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Research Paper

2-Alkenal modification of hemoglobin: Identification of a novel hemoglobin-specific alkanoic acid-histidine adduct

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ABSTRACT

α,β-Unsaturated aldehydes generated during lipid peroxidation, such as 2-alkenals, give rise to protein degeneration in a variety of pathological states. 2-Alkenals are highly reactive toward nucleophilic amino acid residues, such as histidine and lysine, to form Schiff base adducts or Michael addition adducts. In this study, upon the reaction of hemoglobin with 2-octenal, we unexpectedly detected a product corresponding to the reduced form of the 2-octenal-histidine Michael adduct plus 14 mass unit. Based on the LC-ESI-MS/MS analysis of synthetic adduct candidates, the adduct was identified to be N^{τ}-(1-carboxyheptan-2-yl)-histidine (CHH), a novel alkanoic acid-type histidine adduct. The alkanoic acid-histidine adducts were detected in the 2-alkenal-treated hemoglobin and myoglobin, but not in the 2-alkenal-treated cytochrome c and transferrin. The addition of hemin to the reaction mixture, containing a non-heme protein and 2-alkenals, resulted in the formation of the alkanoic acid-histidine adducts, suggesting that a heme iron may play a role in the oxidation of covalently modified proteins. Moreover, using the stable isotope dilution method, we showed evidence for the endogenous formation of CHH in red blood cells exposed to hydrogen peroxide. Thus, this study establishes a novel mechanism for covalent modification of proteins by 2-alkenals, in which heme iron is involved in the formation of the alkanoic acid-histidine adducts. The potential implications of this novel adduct are discussed.

1. Introduction

The lipid peroxidation reaction represents a decomposition process in the body arising from the production and propagation of free radical reactions primarily involving membrane polyunsaturated fatty acids (PUFAs), such as linoleic acid and arachidonic acid. This reaction has been implicated in the pathogenesis of various diseases, such as cancer, diabetes, atherosclerosis, as well as aging [1]. The peroxidative degradation of PUFAs leads to the generation of a broad array of different molecules with diverse chemical and biological properties [2]. In the presence of transition metals, the lipid hydroperoxides, which are the primary products of the peroxidation reaction, can undergo C—C bond cleavage *via* alkoxyl radicals to generate unesterified aldehydes of 3–9 carbons in length, and core-aldehydes (aldehydes still esterified to the parent lipid). Due to the electrophilic properties, these reactive aldehyde molecules readily react with cellular macromolecules, including proteins, to form covalent adducts, leading to the disruption of important biological functions. Among them, α, β-unsaturated aldehydes, such as 2-alkenals, and 4-hydroxy-2-alkenals, are important agents to form the covalent modification of proteins [3,4].

2-Alkenals represent one of the groups of highly-reactive lipid aldehydes possessing two electrophilic centers, carbon positions 1 and 3. It has been suggested that 2-alkenals primarily react with the nucleophilic amino acid residues, such as lysine, cysteine, and histidine, in the proteins [3,5]. The modification of histidine by 2-alkenals primarily consists of the Michael addition reaction of the nitrogen atom in the imidazole moiety to the α, β-unsaturated bond. On the other hand, the modification of lysine by 2-alkenals is known to be quite diverse compared to the histidine modification. 2-Alkenals can undergo a nucleophilic addition reaction of the ε-amino group of the lysine residue at the carbon position 1 (aldehyde moiety) and the carbon position 3 (double bond) to form pyridinium adducts *via* Schiff base adducts, and

Abbreviations: LC-ESI-MS/MS, liquid chromatography with electrospray ionization tandem mass spectrometry; PUFA, polyunsaturated fatty acids; HSA, human serum albumin; PP IX, protoporphyrin IX; RBCs, red blood cells; SRM, Selected reaction monitoring; *m/z*, mass-to-charge ratio; CHH, N^{τ}-(1-carboxyheptan-2-yl)-histidine; LDL, low-density lipoproteins

