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The covalent modification of spectrin in red cell membranes by the lipid peroxidation product 4-hydroxy-2-nonenal

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ABSTRACT

Spectrin strengthens the red cell membrane through its direct association with membrane lipids and through protein–protein interactions. Spectrin loss reduces the membrane stability and results in various types of hereditary spherocytosis. However, less is known about acquired spectrin damage. Here, we showed that α- and β-spectrin in human red cells are the primary targets of the lipid peroxidation product 4-hydroxy-2-nonenal (HNE) by immunoblotting and mass spectrometry analyses. The level of HNE adducts in spectrin (particularly α-spectrin) and several other membrane proteins was increased following the HNE treatment of red cell membrane ghosts prepared in the absence of MgATP. In contrast, ghost preparation in the presence of MgATP reduced HNE adduct formation, with preferential β-spectrin modification and increased cross-linking of the HNE-modified spectrins. Exposure of intact red cells to HNE resulted in selective HNE–spectrin adduct formation with a similar preponderance of HNE–β-spectrin modifications. These findings indicate that HNE adduction occurs preferentially in spectrin at the interface between the skeletal proteins and lipid bilayer in red cells and suggest that HNE–spectrin adduct aggregation results in the extrusion of damaged spectrin and membrane lipids under physiological and disease conditions.

Introduction

Spectrin, a major constituent of the human red blood cell (RBC) membrane skeleton, acts as a scaffolding protein and mediates the organization and assembly of a diverse set of proteins in specialized membrane domains [1]. Spectrin is a flexible, rod-like protein formed by two end-to-end associated heterodimers composed of α and β subunits, with sizes of 280 and 246 kDa, respectively. In RBCs, the spectrin–actin network is linked to the lipid bilayer through protein–protein interactions, specifically spectrin–protein 4.1R-glycophorin C and spectrin–ankyrin-band 3 associations, and through direct association of spectrin with phosphatidylserine (PS) in the inner bilayer leaflet [2–4]. Such vertical interactions between the spectrin–actin skeleton and lipid bilayer play pivotal roles in maintaining RBC membrane stability [3,5,6]. Spectrin loss reduces the membrane mechanical resilience, leading to spherocyte formation in various types of hereditary spherocytosis [4,7]. In contrast with its role in congenital disorders, less is known about acquired spectrin damage. Previous studies have focused on the oxidation of spectrin sulfhydryl groups, revealing that RBC exposure to oxidative conditions causes morphological and mechanistic changes, principally through spectrin oxidation [8,9].

In contrast, membrane phospholipid peroxidation generates various cytotoxic aldehydes that can damage proteins and other cellular constituents [10–12]. 4-Hydroxy-2-nonenal (HNE) is the most abundant and toxic aldehyde generated by the oxidation of plasma membrane polyunsaturated fatty acids, such as arachidonic acid. HNE is a highly reactive electrophile that reacts with the side chains of various amino acid residues, including Cys, His, and Lys, to form Michael adducts [10,13]. Protein modification by HNE causes serious detrimental effects in the cell, due to the resulting functional defects and cross-linking of proteins [14–18]. HNE also accumulates in RBCs [19], leading to the covalent modification of membrane proteins [20]. However, the target proteins for HNE modification remain unknown.

These findings have suggested that oxidative environments under physiological or disease conditions may cause spectrin modifications by HNE, leading to deleterious changes in the plasma membrane mechanical properties. Therefore, here we examined the presence and significance of covalent spectrin modifications by HNE in human RBCs. We used anti-HNE antibodies and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) to identify HNE-modified proteins in RBC membranes.

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Materials and methods

Preparation of RBC membrane ghosts and spectrin–actin extracts. After obtaining informed consent, blood was obtained from healthy volunteers. Preparation of RBC membrane ghosts and extraction of spectrin–actin were performed as described previously [6]. In some experiments, RBC ghosts were prepared by hemolysis in hypotonic buffer (5 mM Tris/Cl [pH 7.4], 5 mM KCl, and 1 mM $MgCl_2$) with or without 0.6 mM MgATP (Sigma), as described previously [3]. We also obtained ghosts from RBCs separated into different fractions using a discontinuous arabinogalactan gradient [21].

SDS–PAGE and immunoblotting. Membrane proteins were separated by SDS–PAGE on 6.5% or 10% SDS–gels followed by staining with Coomassie brilliant blue or immunoblotting. Immunoblotting was performed as described previously [22] using antibodies against HNE-Michael adducts (Calbiochem), HNE–His (monoclonal antibody HNEJ-2; Nikken SEIL Co., Fukuroi, Japan), and spectrin [6].

MALDI-TOF MS analysis of spectrins. After separation by SDS–PAGE, proteins in the gel slices were reduced with $NaBH_4$ for 30 min at an ambient temperature unless otherwise indicated. This was done to detect unstable reversibly formed adducts, such as HNE–Lys–Michael adducts [23]. The proteins were subsequently stained with Coomassie brilliant blue and digested in gel slices with 10 μg/ml of trypsin Gold (Promega) for 16 h at 37 °C. Prior to protease digestion, the Cys residues were reduced with 10 mM dithiothreitol and alkylated with 55 mM iodoacetamide. Peptides were eluted from gels, desalted using C18 ZipTips (Millipore), and crystallized using saturated α-cyano-4-hydroxy-cinnamic acid (Bruker Daltonics) as a matrix. Full-scan mass spectra of the tryptic peptides from 800 to 3000 *m/z* were collected in positive mode by averaging 100–250 spectra using a Bruker autoflex MALDI-TOF mass spectrometer. Measured peptide masses were used to search the NCBI and Swiss-Prot sequence database for protein identification using MASCOT software.

