Structural Basis of Protein-bound Endogenous Aldehydes

CHEMICAL AND IMMUNOCHEMICAL CHARACTERIZATIONS OF CONFIGURATIONAL ISOMERS OF A 4-HYDROXY-2-NONENAL-HISTIDINE ADDUCT

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4-Hydroxy-2-nonenal (HNE), a major racemic product of lipid peroxidation, reacts with histidine to form a stable HNE-histidine Michael addition-type adduct possessing three chiral centers in the cyclic hemiacetal structure. In the present study, we characterized configurational isomers of a HNE-N\(^{\text{α}}\)-acethylhistidine adduct by NMR spectroscopy and by molecular orbital calculations. In addition, we raised monoclonal antibodies against (R)-HNE-histidine and (S)-HNE-histidine adducts, characterized their specificities, and examined in vivo localizations of each adduct under oxidative stress. To facilitate structural characterization of the configurational isomers of an HNE-histidine adduct, we prepared the (R)-HNE-histidine and (S)-HNE-histidine adducts by incubating N\(^{\text{α}}\)-acetylhistidine with each HNE enantiomer, both of which provided two peaks (Ra and Rb from (R)-HNE-histidine and Sa and Sb from (S)-HNE-histidine adducts) in reversed-phase high-performance liquid chromatography. The NMR analysis showed that each peak was a mixture of two diastereomers. In addition, the analysis of the nuclear Overhauser effect enabled the determination of configurations of the eight isomers. The relative amounts of these isomers in the NMR analysis correlated with the relative energies calculated by molecular orbital methods. On the other hand, using (R)-HNE-modified and (S)-HNE-modified keyhole limpet hemocyanins as the antigens, we raised the monoclonal antibodies, mAbR310 and mAbS412, which enantioselectively recognized the (R)-HNE-histidine and (S)-HNE-histidine adducts, respectively. Among the mixtures (Ra, Rb, Sa, and Sb) of diastereomers, mAbR310 showed the highest immunoreactivity to Rb (the mixture of 2R,4S,5R and 2S,4S,5R isomers), whereas mAbS412 preferentially recognized Sa (the mixture of 2R,4S,5S and 2S,4S,5S isomers). The presence of (R)-HNE and (S)-HNE epipods in vivo was immunohistochemically examined in the kidney of rats exposed to the renal carcinogen, ferric nitritriacetate, by which nuclear and cytosolic stainings with mAbR310 and mAbS412, respectively, were detected.

Several lines of evidence indicate that the oxidative modification of proteins and the subsequent accumulation of the modified proteins have been found in cells during aging, oxidative stress, and in various pathological states, including premature diseases, muscular dystrophy, rheumatoid arthritis, and atherosclerosis (1, 2). The important agents that give rise to the modification of a protein may be represented by lipid metabolites, such as 4-hydroxy-2-alkenals (3, 4). These metabolites are considered important mediators of cell damage due to their ability to covalently modify biomolecules, which can disrupt important cellular functions and can cause mutations (3). Furthermore, the addition of aldehydes to apolipoprotein B in low density lipoproteins has been strongly implicated in the mechanism by which low density lipoprotein is converted to an atherogenic form that is taken up by macrophages, leading to the formation of foam cells (5, 6).

4-Hydroxy-2-nonenal (HNE), a major product of lipid peroxidation (3, 7–9) and believed to be largely responsible for cytopathological effects observed during oxidative stress (3). HNE exerts these effects because of its facile reactivity with biological materials, particularly the sulf-hydryl groups of proteins (10). The reaction of HNE with sulf-hydryl groups leads to the formation of thiether adducts that further undergo cyclization to form cyclic hemiacetals (3, 11). Formation of thiol-derived Michael adducts, stabilized as cyclic hemiacetals, was initially considered to constitute the main reactivity of HNE (3). However, other studies led to the realization that HNE could form Michael adducts also with the imidazole moiety of histidine residues (12, 13) and the e-amino group of lysine residues (14). The HNE-histidine Michael adduct is readily isolable and is stabilized toward retro-Michael reaction, because of the poorer leaving group ability of imidazole over amine at neutral conditions.

Because HNE generated in lipid peroxidation is a racemic mixture of 4R- and 4S-enantiomers (15), the HNE-histidine Michael adduct, possessing three chiral centers at C-2, C-4, and C-5 in the tetrahydrofuran moiety, is presumed to be composed of at least eight isomers (see Scheme 1). However, the structures of HNE-histidine adducts in solution have not been fully characterized as yet with complicating 1H NMR diastereoscopic splittings by three chiral centers. In the present study, we

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characterized the configurational isomers of the HNE-N'-acetylhistidine adduct by NMR spectroscopy and by molecular orbital calculations. Moreover, we raised monoclonal antibodies against (R)-HNE-histidine and (S)-HNE-histidine adducts, characterized their specificities, and investigated the in vitro distributions of each adduct under oxidative stress.