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Michael adducts including the β-substituted and 3-formyl-3,4-dehydropiperidino adduct, respectively [6]. The reactions of protein with 2-alkenals have been mainly studied with acrolein, crotonaldehyde, and 2-nonenal. Pocker and Janjic [7] identified the formylethylated histidine derivative as the predominant histidine adduct using the N^{α}-acetylhistidine and imidazole derivative. Uchida and co-workers reported the modifications of lysine with acrolein. Upon incubation of N^{α}-acetyllysine with acrolein, major adduct was detected by HPLC analysis. After purification, they identified N^{ϵ}-(3-formyl-3,4-dehydropiperidino)lysine, which requires attachment of two acrolein molecules to one lysine side chain, using 1H and 13C NMR and LC-MS [8]. This and the fact that crotonaldehyde also forms a similar adduct, N^{ϵ}-(2,5-dimethyl-3-formyl-3,4-dehydropiperidino)lysine [9], suggest that this type of condensation reaction is characteristic of the reaction of 2-alkenals with primary amines. In addition, Furuhata et al. [10] characterized the acrolein modification of a model peptide (the oxidized B chain of insulin) by LC-ESI-MS/MS and established a novel acrolein-lysine condensation reaction. Based on the analysis of both acrolein-modified peptide and lysine derivatives, they identified the pyridinium-type lysine adduct, N^{ϵ}-(3-methylpyridinium)lysine, as the novel acrolein-lysine adduct. Moreover, Ishino et al. identified *cis*- and *trans*-N^{ϵ}-3-[(hept-1-enyl)-4-hexylpyridinium]lysine as major 2-nonenal-lysine adducts [11]. They also demonstrated that these adducts were indeed formed during the lipid peroxidation-mediated modification of protein *in vitro* and *in vivo* by the stable isotope dilution-based LC-ESI-MS/MS and immunohistochemical techniques with the specific antibody.

In the present study, to gain further structural insight into covalent modification of the proteins by lipid peroxidation products, we carried out a comprehensive analysis of the histidine adducts in the 2-alkenal-modified proteins and identified an adduct specifically generated in the 2-alkenal-modified heme proteins.

2. Materials and methods

2.1. Materials

Human serum albumin (HSA) and human hemoglobin was purchased from Sigma. According to the supplier, because the hemoglobin protein was exposed to air during purification and packaging, it would be predominantly methemoglobin. 2-alkenals (acrolein, crotonaldehyde, 2-pentenal, 2-hexenal, 2-heptenal, 2-octenal, 2-nonenal, 2-decanal, 2-undecanal, 2-dodecanal) were purchased from Tokyo Chemical Industry (Tokyo, Japan) and Wako Pure Chemical Industry (Osaka, Japan).

2.2. Preparation of 2-alkenal-treated proteins and acidic hydrolysis

Human hemoglobin or HSA (1 mg/ml) was treated with 1 mM 2-alkenals at 37 °C for 24 h in PBS. The reaction mixture was reduced by the addition of 100 mM $NaBH_4$ at 4 °C overnight and then treated with a quarter volume of 50% trichloroacetic acid on ice for 1 h following neutralization by HCl. After centrifugation, the proteins were washed with cold acetone, then hydrolyzed under acidic conditions for 24 h at 110 °C, and dried under vacuum conditions in a desiccator. The dried sample was dissolved in ethanol and subjected to analysis.

2.3. Preparation of red blood cells from human blood

Blood from a healthy human volunteer was immediately treated with EDTA and fractionated the plasma and red blood cells by centrifugation (1200 × *g*, 4 °C, 10 min). The RBC fraction (precipitate) was washed three times with equal volumes of PBS.

2.4. Adductome analysis of protein using LC-ESI-MS/MS

The hydrolyzed samples were subjected to the adductome analysis using a TQD triple stage quadrupole mass spectrometer (Waters) equipped with an ACQUITY ultra-performance LC system (Waters) on a reverse-phase column (Develosil HB-C30-UG 3-μm column (100 × 2.0 mm), Nomura Chemical). Elution was performed using mobile phase A (0.1% formic acid) and B (methanol) at the flow rate of 0.3 ml/min with a discontinuous gradient as follows: 1% B at 0 min, 1% B at 1 min, 99% B at 15 min, and 99% B at 20 min. Selected reaction monitoring (SRM) was performed in the positive ion mode using nitrogen as the nebulizing gas under the following conditions: ion source temperature, 120 °C; desolvation temperature, 350 °C; cone voltage, 25 V; collision energy, 25 eV; desolvation gas flow rate, 700 L/h; cone gas flow rate, 50 L/h; collision gas, argon. The strategy was designed to detect the product ion of *m/z* 110.0 from the positively ionized histidine adducts by monitoring the sample transmitting their $[M + H]^+ > 110.0$ transitions. The MS data could be visualized as a two-dimensional image, in which the *x* axis represents the retention time (min), *y* axis represents the mass-to-charge ratio (*m/z*) for the individual detected adducts. The adductome maps are shown with a size of circle encoding the relative abundance.

