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Covalent Attachment of 4-Hydroxy-2-Nonenal to Erythrocyte Proteins

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It has been established that the covalent modification of proteins occurs *in vivo* as a consequence of reaction with reactive lipids such as 4-hydroxy-2-nonenal (HNE). The fact that HNE occurs in blood under physiological as well as pathophysiological conditions suggests that erythrocytes undergo modification by HNE. Here we describe the immunochemical characterization of HNE-treated erythrocytes by demonstrating the Michael-type HNE addition to both membrane and cytosolic proteins. Exposure of erythrocytes to HNE (0-0.5 mM) for 2 h resulted in HNE labeling of multiple membrane proteins. Pretreatment of erythrocytes with a sulfhydryl reagent, *N*-ethylmaleimide (NEM), resulted in a significant decrease of HNE attached to the proteins, suggesting that HNE primarily reacts with the sulfhydryl groups of erythrocyte membrane proteins, whereas enhanced HNE labeling of the membrane proteins was observed when the erythrocytes were pretreated with H_2O_2 (0.1-5 mM) for 15 min. On the other hand, highly selective modification of a 30 kDa protein was observed in the hemolysates of erythrocytes treated with HNE. The protein, which represents a major intracellular target of HNE in erythrocytes, was identified as carbonic anhydrase, based on the observations that (i) a reverse-phase HPLC analysis of the chloroform/ethanol extract of HNE-treated erythrocytes detected two major proteins, which cross-reacted with anti-carbonic anhydrase antibody as well as with the anti-HNE adducts antibody, (ii) the chloroform-ethanol extraction of authentic carbonic anhydrase gave a similar HPLC pattern, and (iii) the HNE treatment of erythrocytes resulted in the partial inhibition of the carbonic anhydrase activity.

Key words: carbonic anhydrase, erythrocyte, 4-hydroxy-2-nonenal, protein sulfhydryl group.

There is increasing evidence that aldehydes generated endogenously during lipid peroxidation are causally involved in most of the pathophysiological effects associated with oxidative stress in cells and tissues (*1*). A number of reactive lipid peroxidation-derived aldehydes have been implicated as causative agents in cytotoxic processes initiated by the exposure of biological systems to oxidizing agents (*2*). Compared to free radicals, the aldehydes are stable and can diffuse within or even escape from the cell and attack targets far from the site of the original free radical-initiated event. The aldehydes, therefore, are not only end products and remnants of lipid peroxidation processes, but also may act as "second cytotoxic messengers" for the primary free radicals that initiated lipid peroxidation.

Among the aldehydes which originate from the peroxidation of cellular membrane lipids, 4-hydroxy-2-nonenal (HNE) is believed to be largely responsible for the cytopathological effects observed during oxidative stress *in vivo* (*2*). HNE exhibits a wide range of biological activities including inhibition of protein and DNA synthesis, inactivation of enzymes, stimulation of phospholipase C, reduction of gap-junction communication, and stimulation of neutrophil migration (*2*). Many of these *in vitro* effects, which are observed at low micromolar or even submicromolar concentrations of HNE, have been attributed to the modification of cellular proteins with HNE.

It has been demonstrated that the level of HNE increases in whole blood and plasma in relation to extensive aortic atherosclerosis (*3*). In that experiment, about 25% of blood HNE appeared in the plasma, with the majority apparently associated with the erythrocytes. Ando *et al.* (*4*) have demonstrated that lipid peroxidation products, including HNE, accumulate during the aging of erythrocytes in the circulation. In addition, erythrocytes from shock patients have been reported to contain increased amounts of various aldehydes, including HNE, ascribed to *in vivo* lipid peroxidation in erythrocytes (*5*). Our *in vitro* experiments have revealed that a considerable amount of HNE was released from human plasma low-density lipoproteins treated with metal ions or endothelial cells (*6*). These accumulating data suggest that erythrocytes may undergo modification by HNE *in vivo*. Indeed, erythrocyte membrane proteins are known to be susceptible to covalent damage by lipid peroxidation products (*7*). Sambrano *et al.* (*8*) have suggested that modification of erythrocytes with lipid

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Abbreviations: HNE, 4-hydroxy-2-nonenal; GSH, glutathione; NEM, *N*-ethylmaleimide; PVDF, polyvinylidene difluoride; SDS-PAGE, sodium dodecylsulfate-polyacrylamide gel electrophoresis; BSA, bovine serum albumin; PBS, phosphate-buffered saline; TBS, Tris-buffered saline.

peroxidation-derived aldehydes generates a ligand recognized by receptor(s), which is closely related or identical to those that recognize oxidized erythrocytes as well as oxidized low-density lipoproteins. These findings led us to investigate in this study the covalent attachment of HNE to erythrocyte proteins.