EXPERIMENTAL PROCEDURES

Materials—N'-Acetyl-L-histidine and bovine serum albumin (BSA) were obtained from Sigma. Keyhole limpet hemocyanin (KLH) was obtained from Pierce. Ferric nitrate nonahydrate and sodium carbonate were from Wako (Osaka, Japan); nitrolotriacetic acid disodium salt was from Calbiochem (La Jolla, CA). Anti-HNE-histidine monoclonal antibody (mAbHNE-2) was kindly provided by Nihon Oil Factory Co. (Tokyo, Japan) (16). Horseradish peroxidase-linked anti-rabbit IgG immunoglobulin and ECL (enhanced chemiluminescence) Western blotting detection reagents were obtained from Amersham Biosciences (Buckinghamshire, UK).

Racemic and Enantioisomeric HNE—The stock solutions of HNE were prepared by the acid-treatment (1 mm HCl) of 4-hydroxy-2-nonenal dimethylacetal, which was synthesized according to the procedure of De Montarby et al. (17). (R)- and (S)-HNEs were prepared by the enzymatic resolution of racemic HNE (18) and purified by a chiral-phase HPLC on a ChiralPak AD-H column (0.46 × 15 cm) (Daicel Chemical Industries, Ltd., Osaka, Japan) eluted with a linear gradient of acetonitrile/water/2-acetic acid (90/10/0.01, v/v) (solvent A)—acetonitrile (solvent B) (time = 0–5 min, 100% A; 60 min, 0% A), at a flow rate of 0.8 ml/min. The elution profiles were monitored by UV absorbance at 224 nm. The concentrations of racemic and enantioisomeric HNE stock solutions were determined by the measurement of UV absorbance at 224 nm (19).

Reaction of N'-Acetylhistidine with Racemic or Enantioisomeric HNE—The reaction mixture (10 ml) containing 40 mm N'-acetylhistidine was incubated with 20 mM (R,S)-HNE, (R)-HNE, or (S)-HNE in 50 mM sodium phosphate buffer (pH 7.2). After incubation for 24 h at 37 °C, the reaction mixtures were analyzed with a reverse-phase HPLC on a Dovenlab ODS-HG-5 column (4.6 × 250 mm, Nomura Chemicals, Aichi, Japan) eluted with a linear gradient of acetonitrile/water/trifluoroacetic acid (90/10/0.01, v/v) (solvent A)—acetonitrile/trifluoroacetic acid (100/0.01, v/v) (solvent B) (time = 0–5 min, 100% A; 60 min, 0% A), at a flow rate of 1.0 ml/min. The elution profiles were monitored by absorbance at 280–400 nm.

In Vitro Modification of BSA—Modification of protein by HNE enantiomers was performed by incubating BSA (1.0 mg/ml) with 1–10 mM (R)-HNE or (S)-HNE in 1 ml of 0.1 M sodium phosphate buffer (pH 7.2) at 37 °C for 24 h.

General Procedures—NMR spectra were recorded using a Bruker AMX600 (600 MHz) instrument. Liquid chromatography-mass spectrometry was carried out with a Jasco PlatformII-LC instrument. Liquid chromatography-mass spectrometry was carried out with a Jasco PlatformII-LC instrument. Liquid chromatography-mass spectrometry was carried out with a Jasco PlatformII-LC instrument. Liquid chromatography-mass spectrometry was carried out with a Jasco PlatformII-LC instrument.

Molecular Orbital Calculations—The restricted Hartree-Fock (RHF) and density functional theory (DFT) with B3LYP exchange-correlation functional calculations for eight isomers of the model compound (5'-methyl-N'-2-hydroxy-5-methyltetrahydrofuran-4-yl)(imidazole) were performed by using the Gaussian 98 program package. All molecular geometries were fully optimized with RHF/8–31G* and DFT/P-31G* levels at C3 symmetry, respectively. After molecular geometries of each species were optimized, vibrational analysis calculations were also carried out to check whether they were correctly energy-minimized structures. All calculations were made on the HIT alpha 687d computer at Gifu University.

Antibody Preparation—Female BALB/c mice were immunized three times with (R)-HNE-treated or (S)-HNE-treated KLH. Spleen cells from the immunized mice were fused with P3.U1 myeloma cells and cultured in hypoxantine/aminopterin/thymidine selection medium. Culture supernatants of the hybridoma were screened using ELISA, employing pairs of wells of microtiter plates on which were absorbed (R)-HNE-treated or (S)-HNE-treated KLH. Hybridoma cells, corresponding to the supernatants that were positive on (R)-HNE-modified or (S)-HNE-modified BSA, were then cloned by limiting dilution. After repeated screening, four clones were obtained. Among them, clones R310 and S412 showed the most distinctive recognition of the (R)-HNE-modified and (S)-HNE-modified BSA, respectively.

