recently, Wycrecka et al. [31] demonstrated that α-enolase expressed on the cell-surface in human blood monocytes and U937 leukemic cells enhanced migration of these cells through epithelial monolayers. These effects were abrogated by antibodies directed against the plasminogen-binding site of α-enolase, thus demonstrating that they were mediated by plasminogen binding to α-enolase. Since HNE inhibited the binding of plasminogen to α-enolase, we investigated the adhesion of HL-60 cells to human umbilical vein endothelial cells (HUVECs) after HNE treatment. As a negative control, we used cells treated with an anti-α-enolase antibody capable of inhibiting plasminogen binding, as previously tested (data non shown). Treatment with 10 µM HNE reduced the adhesion
of HL-60 cells to a HUVEC monolayer by 56±7%, after 15 min. When the cells were maintained in the presence of 1 µM HNE for longer times, through 10 repeated treatments, the inhibition of cell adhesion was again evident (by 53±4%) until 7.30 hours. The percent of inhibition produced by antibodies directed against the plasminogen-binding site of α-enolase was 70 ± 5%.

DISCUSSION

The results obtained in the present study indicate that HNE-α-enolase adduct(s) represent the most relevant adducts detectable after 15 min of HNE addition to HL-60 human leukemic cells. This kind of adducts was detectable by 2-DE and MALDI-TOF/MS, despite the low HNE concentration used (10 µM). HNE-α-enolase adducts were detected quite early (15 min) after the beginning of treatment with HNE, indicating a high degree of exposure of α-enolase, on the surface of cells, to HNE. The time course of these events, observed by confocal microscopy, demonstrated that the binding between HNE and α-enolase was probably the first step of the HNE penetration into the cells and that, after 2 hours, the aldehyde could react with other cellular targets. The localization of HNE and α-enolase at 15 min and 2 hours has been confirmed by electron microscopy, even though the presence of the HNE-α-enolase adduct could not be demonstrated by this analysis, because of the steric encumbrance of the colloidal gold goat antibodies. The binding between HNE and plasma membrane proteins has been previously reported by Pompella et al. [47], by using confocal laser scanning microscopy in human peripheral blood lymphocytes, after 5-20 min of treatment with 100 µM HNE.

Our results indicate that α-enolase is expressed at high levels in HL-60 cells, and that the treatment with HNE did not alter its expression, nor its enzymatic activity. This may be due to the dimeric structure of the α-enolase enzyme [20], which may protect the active site amino acyl side-chains from modification by HNE. For this reason, we focused our attention onto the transcriptional and the receptorial roles played by this molecule. The inhibition of c-myc expression, after HNE treatment, has been demonstrated by our and other research groups, in a wide variety of cell types [8]. Moreover, some experiments indicated that HNE inhibited the transcription start from the P2 c-myc promoter [16]. MBP-1 was found to bind to the minor groove of the P2 c-myc promoter, thus preventing the formation of the transcription initiation complex [25]. For these reasons, we investigated whether HNE treatment could interfere with the expression or localization of MBP-1. We observed that the expression of a 37-kDa fragment recognized by anti-α-enolase antibodies, and possibly corresponding to MBP-1, was very low in the total protein extract of HL-60 cells and its expression did not vary after HNE treatment. Since it was shown in HeLa cells that MBP-1 is predominantly located in the nuclear fraction [24], we investigated the nuclear localization of the 37-kDa protein in control and in HNE-treated cells. Interestingly, we found that this 37-kDa protein in HL-60 cells was preferentially located in the post-nuclear supernatant (cytosol and membranes) and not in the nuclear fraction, and that its localization did not change after HNE treatment, suggesting a secondary role of MBP-1 in the control of c-myc expression in the HL-60 cell line.