EXPERIMENTAL PROCEDURES

Materials—The stock solution of *trans*-4-hydroxy-2-nonenal was prepared by acid treatment (1 mM HCl) of HNE diethylacetal, which was synthesized according to the procedure of De Montarby *et al.* (*9*). The concentration of the HNE stock solution was determined by measurement of UV absorbance at 224 nm.

Erythrocyte Treatment—Normal rabbit erythrocyte suspensions were prepared from heparinized fresh rabbit blood obtained from Wako (Osaka). After centrifugation to remove the plasma and leukocytes, the erythrocytes were washed three times in 10 mM phosphate-buffered saline (pH 7.4). The washed, packed red cell-rich fraction was adjusted to 10% (v/v) in phosphate-buffered saline and incubated with varying concentrations (0.1–1 mM) of HNE for 2 h at 37°C. The erythrocytes were incubated with 5 mM *N*-acetylcysteine to remove non-reacting HNE, then lysed in 10 mM phosphate buffer (pH 7.4) and fractionated to obtain the membranes and the hemolysate by centrifugation at 20,000 × *g* for 40 min. The membranes were washed twice with 10 mM phosphate buffer (pH 7.4).

Assay of Carbonic Anhydrase—Carbonic anhydrase activity was measured by CO_2 hydration (*10*). The assay mixture (8 ml), maintained at 0°C, contained 0.02 M Tris-HCl and a sample, at pH 8.0. Reaction was initiated by the addition of chilled CO_2-saturated water, and the hydration rate was recorded. Allowance was made for the rate of hydrolysis in the absence of sample.

HPLC Analysis of Carbonic Anhydrases—To one volume of hemolysate of the erythrocyte exposed to 1 mM HNE for 2 h, one volume of 40% ethanol and 0.5 volume of chloroform (chloroform/ethanol mixture) were added. The mixture was extracted by vigorous shaking for 5 min and then allowed to stand for 30 min. After centrifugation at 2,000 rpm for 15 min, the ethanol layer was taken and dialyzed against 3 mM sodium phosphate buffer (pH 7.0). The carbonic anhydrase isozymes were analyzed by reverse-phase HPLC on a Develosil ODS-HG5 column (0.46 × 25 cm). The column had previously been equilibrated with 0.1% trifluoroacetic acid and developed using a linear gradient of acetonitrile containing 0.1% trifluoroacetic acid (2%/min) at a flow rate of 0.8 ml/min.

Antibodies—Polyclonal antiserum that recognizes the HNE-protein epitopes was raised by immunizing New Zealand White rabbits with HNE-treated KLH as previously reported (*11*). For the preparation of an antibody specific for Michael addition-type HNE-amino acid adducts, the antiserum was partially purified by affinity chromatography on an HNE-histidyl peptide column. Affi-gel 10 was derivatized by incubation of the gel slurry with the HNE-derivatized histidine-containing heptapeptide amide (NH_2-Ala-Ala-Ala-His-Ala-Ala-Ala-CO-NH_2) in 0.1 M Hepes, pH 8.0, for 20 h at 4°C. The antiserum (2 ml) was diluted to 20 ml with 0.1 M Hepes buffer, pH 8.0, and passed three times over a column containing 1 ml of affinity resin. Unbound proteins were removed by washing with 5 ml of 0.1 M Hepes buffer, pH 8.0, followed by 5 ml of 100 mM NaCl, and bound antibodies were eluted with 5 ml of 0.1 M glycine, pH 2.5. The eluate was immediately neutralized with 1 M TrisHCl, pH 8.0, and stored at −70°C. The anti-rabbit carbonic anhydrase antibody was obtained from Sigma.