Enzyme-linked Immunosorbutant Asssay—Cross-reactivity of antibodies for aldehyde-treated proteins was determined by a non-competitive ELISA. A coating antigen was prepared by incubating 1 mg of BSA with 2 mM aldehyde in 1 ml of 50 mM sodium phosphate buffer, pH 7.2, for 2 h at 37 °C. A 100-μl aliquot of the antigen solution was added to each well of a 96-well microtiter plate and incubated for 2 h at 37 °C. The antigen solution was then removed, and the plate was washed with Tris-buffered saline (TBS) containing 1% Tween 20 (TBS/Tween). Each well was incubated with 200 μl of 1% BSA in TBS/Tween for 30 min at 37 °C in a moist chamber to block the unsaturated plastic surface. The plate was then washed once with TBS/Tween. A 100-μl aliquot of antibody was added to each well and incubated for 1 h at 37 °C. After discarding the supernatants and washing three times with TBS/Tween, 100 μl of a 5 × 10−4 dilution of goat anti-rabbit IgG conjugated to horseradish peroxidase in TBS/Tween was added. After incubation for 1 h at 37 °C, the supernatant was discarded, and the plates were washed three times with TBS/Tween. Enzyme-linked antibody bound to the well was revealed by adding 100 μl/well of 1.2-phenylenediamine in 0.1 M citrate/phosphate buffer (pH 5.0) containing 0.003% hydrogen peroxide. The reaction was terminated by the addition of 50 μl of 2% sulfuric acid, and absorbance at 492 nm was read on a micro-ELISA plate reader.

In a competitive ELISA study, a competitor was incubated with antibody for 20 h at 4 °C to yield competitor/antibody mixtures containing antibody at 25 ng/ml and variable concentrations of the competitor. A 100-μl aliquot of competitor/antibody mixtures was added to each well and incubated for 1 h at 37 °C. After discarding the supernatants and washing three times with TBS/Tween, the second antibody was added and the enzyme-linked antibody bound to the well was revealed as described above. Results were expressed as the ratio, B/B0, where B = absorbance in the presence of competitor — background absorbance (no antibody) and B0 = absorbance in the absence of competitor — background absorbance.

SDS-PAGE—SDS-PAGE was performed according to Laemmli (20). The protein was stained with Coomassie Blue.

Immunoblot Analysis—The gel was transblotted onto a nitrocellulose membrane, incubated with Block Ace (40 mg/ml) for blocking, washed, and treated with the primary antibody. This procedure was followed by the addition of horseradish peroxidase conjugated to a goat anti-mouse IgG F(ab′)2 fragment and ECL reagents (Amersham Biosciences, Buckinghamshire, UK). The bands were visualized by exposure of the membranes to autoradiography film.

Animal Experiments—The ferric nitrolotriacetate (Fe3−-NTA) solution was prepared immediately before use by the method described by Toyokuni et al. (21) with a slight modification. Briefly, ferric nitrate enneahydrate and the nitrolotriacetic acid disodium salt were each dissolved in deionized water to form 80 and 160 mM solutions, respectively. They were mixed at the volume ratio of 1:2 (molar ratio, 1:4), and the pH was adjusted with sodium hydroxide carbonate to 7.4. Male SPF slc: Wistar rats (Shizuoka Laboratory Animal Center, Shizuoka), weighing 130–150 g (6 weeks of age), were used. They were kept in a stainless steel cage and given commercial rat chow (Funabashi F-2, Chiba) as well as deionized water (Millipore Japan, Osaka) ad libitum.
Animals received a single intraperitoneal injection of Fe\(^{3+}\)-NTA (15 mg of Fe\(^{3+}\)/kg body weight). They were sacrificed at 0, 24, and 48 h after the administration. The animals were sacrificed by decapitation. Both kidneys of each animal were immediately removed. One of them was fixed in Bouin’s solution, embedded in paraffin, cut at 3-μm thickness, and used for immunohistochemical analyses by an avidin-biotin complex method with alkaline phosphatase (22). Briefly, after deparaffinization with xylene and ethanol, normal rabbit serum (Dako Japan Co., Ltd., Kyoto; diluted to 1:75) for the inhibition of the nonspecific binding of the secondary antibody, a monoclonal antibody (mAbR310 or mAbS412) (0.5 μg/ml), biotin-labeled rabbit anti-mouse IgG serum (Vector Laboratories; diluted 1:300), and avidin-biotin complex (Vector; diluted 1:100) were sequentially used. Procedures, using phosphate-buffered saline or the IgG fraction (0.5 g/ml) of normal mouse serum instead of mAbR310 and mAbS412 antibodies, showed no or negligible positive responses.