In vitro formation of HNE–protein adducts in RBCs and RBC ghosts. A packed 10% cellular volume of RBCs was suspended in PBS containing 5 mM glucose. After incubation for various times (0–6 h) at 37 °C in the presence or absence of 0.1–1.0 mM HNE (Calbiochem), the cells were washed with PBS. Membrane ghosts were then prepared as described above. Ghost proteins were labeled with HNE by incubating PBS-suspended ghosts (1 mg protein/ml) with 0.01–1.0 mM HNE at 37 °C for appropriate time periods (0–60 min), followed by SDS–PAGE and immunoblotting. In some experiments, incubation was performed in hypotonic buffer with 0.1 mM MgATP.

Statistical analysis. Paired Student *t* test was used to assess statistical significance.

Results

HNE modification of α- and β-spectrin in human RBC membranes

Immunoblotting analysis revealed the presence of polypeptides that had reacted with the anti-HNE antibody. These polypeptides were located at positions corresponding to α- and β-spectrin in the freshly prepared human RBC membranes and in the crude spectrin–actin preparation extracted from the ghosts (Fig. 1A). These signals were weak and required >15 s for detection when 5–10 μg of membrane proteins were loaded. Spectrin, especially β-spectrin, also reacted with the anti-HNE–His adducts, suggestive of a predominance of HNE–His adducts in β-spectrin (Fig. 1A).

We then examined the contents of HNE-modified spectrin in density-separated RBCs to examine if these adducts accumulate during RBC aging. The lightest/youngest cell fraction showed

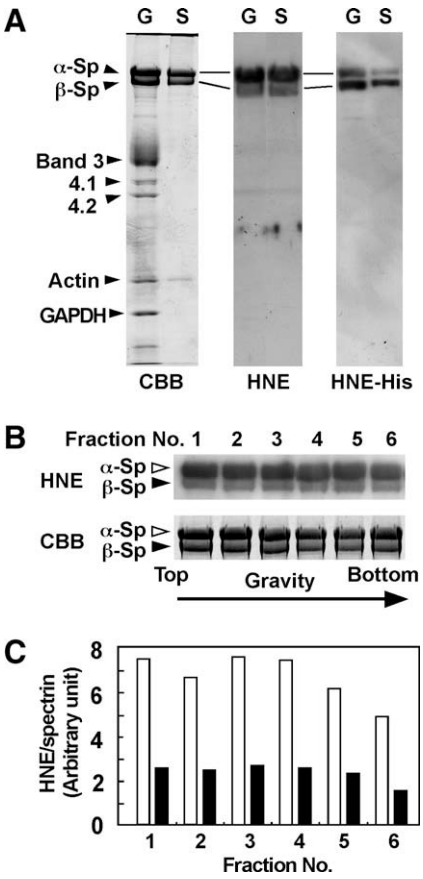

Fig. 1. HNE–spectrin adducts in human RBC membranes. (A) Ghost membrane proteins (*G*, 8 μg/lane) and the crude spectrin/actin extracted from ghosts (*S*, 2–3 μg/lane) were separated on SDS–PAGE gels. Protein components were analyzed by Coomassie brilliant blue staining (*CBB*) and HNE–protein adduct formation was assessed by immunoblotting using anti-HNE (*HNE*) or anti-HNE–His (*HNE–His*) adduct antibodies. In immunoblotting, the membranes were exposed to the films for 1 min. The migrating positions of the major membrane proteins, including α- and β-spectrin (α-*Sp* and β-*Sp*, respectively), are indicated. (B) Human RBCs were separated into fractions 1–6 by centrifugation on an arabinogalactan density gradient [21]. Fractions were examined for their HNE–spectrin and whole spectrin contents as described above (8 μg/lane, exposed for 1 min). (C) Contents of HNE-modified α-spectrin (hatched columns) and β-spectrin (unhatched columns) shown in B were quantified by densitometric scanning, normalized by the corresponding spectrin, and shown as the relative abundance of the HNE–spectrin (*HNE/Spectrin*). Data represent a typical result from several independent experiments.

HNE/spectrin ratios equivalent to those in fractions 3 and 4, which comprised ~90% of total cell counts (Fig. 1B). No statistically significant differences in the HNE levels of α- and β-spectrin were observed among fractions 1–6, although the densest fractions (fractions 5 and 6) had reduced HNE/spectrin ratios; an increase would have been expected if there was accumulation of HNE–spectrin adducts during RBC senescence. These data suggested that human RBCs possess HNE-modified spectrin throughout their life-span.

We analyzed the positions where these spontaneous modification with HNE occurred in spectrin polypeptides from freshly prepared RBC ghosts. MALDI-TOF MS detected 50~70 tryptic peptides that covered ~25–35% of the total amino acid residues of α- and β-spectrin. Several independent analyses consistently showed that a dozen or more distinct peptides derived from either α- and β-spectrin contained one or more Michael-type HNE-amino acid adduct and Schiff base conjugate (Fig. 2 and Supplementary Table 1). For example, in β-spectrin reduced with $NaBH_4$, the MALDI-TOF MS consistently detected an HNE-modified species at 2622.4 *m/z* for the peptides IHCLENVDKALQFLKEQR (amino acid residues 110–127) derived from the N-terminal actin-binding domain of this