Western Blot—The HNE-treated and untreated erythrocytes were rinsed twice with PBS (pH 7.0) and lysed with 0.1% Triton X-100 in PBS. Each cell lysate was then treated with Laemmli sample buffer for 5 min at 100°C (*12*). The samples (50 μg protein) were run on 10% SDS-PAGE slab gels. One gel was used for staining with Coomassie Brilliant Blue, and the other was transblotted to an Immobilon PVDF membrane, incubated with Block Ace (40 mg/ml) for blocking, washed, and treated with the antibody. This procedure was followed by the addition of horseradish peroxidase conjugated to goat anti-rabbit IgG immunoglobulin and ECL reagents. The bands were visualized by exposure of the membrane to an autoradiography film.

GSH Assay—Measurement of GSH in the erythrocytes was fluorometrically performed according to the method of Hissin and Hilf (*13*). In brief, the erythrocytes were exposed to various concentrations of HNE for the indicated times and, at the end of the incubation period, the erythrocytes were washed twice with PBS (pH 7.0) and extracted with 25% (w/v) metaphosphoric acid solution containing 5 mM EDTA. After ultracentrifugation (105,000 × *g*, 30 min), 1.8 ml of 0.1 M phosphate solution (pH 8.0) containing 5 mM EDTA and 100 μl of the *o*-phthalaldehyde solution (1 mg/ml) were added to the resulting supernatant (100 μl), and the fluorescence intensity at 420 nm was determined with excitation at 350 nm.

RESULTS

Covalent Attachment of HNE to Erythrocyte Membrane Proteins—Proteins in the membrane fraction of HNE-treated erythrocytes were separated by SDS-PAGE, and HNE-protein conjugates were then visualized on the Western blots with an anti-HNE adducts antibody. As shown in Fig. 1A, the immunoreactivity of the various protein bands clearly depended upon the HNE concentration used. Exposure of erythrocytes to HNE (0–0.5 mM) for 2 h resulted in Michael-type HNE addition to multiple membrane proteins. Although HNE and other aldehydic lipid peroxidation products have been shown to increase the osmotic fragility of the erythrocyte membrane (*14*), hemolysis was scarcely observed even at the highest concentration (1 mM) of HNE.

It has been demonstrated that the maintenance of optical membrane function depends on the preservation of specific membrane sulfhydryl groups in the reduced state (*15*). The fact that HNE is an efficient sulfhydryl-alkylating reagent suggests that HNE mediates membrane alteration *via* sulfhydryl modification. Hence, we determined the degree of HNE modification of the sulfhydryl groups in membrane proteins. To this end, erythrocytes were treated with NEM (0.05–1 mM) prior to the treatment with 0.1 mM HNE, and erythrocyte membrane proteins were analyzed by Western blotting. As shown in Fig. 1B, pretreatment of erythrocytes with NEM resulted in a decreased HNE labeling of

membrane proteins, suggesting that the HNE reacted primarily with the sulfhydryl groups of erythrocyte membrane proteins. It is noteworthy that the HNE labeling of two major proteins, indicated by the arrowheads in Fig. 1B, was scarcely affected by the blockage of sulfhydryl groups with NEM.

It has been shown that the treatment of erythrocytes with H_2O_2 is associated with alterations in cell shape, decreased membrane deformability, and increased recognition by anti-IgM immunoglobulin (*16*). A previous finding that H_2O_2 increases phospholipid spacing (*17*) suggests that these membrane alterations in the erythrocytes influence the sensitivity to HNE. Hence, we examined the effect of oxidant treatment on the sensitivity of erythrocyte membrane proteins to HNE modification. For this purpose, the erythrocytes were treated with H_2O_2 for 15 min at a dose of 0.1-5 mM and then treated with 0.1 mM HNE for 2 h. The erythrocyte GSH levels were not appreciably affected by short-term incubations with H_2O_2 (data not shown). However, as shown in Fig. 2, the H_2O_2 treatment resulted