RESULTS

Reactions of N\(^{\text{acetyl}}\)-Acetylhistidine with Racemic and Enantiomeric HNE—To facilitate structural characterization of configurational isomers of the HNE-histidine adduct, we prepared (R)-HNE-histidine and (S)-HNE-histidine adducts by incubating N\(^{\text{acetyl}}\)-acetylhistidine with (R)-HNE and (S)-HNE, respectively. As shown in Fig. 1, reversed-phase HPLC demonstrated that the reaction of N\(^{\text{acetyl}}\)-acetylhistidine with (S)-HNE in sodium phosphate buffer (pH 7.2) for 24 h at 37 °C gave two peaks (Sa and Sb). The reaction with (R)-HNE also provided two peaks (Ra and Rb). These HPLC profiles were similar to those observed in the reaction of N\(^{\text{acetyl}}\)-acetylhistidine with racemic HNE, providing RSa and RSb. The liquid chromatography-mass spectrometry analysis of these peaks gave a pseudomolecular ion peak at m/z 354 (M+H)\(^{+}\) (data not shown), which would be expected from the Michael addition-type HNE-histidine adducts, suggesting that they all represent the products derived from the Michael addition of the imidazole nitrogen atom to the C-3 of HNE.

NMR Characterization of HNE-Histidine Michael Adducts—The four peaks (Ra, Rb, Sa, and Sb) obtained from (R)-HNE-histidine and (S)-HNE-histidine adducts were then subjected to characterization by 600-MHz NMR. The assignment of the proton signals and stereochemistry could be made by analyzing \(^1\)H-\(^1\)H-correlation spectroscopy (COSY), \(^1\)H-detected multiple-bond heteronuclear multiple quantum coherence (HMBC), \(^1\)H-detected multiple-bond heteronuclear multiple quantum coherence (HMQC), and nuclear Overhauser and exchange spectroscopy (NOESY) spectrum recorded in D\(_2\)O. In all samples, we observed the correlation between the methine proton (C-4) of the tetrahydrofuran moiety and two imidazole vinyls (C-2’ and C-4’) in the HMBC spectrum (data not shown), indicating that the reaction exclusively occurred at the N’ position of the imidazole ring. This agrees with the previous assignment on the basis of the coupling constant between two imidazole vinyls (23). The COSY spectrum of Sa suggested that the peak was a mixture of two diastereomers (Sa1 and Sa2) (Supplementary Data Fig. 1). Similarly, other peaks (Ra, Rb, and Sb) also appeared to contain a pair of diastereomers (Supplementary Data Tables I and II). Identification of the proton signals of the tetrahydrofuran moiety in Ra, Rb, Sa, and Sb follows from the analysis of NOESY spectra collected in D\(_2\)O. In Sa, proton signals at 4.24 and 4.00 ppm, which were assigned to the methine protons at C-5 of Sa1 and Sa2, respectively, displayed NOE cross-peaks to the methine proton at C-4, whereas there were no such NOE cross-peaks in Sb (Sb1 + Sb2) (Supplementary Data Fig. 2), suggesting that the configurations at C-4 of Sa and Sb are S and R, respectively. Absolute configurations at C-2 and C-3 were determined by the NOE connectivity between the C-2 methine proton and C-3 methylene.
protons (H-3α and H-3β). We observed NOE cross-peaks between C-4 proton and C-3 proton (H-3β) and between C-3 proton (H-3α) and C-2 proton in Sa1, whereas the presence of NOE cross-peaks between the C-4 proton and C-3 (H-3α) proton and between the C-3 proton (H-3α) and C-2 proton were observed in Sa2. These data suggest that Sa1 and Sa2 correspond to 2R,4S,5S and 2S,4S,5S isomers, respectively. Similarly, the absolute configuration of the tetrahydrofuran moiety of Sb1 and Sb2 was determined to be 2S,4R,5S and 2R,4R,5S, respectively. Based on the assignment of these signals, relative amounts (% of Sb1, Sb2, Sb1, and Sb2 in D2O) were found to be 25, 8, 25, and 42, respectively. In a similar manner to the assignment of the (S)-HNE-histidine isomers, the absolute configuration of the four isomers (Ra1, Ra2, Rb1, and Rb2) of the (R)-HNE-histidine adduct were determined to be 2S,4R,5R, 2R,4R,5R, 2R,4S,5R, and 2S,4S,5R, respectively. In addition, their relative amounts (% of (R)-HNE-histidine isomers (Ra1: Ra2: Rb1: Rb2 = 25:8:25:42) were exactly comparable to those of the (S)-HNE-histidine isomers. Fig. 2 summarizes the configurations of the eight isomers of the HNE-N-acetylhistidine adduct. For each of the isomers, the (R)-HNE-histidine adduct structure is essentially a mirror image of the (S)-HNE-histidine adduct. Furthermore, the NMR data on the eight isomers showed assignment of the signals in the one-dimensional 1H-NMR analysis of the (R,S)-HNE-N-acetylhistidine adduct (Supplementary Data Fig. 3).