2.5. Preparation of N^{τ}-(1-carboxyheptan-2-yl)-histidine (CHH) and its stable isotope

N^{τ}-(1-Carboxyheptan-2-yl)-histidine (CHH) and its stable isotope-labeled sample were prepared by a previously described method [12]. Briefly, the 10 mM histidine or [$^{13}C_6$] histidine stable isotope was reacted with 1 mM 2-octenal in PBS. After incubation for 6 h at 37 °C, the reaction mixtures were treated with $NaClO_2$ and the oxidized product, CHH, was purified by reverse-phase HPLC with a Develosil HB C30-UG-5 column (100 mm × 8.0 mm, Nomura Chemical, Japan), eluted with a linear gradient of water containing 0.1% trifluoroacetic acid (solvent A)-acetonitrile containing 0.1% trifluoroacetic acid (solvent B) (time = 0 min, 0% B; 40 min, 100% B) at a flow rate of 2.0 ml/min.

2.6. LC-ESI-MS/MS analysis of 2-alkenal-histidine Michael adduct and 2-alkanoic acid histidine adduct

A mass spectrometric analysis was performed using the ACQUITY TQD system (Waters) equipped with an ESI probe and interfaced with a UPLC system (Waters). The sample injection volume of 10 μl each was separated on a Develosil HB C30-UG-3 (100 mm × 2.0 mm, Nomura Chemical, Japan) at the flow rate of 0.3 ml/min under the following elution conditions: mobile phase A (0.1% formic acid) and B (methanol) with a linear gradient from 1% to 99% mobile phase B in 6 min. A mass spectrometric analysis in the positive ion mode was performed with the SRM mode (cone potential 40 eV/collision energy 30 eV) and product ion scan mode (precursor ion; *m/z* 298, cone potential 30 eV/collision energy 30 eV). The monitored SRM transitions were as follows: [$^{13}C_6$]-CHH, *m/z* 304 > 116, and CHH, *m/z* 298 > 110. The quantification of CHH was performed by the ratio of the peak area of the target adduct and of the stable isotope labeled CHH.

2.7. Quantification of CHH in 2-octenal or H_2O_2 treated red blood cells (RBCs)

The RBCs were treated with 1 mM 2-octenal or 10 mM H_2O_2 containing 100 μM sodium nitrite at 37 °C in 10 mM sodium phosphate buffer (pH 7.4) supplemented with 152 mM NaCl. After 24 h of incubation, the RBCs were reduced under alkaline conditions by 100 mM $NaBH_4$ at 4 °C overnight, then treated with a quarter volume of 50% trichloroacetic acid on ice for 1 h following neutralization by HCl. After centrifugation, the proteins were washed with cold acetone and hydrolyzed under acidic conditions for 24 h at 110 °C, then dried under vacuum conditions in the desiccator. The dried sample was dissolved in ethanol containing a stable isotope labeled CHH as the internal standard and subjected to a quantitative analysis. The quantitative analysis

was repeated for at least three different preparations.

2.8. Statistical analysis

All experiments for quantification were repeated for at least three deferent preparations. All data are expressed as means ± S.D. Statistically significant differences were determined using the unpaired *t*-test.