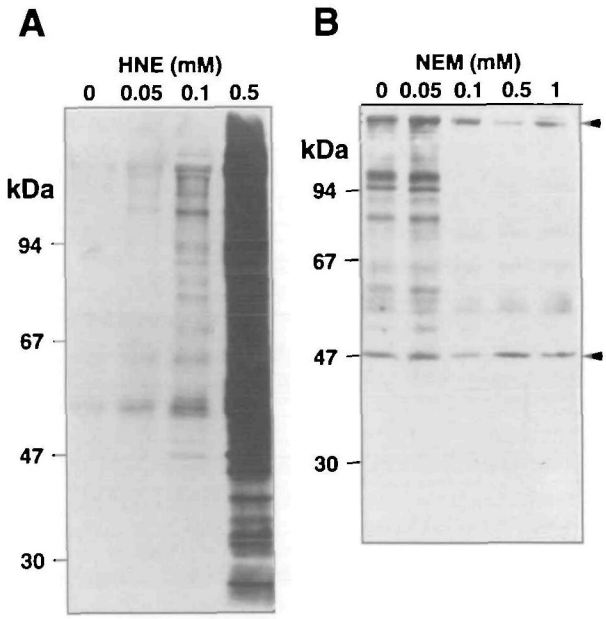

Fig. 1. **Covalent attachment of HNE to erythrocyte membrane proteins (A) and effect of *N*-ethylmaleimide (NEM) pretreatment on the HNE attachment (B).** The erythrocytes, adjusted to 10% (v/v) in phosphate-buffered saline, were incubated with varying concentrations (0.1-1 mM) of HNE for 2 h at 37°C. In B, the erythrocytes were pretreated with NEM (0.05-1 mM) for 15 min at 37°C and then incubated with 0.1 mM HNE for 2 h at 37°C. The arrowheads indicate the proteins that were scarcely affected by the blockage of sulfhydryl groups with NEM. Proteins in the membrane fraction were analyzed by Western blotting with an anti-HNE adducts antibody.

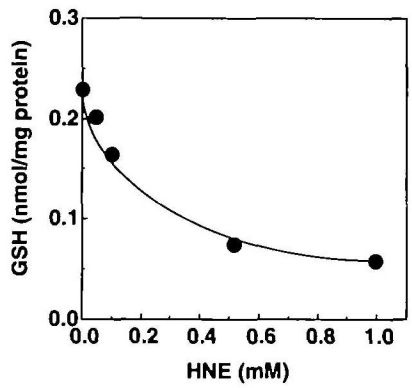

Fig. 3. **Effect of HNE on erythrocyte GSH levels.** The erythrocytes, adjusted to 10% (v/v) in phosphate-buffered saline, were incubated with varying concentrations (0.05-1 mM) of HNE for 2 h at 37°C.

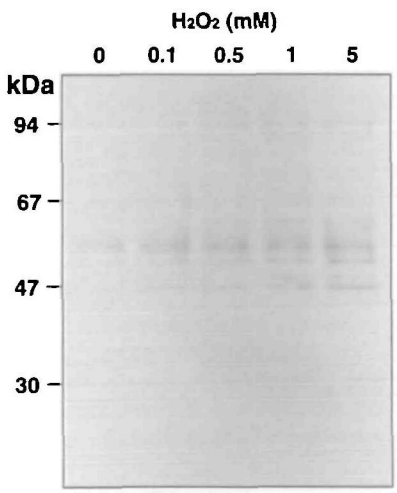

Fig. 2. **Effect of H_2O_2 pretreatment on the HNE attachment to erythrocyte membrane proteins.** The erythrocytes, adjusted to 10% (v/v) in phosphate-buffered saline, were pretreated with varying concentrations (0.1-5 mM) of H_2O_2 for 15 min, followed by treatment with 0.1 mM HNE for 2 h at 37°C. Proteins in the membrane fraction were analyzed by Western blotting with an anti-HNE adducts antibody.