Molecular Orbital Calculations—To elucidate the fundamental structural principles that govern the tetrahydrofuran ring in the histidine adducts and to relate the results to experimental observations in the NMR analysis, molecular orbital calculations on the eight isomers of the model compound (5′-methyl-1′-2-hydroxy-5-methyltetrahydrofuran-4-yl)imidazole) were performed. Table I shows the total energies in atomic mass units and relative energies in kilocalories/mol. There is well with the relative amounts of the isomers of the (R)-HNE-histidine and (S)-HNE-histidine adducts found in the NMR analysis. Fig. 3 shows optimized structures of eight isomers calculated by the DFT/6–31G* method. The values in parentheses are electronic charges on atoms. The instability of each molecule increases in the orders of Rb2, Rb1, Ra1, Ra2 and of Sb2, Sb1, Sa1, Sa2, Sa1, Rb2, Rb1, Ra1, Ra2 and of Sb2, Sb1, Sa1, Sa2, Sa1, Rb2, Rb1, Ra1, Ra2 and of Sb2, Sb1, Sa1, Sa2, Sa1, Rb2, Rb1, Ra1, Ra2 and of Sb2, Sb1, Sa1, Sa2.

The restricted Hartree-Fock (RHF) and density functional theory (DFT) with B3LYP exchange-correlation functional calculations for eight isomers of the model compound (5′-methyl-1′-2-hydroxy-5-methyltetrahydrofuran-4-yl)imidazole were performed by using the Gaussian 98 program package. All molecular geometries were fully optimized at RHF/6–31G* and DFT/6–31G* levels at C2 symmetry, respectively. After molecular geometries of each species were optimized, vibrational analysis calculations were also carried out to check whether they are correctly energy minimized structures.

**TABLE I**

| Models | Total energy | Relative energy |
|--------|--------------|-----------------|
| Ra1    | −607.578532  | 1.45            |
| Ra2    | −607.578264  | 2.87            |
| Rb1    | −607.580414  | 0.27            |
| Rb2    | −607.580840  | 0.00            |
| Sa1    | −607.578532  | 1.45            |
| Sa2    | −607.576264  | 2.87            |
| Sb1    | −607.580413  | 0.27            |
| Sb2    | −607.580840  | 0.00            |

HNE-histidine Michael Adducts—We have previously raised a monoclonal antibody (mAbHNEJ2) against HNE-modified protein and found that it recognizes the HNE-histidine adduct as the epitope (21). This antibody has been widely used for assessing oxidative stress in vitro (24) and in vivo (25–28). We examined the binding of the antibody to the (R)-HNE-histidine and (S)-HNE-histidine adducts and found that it equally recognized both adducts. Hence, to evaluate the distribution of the (R)-HNE-histidine and (S)-HNE-histidine adducts separately, we attempted to raise a monoclonal antibody directed toward each adduct. To this end, mice were immunized individually with (R)-HNE-modified and (S)-HNE-modified keyhole limpet hemocyanins. During the preparation of the monoclonal antibodies, hybridomas were selected by the reactivities of the culture supernatant to the (R)-HNE-modified and (S)-HNE-modified BSA, respectively. We finally obtained the monoclonal antibodies, mAbR310 and mAbS412, which specifically recognized (R)-HNE-modified and (S)-HNE-modified proteins, respectively (Fig. 4, A and B). The ELISA study attested that, among reactive aldehydes tested, only (R)-HNE and (S)-HNE generated the epitopes that could be recognized by mAbR310 and mAbS412, respectively (Fig. 4C).

Then, we characterized the antibody’s ability to recognize specific molecular targets in their native three-dimensional structure. As shown in Fig. 5A, binding of the HNE-modified protein to mAbR310 and mAbS412 was scarcely inhibited by the HNE-cysteine and HNE-lysine adducts but significantly inhibited by the HNE-histidine adduct. Approximately 5 nmol of the HNE-histidine adduct per well (100 μl) caused 50% inhibition of antibody binding to the HNE-modified protein, whereas at least 10-fold higher concentrations of HNE-lysine or HNE-cysteine adduct were necessary for the same inhibition. These data indicate that both antibodies represent the anti-HNE-histidine monoclonal antibodies. To further examine whether configuration of the tetrahydrofuran moiety of HNE-histidine is involved in the antibody binding, immunoreactivity of mAbR310 and mAbS412 to the mixtures (Ra, Rb, Sa, and Sb) of two diastereomers was tested. As shown in Fig. 5B, mAbR310 showed the highest immunoreactivity to Rb (the mixture of 2R,4S,5R and 2S,4S,5R isomers), whereas mAbS412 preferentially recognized Sa (the mixture of 2R,4S,5S and 2S,4S,5S isomers). Based on the common configurations in each sample, the 4S,5R and 4S,5S configurations of the tetrahydrofuran moiety were suggested to be critical in the binding of mAbR310 and mAbS412, respectively.