3. Results

3.1. Comprehensive analysis of histidine adducts in the 2-alkenals-modified proteins

Glycated hemoglobins have been widely used as a useful biomarker for diabetic complications [13,14]. Based on our speculation that hemoglobin might undergo covalent modification by lipid peroxidation products, we attempted to identify a hemoglobin-specific adduct using the adductome approach [15]. Both HSA and hemoglobin were incubated with 1 mM 2-alkenals (2-octenal, 2-nonenal, and 2-decenal) at 37 °C for 24 h in PBS. After reduction with $NaBH_4$, they were hydrolyzed under the conventional acidic conditions, then subjected to the adductome analysis against the histidine residue using LC-ESI-MS/MS (Fig. 1). As shown in Fig. 2, most of the peaks, corresponding to the 2-alkenal-histidine Michael adducts, namely 2-octenal-histidine (*m/z* 284), 2-nonenal-histidine (*m/z* 298) and 2-decenal-histidine (*m/z* 312), were detected in both modified proteins. However, the adducts X_{C8} (*m/z* 298), X_{C9} (*m/z* 312), and X_{C10} (*m/z* 326), corresponding to the reduced form of the 2-alkenal-histidine Michael adducts plus 14 mass unit, were detected only in the 2-alkenal-modified hemoglobin, but not in the modified HSA. The data suggested the formation of histidine adducts unique to the 2-alkenal-modification of hemoglobin.

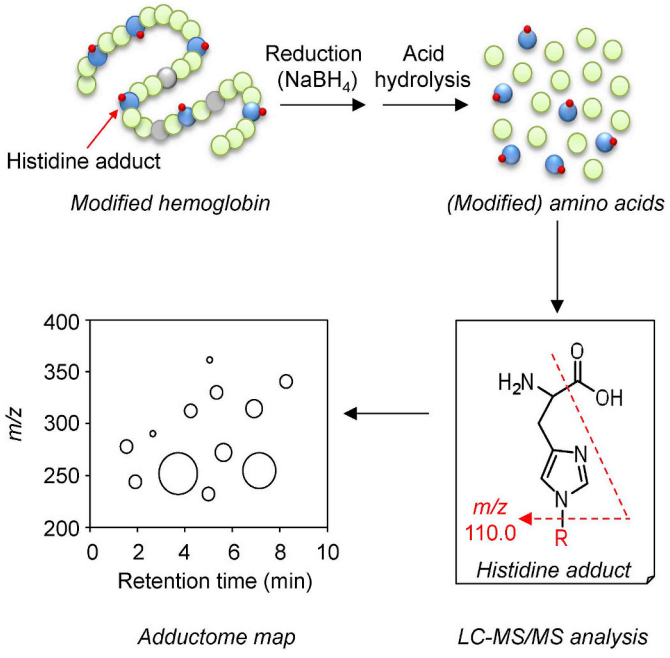

Fig. 1. Schematic illustration of strategy for histidine adductome analysis. Hemoglobin samples were reduced with $NaBH_4$ to stabilize the unstable adducts and then hydrolyzed to free (modified) amino acids under the conventional acidic conditions. The resulting free amino acids mixture was subjected to LC-ESI-MS/MS analyses. The strategy was designed to detect the product ion of *m/z* 110.0 (immonium ion of histidine) from the positively ionized histidine adducts by LC-ESI-MS/MS.

3.2. Identification of a histidine adduct unique to the 2-alkenal-modified hemoglobin

When the kinetics for the formations of the 2-octenal-histidine Michael adduct and X_{C8} were determined by LC-ESI-MS/MS, the formation of X_{C8} steadily increased up to 24 h, whereas the 2-octenal-histidine Michael adduct increased in the early phase and thereafter decreased (Fig. 3A). The data suggest a possibility that the 2-octenal-histidine Michael adduct may be a precursor of X_{C8}. In addition, the high resolution ESI-MS of X_{C8} showed a molecular ion peak at *m/z* 298.1765, $[M+H]^+$, corresponding to the molecular formula of $C_{14}H_{23}N_3O_4$. These and the fact that the molecular formula of the reduced form of the 2-octenal-histidine Michael adduct is $C_{14}H_{25}N_3O_3$ suggested that the aldehyde group of the 2-octenal-histidine Michael adduct might be oxidized to form the corresponding carboxylic acid. Thus, it was speculated that the adduct might be an oxidized form of the 2-octenal-histidine Michael adduct, N^{τ}-(1-carboxyheptan-2-yl)-histidine (CHH) (Fig. 3B). To prove this hypothesis, CHH was prepared by the oxidation of the 2-octenal-histidine Michael adduct with sodium chlorite ($NaClO_2$) and the product was analyzed by LC-ESI-MS/MS. Fig. 3C shows that X_{C8} was indistinguishable from the oxidized form of the 2-octenal-histidine Michael adduct. The chemical structure of adduct was elucidated by the comparison of fragment ion pattern of authentic standard with that of X_{C8} from 2-octenal-treated hemoglobin. As shown in Fig. 3D, the fragment pattern of X_{C8} was in agreement with that of authentic CHH. In a manner similar to X_{C8}, X_{C9} and X_{C10} were also identified as N^{τ}-(1-carboxyoctan-2-yl)-histidine and N^{τ}-(1-carboxynonan-2-yl)-histidine, respectively. Other 2-alkenals also generated similar alkanoic acid-histidine adducts upon the reaction with hemoglobin (Fig. 4). These results suggest that 2-alkenals may ubiquitously generate the oxidized forms of the 2-alkenal-histidine Michael adducts, namely, alkanoic acid-histidine adducts, upon the reaction with hemoglobin.