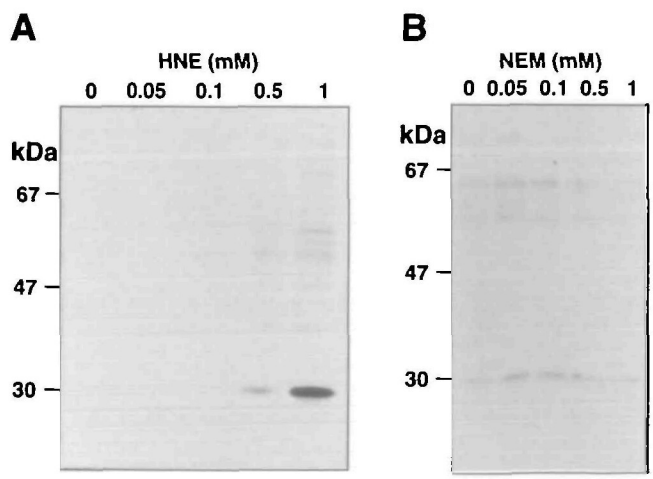

Fig. 4. **Covalent attachment of HNE to intracellular erythrocyte proteins (A) and effect of *N*-ethylmaleimide (NEM) pretreatment on the HNE attachment (B).** The erythrocytes, adjusted to 10% (v/v) in phosphate-buffered saline, were incubated with varying concentrations (0.1-1 mM) of HNE for 2 h at 37°C. In B, the erythrocytes were pretreated with NEM (0.05-1 mM) for 15 min at 37°C and then incubated with 0.1 mM HNE for 2 h at 37°C. Proteins in the hemolysate fraction were analyzed by Western blotting with an anti-HNE adducts antibody.

in the enhanced HNE labeling of the erythrocyte membrane proteins. The HNE-labeled membrane proteins seemed to be almost identical to those detected in the erythrocytes exposed to HNE following NEM treatment (Fig. 1B), suggesting that the major action of H_2O_2 on erythrocyte membrane proteins is sulfhydryl modification.

Intracellular Target of HNE in the Erythrocytes—It has been established that HNE shows the highest reactivity with sulfhydryl materials such as reduced glutathione (GSH), which is present in the cell in millimolar amounts and provides a biologically significant pathway for protection against HNE (*18*). Based on the finding that membrane sulfhydryl groups are major targets of HNE (Fig. 1B), it was assumed that erythrocyte GSH levels could be affected by incubation with HNE. Indeed, the GSH levels were readily reduced by treatment with HNE in a dose-dependent manner (Fig. 3). Interestingly, the reduction of the GSH level was induced by HNE at concentrations lower than 0.1 mM, suggesting that GSH represents a primary target of HNE in the erythrocytes.

Accompanied with the loss of GSH, a 30 kDa protein with strong immunoreactivity was detected in the hemolysate of erythrocytes treated with HNE (Fig. 4A). It should be noted that HNE modification of hemoglobin was scarcely observed and that the pretreatment of erythrocytes with NEM had no significant effect on the formation of the HNE adduct of 30 kDa protein (Fig. 4B). Based on its molecular weight, it was assumed that the band corresponds to the major erythrocyte 30 kDa protein, carbonic anhydrase. To confirm that the protein is identical to carbonic anhydrase, erythrocytes exposed to 1 mM HNE for 2 h were treated with a chloroform/ethanol mixture as described under "EXPERIMENTAL PROCEDURES," and the extract was then analyzed by reverse-phase HPLC. As shown in Fig. 5A, the HPLC analysis of the chloroform/ethanol extract of HNE-treated erythrocytes detected two major components. When both components were subjected to automated Edman degradation, no cleavage occurred. This is consistent with the fact that carbonic anhydrases are generally acetylated at their amino terminus (*19*), preventing Edman degradation. Moreover, a chloroform/ethanol extraction of authentic rabbit carbonic anhydrase gave similar peaks superimposable on an HPLC chromatogram (Fig. 5A). It is, therefore, likely that the two major peaks represent isozymes of rabbit erythrocyte carbonic anhydrase (*10*). Finally, to confirm that the two peaks represent HNE-modified carbonic anhydrase, we purified the proteins and performed Western blot analysis using anti-carbonic anhydrase antibody and anti-HNE adducts antibody. As shown in Fig. 5, B and C, both proteins cross-reacted with anti-carbonic anhydrase antibody, as well as with the anti-HNE adducts antibody, confirming the identification initially made from a consideration of the molecular weight. We have also examined the effect of HNE on the erythrocyte carbonic anhydrase activity and found that even the highest concentration (1 mM) of HNE mediated only a modest loss (~30%) of the enzyme activity. This was also the case for the authentic rabbit carbonic anhydrase, which was only partially inhibited by HNE, in spite of a significant formation of HNE adducts in the enzyme (data not shown). These observations suggest that the covalent attachment of HNE to erythrocyte carbonic anhydrase is not related to the immediate loss of the enzyme activity.