* M. Hashimoto and K. Uchida, unpublished data.
In Vivo Distribution of (R)-HNE and (S)-HNE-derived Epitopes—Formation of (R)- and (S)-HNE-derived epitopes in vivo was immunohistochemically assessed in a rat renal carcinogenesis model with Fe^{3+}-NTA. The monoclonal antibody mAbHNEJ2 (16), which recognizes the (R)-HNE-histidine and (S)-HNE-histidine adducts equally, was also used for comparison. It has been shown that iron overload using Fe^{3+}-NTA induces acute renal proximal tubular necrosis, a consequence of oxidative tissue damage, that eventually leads to a high incidence of renal adenocarcinoma in rodents (29, 30). The kidneys were excised at the time of sacrifice and then fixed with Bouin’s fixative. The hematoxylin and eosin-stained sections of the paraffin-embedded tissues were analyzed for histological damage. The morphological changes in the kidneys of rats treated with Fe^{3+}-NTA versus time are very similar to previous reports on ddY mice (21, 31).
control rat kidney, an almost negligible level of immunoreactivity was observed (data not shown). The immunoreactivities appeared in some of the renal proximal tubular cells 3 h after the administration of 15 mg of Fe^{3+}/kg of body weight of Fe^{3+}-NTA, whereas the patterns of distribution of (R)- and (S)-HNE epitopes appeared to be significantly different. As shown in Fig. 6 (Top), consistently with the previous observation (21), the (R,S)-HNE epitope immunoreactive with mAbHNEJ2e were mainly detected in the cytoplasm and in some of the nuclei. This pattern of distribution in the rat kidney was consistent with that of the distribution of other lipid peroxidation products and their conjugates with cytosolic proteins (32, 33), suggesting a correlation between the production of racemic HNE and oxidative stress. Similarly to the (R,S)-HNE epitope, the distribution of (S)-HNE epitopes in the proximal tubular cells is consistent with that of the (R,S)-HNE epitope (Fig. 6, bottom). However, the (R)-HNE epitopes were mainly located in the nuclei (Fig. 6, middle). Pre-absorption of the antibodies with HNE-histidine adducts completely abolished the immunostainings (data not shown), indicating the specific reactivity of these antibodies with their epitopes. These data suggest that (R)- and (S)-HNE may exert distinct effects on cellular functions under oxidative stress. The nuclear staining of (R)-HNE epitope, in particular, may reflect the mutagenic and cytotoxic potential of HNE.

**DISCUSSION**

Structural information on the nature of the HNE modification of histidine side chain was first reported by Uchida and Stadtman (12). The observation, that reduction of the aldehyde group of the primary Michael addition product with sodium borohydride converts them to the hydroxy derivatives that are stable to strong acid hydrolysis, formed the basis of methods for the identification and quantification of the HNE-histidine Michael adduct of proteins by conventional amino acid analytical techniques (12). It was shown by means of these techniques that at least 80% of the histidine residues that were lost when human plasma low density lipoprotein was treated with HNE were accounted for by the Michael addition product (34). Based on these findings, it had been proposed that the Michael addition of imidazole groups to the double bond of HNE represents the dominant reaction pathway for the histidine residue, existing as a cyclic hemiacetal. Later, definitive evidence for the structure of the HNE-histidine Michael adduct was provided (23). However, until this study, the stereochemistry of the HNE-histidine adduct in solution had remained to be investigated.