Afterwards, we focused our attention onto the plasminogen receptor role played by α-enolase. HL-60 cells, as well as other leukemic cells, display enhanced plasminogen binding, which may contribute to an enhanced fibrinolytic state in leukaemic patients [20]. Treatment with HNE strongly inhibited the binding between plasminogen and α-enolase at the surface of HL-60 cells, most probably as a consequence of HNE addition to lysyl residues of α-enolase involved in plasminogen binding [48]. The inhibition of plasminogen binding was apparent even at HNE concentrations almost as low as those detected in normal tissues and plasma (1 µM). The observed fading of the inhibition of plasminogen binding in time was not surprising. Most of the HNE effects in vitro, such as the inhibition of c-myc expression and telomerase activity [8], were reversible, and this was attributed to the intracellular degradation of HNE, since HL-60 cells possess various
enzymes, able to catabolise the aldehyde [49]. Moreover, HNE did not remain at the cell surface. Indeed, after 2 h from its addition, it diffused toward the interior of the cells. However, in the experiments performed by repeatedly treating the cells with 1 µM HNE, in order to prolong the exposure of cells to HNE, the inhibition of plasminogen binding to α-enolase persisted until the end of treatments. Nevertheless, we can envision that, under persisting pro-oxidant conditions in vivo, tumour cells may be continuously exposed to micromolar concentrations of HNE, sufficient to alter the plasminogen binding activity of α-enolase at the cell surface. Our results demonstrate that the binding of HNE to α-enolase determines, as a functional consequence, a strong reduction of HL-60 cell adhesion to HUVECs and that this effect is related to the inhibition of plasminogen binding, as demonstrated by using an anti-α-enolase antibody. Since α-enolase is present on the surface of many invasive pathogens and tumour cells and plays an important role in tissue invasion and metastasis, this protein may represent a therapeutic target in certain infections and in cancer [50]. From this point of view, our results further support the hypothesis that HNE may represent a novel agent for the eradication of leukaemia stem cells [17] and suggest a novel role for HNE and/or other inhibitors of plasminogen binding to α-enolase in the control of tumour growth and invasion.

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LIST OF ABBREVIATIONS

BSA, Bovine serum albumin; CHAPS, 3-[(3-cholamido-propyl)-dimethylammonium] – propansulfonate; 2-DE, two-dimensional gel electrophoresis; ECL, enhancing chemiluminescence; EDTA, ethylene diamine tetraacetic acid; EGTA, ethylene glycol bis(2-aminoethyl ether)-N,N,N',N'-tetraacetic acid; ENO1 α-enolase gene; FCS, foetal calf serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; 4-HCCA, alpha-cyano-4-hydroxycinnamic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HNE, 4-hydroxynonenal; HRP, horseradish peroxidase; IEF, isoelectrofocusing; IKB kinase, inhibitory kappa B kinase; JNKs, c-jun amino-terminal kinases; MALDI-TOF/MS, Matrix-Assisted Laser Desorption Ionization-Time of Flight/Mass Spectrometry; MBP-1, c-myc binding protein; PARP, anti-Poly-ADP Ribose Polymerase; PBS, phosphate buffered saline; PEP, phosphoenolpyruvate. PGA, 2-phospho-D-glycerate; PMSE, phenylmethylsulphonyl fluoride; PVDF, polyvinylidene difluoride; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

REFERENCES


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FIGURES

Figure 1. (panels A, B) 2-D electrophoresis of the proteome of HL-60 cells under basal conditions and (Panels C, D) after exposure to 10 µM HNE for 15 min at 37 °C. Total cell lysates, containing 45 µg of total protein each, were prepared from 10x10^6 cells. The gels in panels A and C were silver-stained, those in panels B and D were transferred to PVDF membranes, probed with a murine anti-HNE histidine mAb, and binding revealed by chemiluminescence. Molecular weight standards in kDa are marked at right in panels A and C. Spots detected by anti-HNE-antibodies in panels B and D, and the corresponding spots, detected by gel alignment, in panels A and C, are marked by numbers. Panel E: MALDI-TOF/MS spectrum of the products of the in-gel tryptic digestion of spot 3, excised from a preparative-scale replica (with 100 µg of protein) of the gel shown in panel C. MALDI-TOF/MS was performed as described in Experimental Procedures. Italic numbers near mass signals indicate the mass-to-charge (m/z) ratios of the pseudo-molecular ions ([M+H] + ) deriving from the ionisation of the tryptic peptides. The masses obtained were searched against the NCBInr protein sequence database, and spot 3 was identified as human enolase-1 (α-enolase, gi/4503571), nineteen peptides of which were identified, whose amino- and carboxy-terminal residue numbers are indicated. Peaks marked with asterisks indicate contaminants peptides of human keratin.

Figure 2. Immunoprecipitation of control and HNE-treated cells, collected after 15 min of treatment. Panel A: Immunoprecipitation (IP) with the anti-α-enolase antibody and western blotting (WB) with the anti-HNE antibody. As a control for errors in gel loading, the blots were stripped and re-exposed to the anti-α-enolase antibody (panel A: lower frame), Panel B: immunoprecipitation with the anti-HNE antibody and western blotting with the anti-α-enolase antibody. As a control for errors in gel loading equal aliquots (25 µg) were separated by gel electrophoresis and exposed to the anti-β-actin antibody (Panel B: lower frame). Bar graphs represent the densitometric scanning of the blots, normalised using the α-enolase and β-actin signals, respectively, and are means ± SD of three independent experiments.