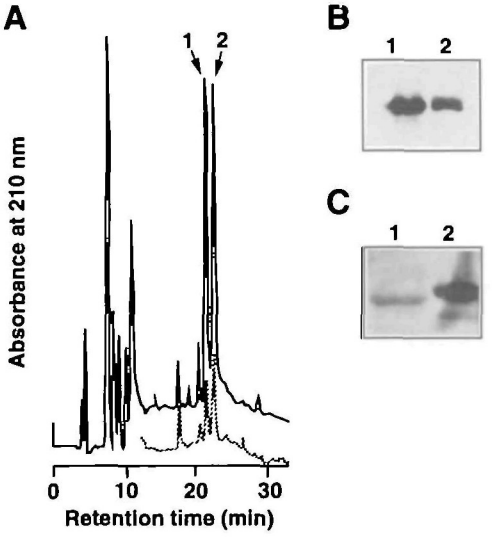

Fig. 5. **Identification of the intracellular target of HNE in the erythrocytes.** The erythrocytes exposed to 1 mM HNE for 2 h or authentic rabbit carbonic anhydrase were treated with a chloroform/ethanol mixture, and the extracts were then analyzed by reverse-phase HPLC (A). Lines: plain, erythrocytes; dashed, authentic carbonic anhydrase. Peaks 1 and 2 were purified and analyzed by Western blotting using anti-carbonic anhydrase antibody (B) and anti-HNE adducts antibody (C). In B and C, lanes 1 and 2 corresponds to peaks 1 and 2 in A, respectively.

DISCUSSION

As far as we know, this is the first time that HNE modification of erythrocyte proteins has been immunochemically characterized *in vitro*. Using a specific antibody (anti-HNE adducts antibody) for HNE bound to proteins, the covalent attachment of HNE to erythrocytes proteins was clearly demonstrated. This antibody was produced by immunizing rabbits against a carrier protein (KLH) treated with HNE, followed by affinity purification of immunoglobulins that recognize the Michael addition-type HNE-histidine adduct. It has been revealed that the antibody specifically recognizes the Michael-type HNE adducts of histidine, lysine, and cysteine as the antigenic sites (*11*). Later, the specificity of the antibody was elucidated to be the 2-$CH_3(CH_2)_n$-5-hydroxytetrahydrofuran ($n \geq 3$) moiety of the adducts (*20*). This antibody has a number of advantages for the analysis of oxidative tissue damage *in vivo*. The presence of HNE-derived epitopes has been assessed in human tissue samples, such as the human aorta with an atherosclerotic lesion (*20*), nigral neurons in Parkinson's disease (*21*), and renal cell carcinomas (*22*).

The observation that the pretreatment of erythrocytes with NEM resulted in a prominent decrease in the level of HNE attached to erythrocyte proteins provided evidence that the HNE modification of erythrocytes can be largely ascribed to the modification of sulfhydryl groups (Fig. 1). HNE is an electrophilic agent that rapidly reacts with the sulfhydryl groups of protein. Esterbauer *et al.* (*23*) have demonstrated that HNE is more reactive with thiol groups than are the α,β-unsaturated aldehydes lacking the C-4 hydroxy group. The electron-withdrawing 4-hydroxy group has been postulated to make the C-3 of HNE more electro-

positive and, thus, more susceptible to nucleophilic attack. The reaction of HNE with a mercaptan is expected to yield a saturated aldehyde as a primary product in which the sulfhydryl group of the mercaptan is attached *via* a thioether linkage to the C-3 of the HNE. Subsequently, the free aldehyde moiety of the primary product reacts with the 4-hydroxy group to form a cyclic hemiacetal derivative (*23-26*). Recent studies have demonstrated the deleterious effects of sulfhydryl-reaction agents on the membrane function of erythrocytes: they affect spectrin-ankyrin interaction (*27*), decrease spectrin self-association, resulting in membrane instability (*28*), alter membrane lipid asymmetry (*29*) and membrane deformability (*30*), and cause a decrease *in vivo* erythrocyte survival (*31*). Although the functional consequences of sulfhydryl modification by HNE are not yet clear, it is likely that the HNE modification of the sulfhydryl groups affects the membrane mechanical properties of deformability and mechanical stability.