3.3. Involvement of heme iron in the formation of the alkanoic acid-histidine adducts

The finding that the alkanoic acid-histidine adducts were formed in the 2-alkenal-treated hemoglobin, but not in the 2-alkenal-treated HSA (Fig. 2), suggests the involvement of iron in the formation of the adducts. Hence, we investigated if the alkanoic acid-histidine adducts could be formed in non-heme iron proteins upon incubation with 2-alkenal. A representative non-heme iron protein, transferrin, and three heme iron proteins, namely hemoglobin, cytochrome C, and myoglobin, were treated with 2-octenal for 24 h at 37 °C. CHH was detected in the 2-octenal-treated hemoglobin and myoglobin, which directly bind to an oxygen molecule *via* the ferrous ion of heme (Fig. 5A), whereas the adduct was barely observed in cytochrome C, a heme-containing but not O_2-binding protein, and transferrin, an iron-binding non-heme protein (Fig. 5A). These results suggested that the heme-oxygen complex may contribute to the formation of the alkanoic acid-histidine adducts. To elucidate the possible involvement of iron ion in the generation of alkanoic acid-histidine adducts, we examined the effect of the heme derivatives on the CHH formation in HSA. As shown Fig. 5B, CHH was formed in the presence of hemin, whereas protoporphyrin IX did not enhance the formation of CHH, suggesting that a heme iron may play a role in the oxidation of the histidine adducts in the proteins.

3.4. Formation of CHH in red blood cells

We then examined the formation of the alkanoic acid-histidine adducts in the RBCs treated with the2-alkenals. As shown in Fig. 6, the alkanoic acid-histidine adducts were detected upon the reaction of the RBCs with the 2-alkenals, whereas the adducts were not detected in the plasma fractions treated with the 2-alkenals. To quantify CHH, we established a quantification method using a stable isotope dilution

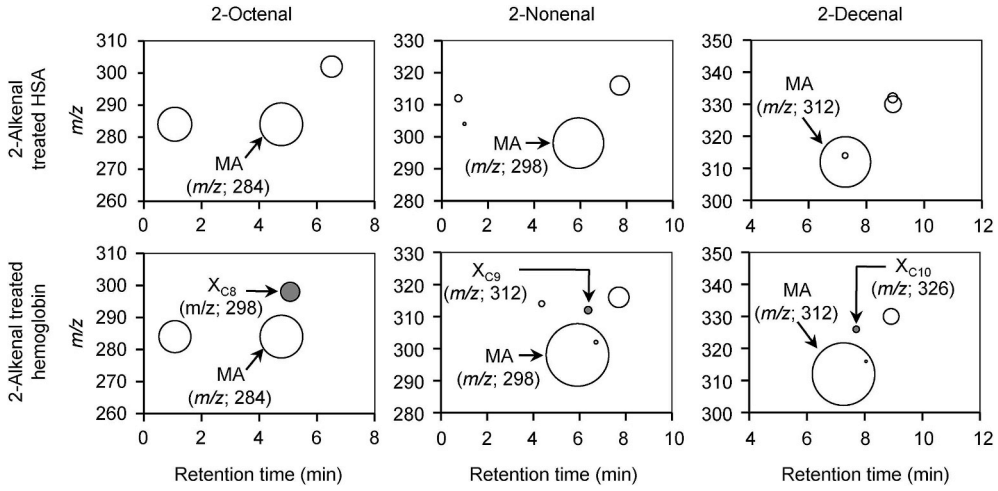

Fig. 2. Comparison of adductome map between 2-alkenal treated HSA and hemoglobin. The adductome maps of 2-alkenal-treated HSA (*upper*) and hemoglobin (*lower*) are shown with a circle encoding the relative abundance. Arrows indicate 2-alkenal-histidine Michael adducts (MA; 2-octenal-histidine (*m/z*; 284), 2-nonenal-histidine (*m/z*; 298) and 2-decenal-histidine (*m/z*; 312)) and 2-alkenal-histidine Michael adduct plus 14 mass unit adducts (X_{C8} (*m/z*; 298), X_{C9} (*m/z*; 312), X_{C10} (*m/z*; 326)).