Due to the presence of three chiral centers at C-2, C-4, and C-5 in the tetrahydrofuran moiety, the HNE-histidine Michael adduct was suggested to be composed of at least eight configurational isomers (Scheme 1) (12, 23). In addition, the previous observations that (i) the HNE-N\(^{\alpha}\)-acetylhistidine Michael adduct was detected as two peaks upon reverse-phase HPLC analysis and (ii) four peaks were detected in the acid-hydrolysis followed by the amino acid analysis of their \(\alpha\)-phenylaldehyde derivatives (12) also suggested the multiplicity of primary products in the HNE-N\(^{\alpha}\)-acetylhistidine reaction. However, with complicating diastereoscopic splittings by three chiral centers, the proton NMR spectrum of (R,S)-HNE-histidine adduct was too complex to analyze directly (23). In the present study, to facilitate the structural characterization of HNE-histidine isomers, we prepared (R)-HNE- and (S)-HNE-N\(^{\alpha}\)-acetylhistidine adducts separately, both of which provided two peaks (Ra and Rb) from (R)-HNE-histidine and Sa and Sb from (S)-HNE-histidine with relative amounts of 1:2 in the reverse-phase HPLC analysis (Fig. 1). With regard to the reactivity of HNE enantiomers toward the histidine derivative, we could not see any differences in the rate of formation of the Michael adducts between two HNE enantiomers, suggesting that the chirality of the C-4 hydroxy group may not affect the reactivity at the C-3 double bond with the imidazole group of the histidine derivative.

The 600-MHz NMR analysis of the four peaks (Sa, Sb, Ra, and Rb) revealed that each peak contained a pair of diastereomers (Sa1 and Sa2, Sb1 and Sb2, Ra1 and Ra2, and Rb1 and Rb2). In addition, we determined the absolute configurations of eight isomers by NOE analysis (Supplementary Data Tables I and II) and finally assigned the signals of the isomers in the one-dimensional proton NMR spectrum of the (R,S)-HNE-N\(^{\alpha}\)-acetylhistidine Michael adduct (Supplementary Data Fig. 3). Our assignment of the eight isomers of the HNE-N\(^{\alpha}\)-acetylhistidine Michael adducts confirms the recent proposal of Nadkarni and Sayre (23) made on the basis of an indirect comparative experiment. The NMR analysis also revealed the presence of ring-opened structures in the four samples dissolved in 100% CD\(_3\)OD.\(^2\) It was observed that, in methanol solution, relative amounts of the ring-opened structure were similar to those of the ring-closed adducts in the fractions Ra and Sa, whereas the ring-opened structure was much more predominant than the closed structure in the fractions Rb and Sb. These observations suggest that the configurations in the ring-opened structure may be critical in the equilibrium between the ring-opened and ring-closed structures.

In molecular orbital calculation of the model compound of the HNE-histidine adduct (5’-methyl-N\(^{\alpha}\)-2-hydroxy-5-methyltetrahydrofuran-4-ylimidazole), there was a correlation between
Competitors were as follows: mAbS412. The HNE-modified BSA was used as the absorbed antigens. With the Michael addition-type HNE adducts. Left, HNE-acetyllysine; □, HNE-N\textsuperscript{2}-acetyllysine; ○, HNE-N\textsuperscript{3}-acetyllysine; ▪, HNE-N\textsuperscript{4}-acetyllysine; ◊, HNE-N\textsuperscript{5}-acetyllysine; ▲, HNE-N\textsuperscript{6}-acetyllysine; □, HNE-N\textsuperscript{7}-acetyllysine. A, competitive ELISA with the four peaks (Ra, Rb, Sa, and Sb) obtained from the (R)-HNE-histidine and (S)-HNE-N\textsuperscript{2}-acetylhistidine adducts. Left, mAbR310; right, mAbS412. The HNE-modified BSA was used as the absorbed antigens. Competitors were as follows: △, Sa; ○, Sb; ▲, Ra; and ◊, Rb.

Fig. 5. Binding of mAbR310 and mAbS412 to the configurational isomers of HNE-histidine adducts. A, competitive ELISA with the Michael addition-type HNE adducts. Left, mAbR310; right, mAbS412. The HNE-modified BSA was used as the absorbed antigens. Competitors were as follows: ◊, HNE-N\textsuperscript{2}-acetylhistidine; ▲, HNE-N\textsuperscript{3}-acetyllysine; ▪, HNE-N\textsuperscript{4}-acetyllysine; ○, HNE-N\textsuperscript{5}-acetyllysine; △, HNE-N\textsuperscript{6}-acetyllysine; □, HNE-N\textsuperscript{7}-acetyllysine. B, competitive ELISA with the four peaks (Ra, Rb, Sa, and Sb) obtained from the (R)-HNE-histidine and (S)-HNE-N\textsuperscript{2}-acetylhistidine adducts. Left, mAbR310; right, mAbS412. The HNE-modified BSA was used as the absorbed antigens. Competitors were as follows: △, Sa; ○, Sb; ▲, Ra; and ◊, Rb.