Figure 3. Left side: Confocal microscopic analysis of control and HNE-treated HL-60 cells. Cells (5x10^4) were harvested before (control) and after 15 and 120 min of treatment with 10 µM HNE. Top row: Cells exposed to the anti-HNE antibody; panel A: control cells; panel B: 15 min of HNE treatment; panel C: 2 h of HNE treatment. Middle row: cells exposed to the anti-α-enolase antibody; panel D: control cells; panel E: 15 min of HNE treatment; panel F: 2 h of HNE treatment. Bottom row: merge; panel G: control cells; panel H: 15 min of HNE treatment; panel I: 2 h of HNE treatment. Right side. Electron microscope images of HL-60 cells treated with HNE 10 µM and harvested after 15 min (plasmamembrane images) and 2 h (cytoplasm/nucleus images) from the treatment. Cells were exposed to anti-α-enolase (white arrows) and anti-HNE-histidine antibody (black arrows) and then prepared for electron microscope image acquisition.

Figure 4. Panel A: MBP-1 expression in control and HNE-treated cells. The analysis revealed an intense 47-KDa band, corresponding to α-enolase, and a weak 37-KDa band, corresponding to MBP-1. Bar graphs represent the densitometric scanning of the blots, normalised using the β-actin signals, and are means ± SD of three independent experiments.

Panel B: Nuclear localization of α-enolase and MBP-1 in control and HNE-treated HL-60 cells. Equal protein loading was confirmed by exposure of the membranes carrying post-nuclear and nuclear extracts to anti-β-actin and anti-PARP antibodies, respectively. Bar graphs represent the densitometric scanning of the blots, normalised using β-actin and PARP signals, respectively, and are means ± SD of three independent experiments.
**Figure 5.** Plasminogen binding to α-enolase. Panel A: Dose dependence of the inhibition of plasminogen binding. HL-60 cells were exposed to different doses (from 1 to 20 µM) of HNE. After 15 min, proteins were extracted and used for the plasminogen binding assay. 10 ng of plasminogen (81-kDa) were loaded, in order to verify the specificity of plasminogen binding to the 47-kDa band, corresponding to α-enolase. Bar graphs represent the densitometric scanning of the blots, normalised using the α-enolase signal and are means ± SD of three independent experiments.

Panel B: Time course of plasminogen binding. HL-60 cells were exposed to 10 µM HNE and harvested after different times. Bar graphs represent the densitometric scanning of the blots, normalised using the α-enolase signal and are means ± SD of three independent experiments.

Panel C: Plasminogen binding after repeated treatments with HNE. HL-60 cells were treated with 1 µM HNE at regular intervals of time (45 min) up to 10 treatments (the overall time of exposure to the aldehyde was 7.5 hours). At the end of experiments cells were harvested and the protein were extracted for plasminogen binding assay. Bar graphs represent the densitometric scanning of the blots, normalised using the α-enolase signal and are means ± SD of three independent experiments.

**Figure 6.** Effect of HNE on HL-60 cell adhesion to HUVECs. HL-60 cells were seeded and let stay for 1 h onto monolayers of HUVECs. Attached cancer cells were visualized as bright dots by fluorescent microscopy. The picture is representative of 1 of 3 individual experiments: panel A: control cells; panel B: cells treated with 10 µM HNE and harvested after 15 min from the treatment; panel C: cells treated with 1 µM HNE at regular intervals of time (45 min) up to 10 treatments and harvested after 7.5 hours from the beginning of treatments; panel D: cells exposed to 50 µg/ml α-enolase mAb (Ab) for 15 min.

Data are presented as percentage of adherent cells versus control value. Control adhesion was 49±4 cells/microscope fields (n = 3). Data are the mean ± SD of three separate experiments.
Fig. 2
**Fig. 4**

A

- **α-enolase**
  - C 15' HNE 2h
  - C 15' HNE

- **MBP-1**
  - 47 kDa

- **β-actin**

B

- **Postnuclear supernatant**
  - 15' C HNE 2h
  - C 15' HNE

- **Nuclear extracts**
  - 15' C HNE 2h
  - C 15' HNE

- **α-enolase**
- **MBP-1**
- **β-actin**
- **PARP**
Fig. 5
Fig. 6

% of adherent cells

A B C D