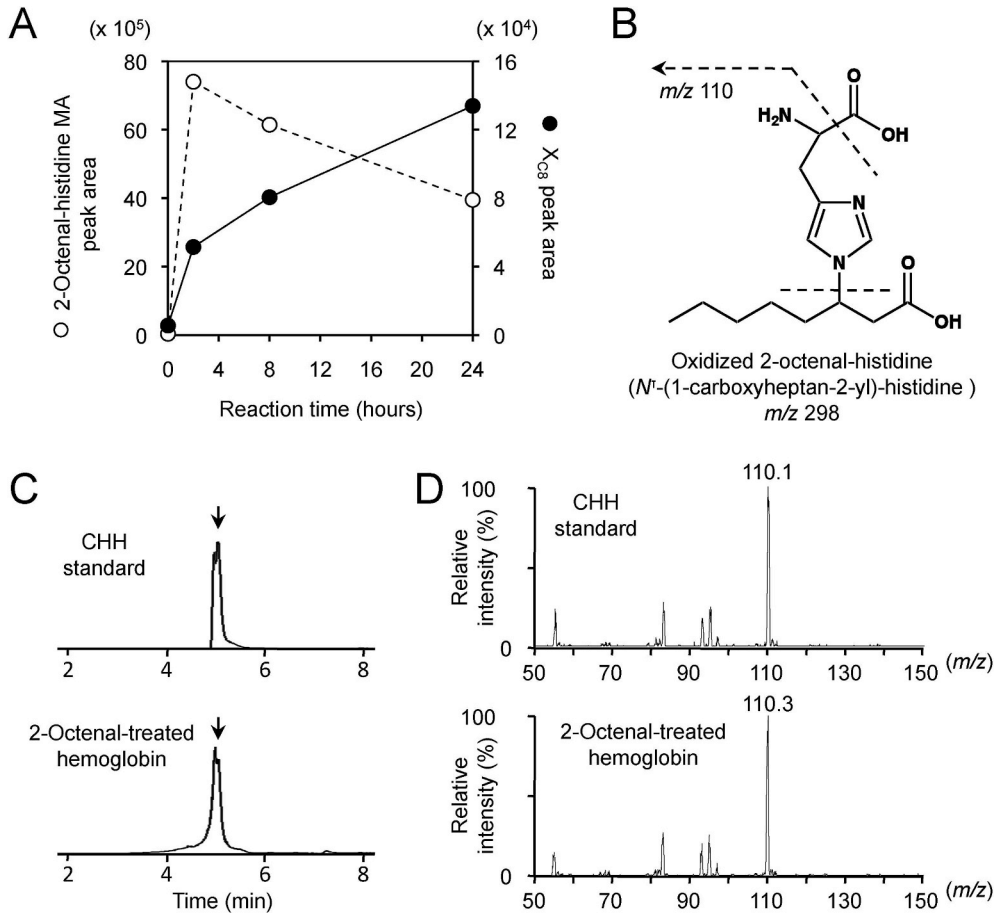

Fig. 3. Identification of 2-octenal-histidine Michael adduct plus 14 adduct. (A) Time-dependent formation of 2-octenal-histidine Michael adduct (*open circle*) and X_{C8} (*closed circle*). (B) Chemical structure of oxidized 2-octenal-histidine Michael adduct, N^{τ}-(1-carboxyheptan-2-yl)-histidine (CHH). (C, D) The authentic CHH and X_{C8} formed in 2-octenal-treated hemoglobin were analyzed by LC-ESI-MS/MS. The mass spectrometric analysis in the SRM mode (C) and product ion scan mode (D) were performed.