Immunological detection is a powerful tool that can be used to evaluate the presence of a desired target and its subcellular localization. Major advantages of this technique over biochemical approaches are the evaluation of small numbers of cells or archival tissues that may otherwise not be subject to analysis. Because of the increasing interest in HNE and HNE modification of proteins under oxidative stress, it seemed useful to prepare an antibody interacting specifically with the HNE moiety or with the HNE-amino acid conjugates in proteins; such antibodies have been prepared by immunizing rabbits with HNE-treated KLH (35), in which HNE adducts of histidine, lysine, and cysteine serve as the antigenic sites. Later, Toyokuni et al. (21) raised the monoclonal antibody (mAbHNEJ2) against HNE-modified KLH and found that the antibody cross-reacted specifically with HNE-modified proteins and had a higher affinity for HNE-histidine adduct than for HNE-lysine and HNE-cysteine adducts (21). However, most of the antibodies, including mAbHNEJ2, directed to the HNE-modified protein have been raised against a protein treated with racemic HNE. Therefore, nothing is known about which of the two enantiomers bound to proteins is generated in vitro and in vivo. Hence, in the present study, we raised novel monoclonal antibodies, mAbR310 and mAbS412, against (R)-HNE-treated and (S)-HNE-modified KLH, respectively. It was observed that mAbR310 and mAbS412 showed the highest affinity for the (R)-HNE-treated and (S)-HNE-treated proteins, respectively (Fig. 4, A and B), and scarcely reacted with the proteins treated with other aldehydes (Fig. 4C). The lack of cross-reactivity of the antibodies for the 2-nonenal-treated protein can be ascribed to the absence of the 4-hydroxy group, which leads the primary Michael adduct to the tetrahydrofuran derivative through an intramolecular cyclization. This and the observation (Fig. 4C) that both antibodies did not cross-react with the proteins that had been treated with the HNE analogs, such as 4-hydroxy-2-pentenal, 4-hydroxy-2-hexenal, 4-hydroxy-2-heptenal, 4-hydroxy-2-octenal, and 4-hydroxy-2-decenal, suggest that both tetrahydrofuran and butyl moieties of the HNE-histidine adduct may be critical for the antibody binding. In addition, the binding of these antibodies to the HNE-treated proteins was selectively inhibited by HNE-histidine adducts, suggesting that the imidazole ring is also involved in the antibody binding. Thus, we propose that mAbR310 and mAbS412 recognize the N\textsuperscript{2}(2-hydroxy-5-butyltetrahydrofuran-4-yl)imidazole as the common epitopes. Furthermore, we characterized the antibodies’ ability to recognize the configurations of the tetrahydrofuran moiety of the HNE-histidine adduct and found that mAbR310 and mAbS412 preferentially reacted with the mixture (Rb) of 2R,4S,5R and 2S,4S,5R isomers and the mixture (Sa) of 2R,4S,5S and 2S,4S,5S isomers, respectively (Fig. 5). Based on the common configurations in each mixture, we suggested that both antibodies might recognize the configurational isomers of 4-Hydroxy-2-nonenal-histidine Adduct.
tions at C-4 and C-5 of the tetrahydrofuran ring in the adduct, i.e. mAbR310 and mAbS412 recognize the 4S,5R and 4S,5S configurations, respectively.

The presence of immunoreactive materials with mAbR310 and mAbS412 in vivo was demonstrated in the kidney of rats exposed to Fe3+-NTA. The iron chelate was originally used for an experimental model of iron overload (36). Repeated intraperitoneal injections of Fe3+-NTA were reported to induce acute and subacute renal proximal tubular necrosis and a subsequent high incidence (60–92%) of renal adenocarcinoma in male rats and mice (28, 29). A single injection of Fe3+-NTA causes a number of time-dependent morphological alterations in the structure and the function of the renal proximal tubular cells and their mitochondria. During the early stage of injury, typical cellular changes are the loss of brush border, cytoplasmic vesicles, mitochondrial disorganization, and dense cytoplasmic deposits in the proximal tubular cells. Most of the damaged epithelia show the typical appearance of necrotic cells, and more than half of the proximal tubular cells are removed. It has been suggested that oxidative stress is one of the basic mechanisms of Fe3+-NTA-induced acute renal injury and is closely associated with renal carcinogenesis (37).

The present study using mAbR310 and mAbS412 demonstrated that, in agreement with the previous observation on racemic HNE (21), both (R)-HNE and (S)-HNE epitopes were mainly detected in the proximal tubules, the target organs of Fe3+-NTA (Fig. 6). However, the intracellular localization of these epitopes was markedly different. The (S)-HNE epitopes were detected in cytosols and in some of the nuclei, whereas the (R)-HNE epitopes were mainly detected in the nuclei. Although the mechanism of the distinct localization of (R)- and (S)-HNE epitopes is currently unknown, these results invite speculation that there may be a differential mechanism for production of (R)- and (S)-HNE or there may be specific targets of each enantiomer in the cells. Clearly, it is important to establish the mechanism for the differential cellular distributions of (R)- and (S)-HNE epitopes in the cells. Furthermore, the characterization of the biological consequences of their distributions also merit immediate attention.

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