On the other hand, it is important to note that some of the erythrocyte proteins underwent HNE modification, despite the sulfhydryl blockage with NEM (Fig. 1B). This may be explained by the reactivity of HNE with non-cysteinyl amino acids, such as lysine and histidine residues (*32-34*). It has been generally accepted that the aldehyde moiety of HNE can react with the ε-amino group of lysine residues of the protein to form Schiff's base conjugates, while recent experiments using glucose-6-phosphate dehydrogenase from *L. mesenteroides* demonstrated that the major reaction product of the lysyl amino group with HNE is a Michael addition-type HNE-lysine adduct resulting from the reaction of the free amino group of lysine with the double bond (C-3) of HNE (*32*). HNE also reacts with the imidazole nitrogen atoms of histidine residues in a Michael-type addition reaction (*33, 34*). As minor reactions, inter- and/or intra-molecular cross-links between cysteine and lysine residues through HNE have been demonstrated (*34*).

Treatment of erythrocytes with HNE leads to the highly selective modification of a single protein of 30 kDa (Fig. 4A). Initially, the protein was tentatively identified as carbonic anhydrase based on its molecular weight. To confirm this, erythrocytes treated with HNE were extracted with a chloroform/ethanol mixture and then analyzed by reverse-phase HPLC (Fig. 5A). Two major components were detected and found to have originated from carbonic anhydrase labeled with HNE. Although further characterization of both proteins has not yet been done, it is likely that they correspond to two major isozymes that have been found in rabbit erythrocytes (*10*). It should be noted that the HNE-bound carbonic anhydrase isozyme (Fig. 5B, lane 2) is apparently less immunoreactive to the anti-carbonic anhydrase antibody than the non-HNE-bound isozyme (Fig. 5B, lane 1). The data suggest that the immunological character of carbonic anhydrase bound to HNE is distinct from that of the native enzyme.

The major physiological role of carbonic anhydrase has been attributed to its catalysis of the ubiquitous interconversion of CO_2 and HCO_3^-. Although it was originally believed that carbonic anhydrase is absolutely specific for this reversible hydration, the high specific activity isozyme from mammalian erythrocytes has been shown to possess a broad catalytic activity covering an extensive list of reactions, including the hydration of numerous aldehydes (*19*). This invites speculation that this protein has a high affinity with aldehydic compounds including HNE and, therefore, could be a critical target for HNE. This may be supported by the previous finding that carbonic anhydrase is covalently inactivated by aclorein, an analog of HNE (*35, 36*). In addition, carbonic anhydrase, particularly isozyme III, is known to be potentially modified by many other processes, including oxidative modification (*37*) and *S*-glutathiolation (*37, 38*), both of which decrease its activity in response to oxidative stress. However, the erythrocyte carbonic anhydrase was found to be only partially inactivated by HNE, probably due to the modification of non-active-site amino acid residues. The determination of the sites of HNE modification is the subject of continuing investigations.

It is believed that GSH-dependent processes represent the most important defense against reactive species such as HNE. Hence, the magnitude of GSH loss directly reflects the magnitude of the decrease in resistance to the toxicity of HNE (*39, 40*). We have previously shown that the cellular GSH level is closely correlated to the sensitivity toward HNE: the cells were significantly sensitized to HNE when they were depleted of GSH, whereas the increase in the cellular GSH levels provided increased cellular resistance to HNE (*18*). The observation (Fig. 3) that GSH in the erythrocytes was significantly depleted by treatment with HNE led us to the assumption that, as the most abundant intracellular sulfhydryl, GSH represents the major target of HNE. The observation that the treatment of erythrocytes with 0.1 mM NEM resulted in ~70% loss of GSH (data not shown), but did not affect the level of HNE attached covalently to either membrane (Fig. 1) or intracellular proteins (Fig. 4) suggests that the erythrocyte GSH levels are not essentially associated with the sensitivity of the erythrocyte proteins to HNE. This may be correlated to the results with sickle cell erythrocytes, which generate an increased amount of active oxygen (*41*), and to the fact that the membranes have undergone oxidation of thiols (*42*), despite the fact that the GSH levels are essentially unchanged.

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