method. The amount of CHH was quantified by the ratio of the peak area of the target and of the stable isotope-labeled internal standard octanoic acid-[$^{13}C_6$] histidine. The limit of the quantification of the CHH is ~200 fmol. Using this method, we quantified the CHH formed in the 2-octenal-treated RBCs. The isolated human RBCs (5×10^8 cells/ml) were incubated with 2-octenal (1 mM) at 37 °C, and subjected to an LC-ESI-MS/MS analysis following reduction with $NaBH_4$ and hydrolyzed under the conventional acidic conditions. As shown in Fig. 7A, CHH was detected in 2-octenal-treated RBCs. The formation of CHH was also confirmed by the fragment ion pattern of CHH formed in 2-octenal-treated RBC (Fig. 7B). The amount of CHH reached at ~300 pmol/mg RBC protein (Fig. 7C).

Finally, we examined the formation of CHH in RBCs exposed to oxidative stress. After H_2O_2 (10 mM) treatment in the presence of nitrite (100 μM) for 24 h, the RBCs were lysed with RIPA buffer, and subjected to an LC-ESI-MS/MS analysis. The CHH levels in the H_2O_2-treated RBCs were significantly higher than those of the control RBCs. The amount of CHH in the H_2O_2-treated RBCs was about 6 pmol/mg protein (Fig. 7D). These results suggest that the alkanoic acid-histidine adducts could be formed in the RBCs under oxidative stress.

4. Discussion

Protein modifications are generally catalyzed by a specific enzyme,

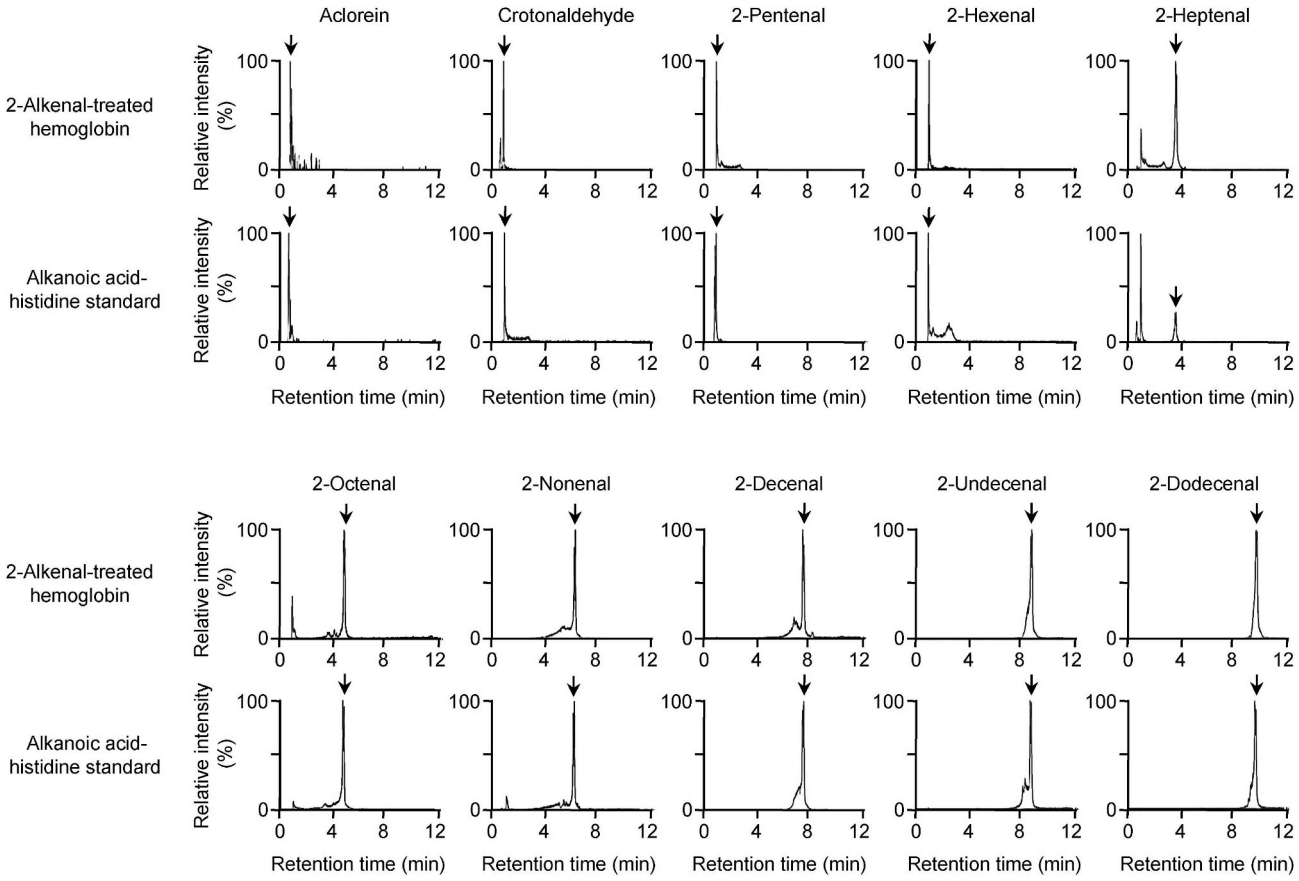

Fig. 4. Formation of alkanoic acid-histidine adducts in hemoglobin treated with 2-alkenals. The 2-alkenal-treated hemoglobins were reduced with $NaBH_4$, acid-hydrolyzed, and analyzed by LC-ESI-MS/MS in the SRM mode.

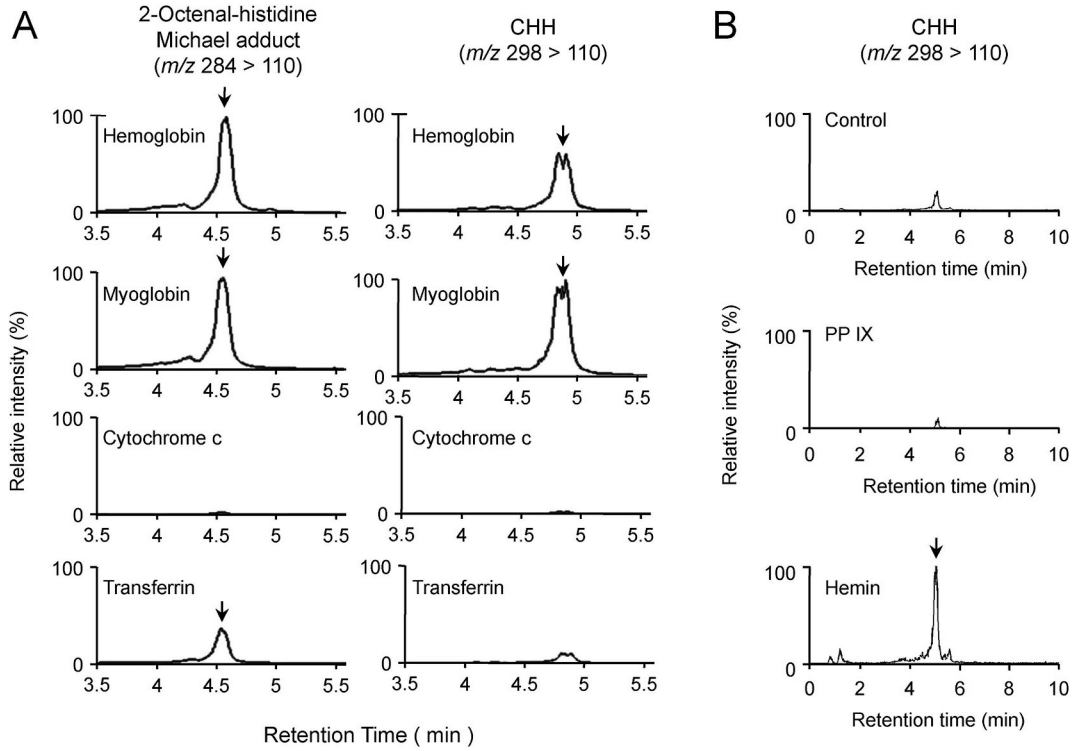

Fig. 5. Heme-dependent formation of CHH. (A) 2-octenal-treated iron-containing proteins (hemoglobin, myoglobin, cytochrome c and transferrin) were analyzed by LC-ESI-MS/MS in the SRM mode following $NaBH_4$ reduction and acid hydrolysis. (B) HSA was treated with 2-octenal in the presence of protoporphyrin IX (PP IX) or hemin, then analyzed by LC-ESI-MS/MS in the SRM mode following $NaBH_4$ reduction and acid hydrolysis.

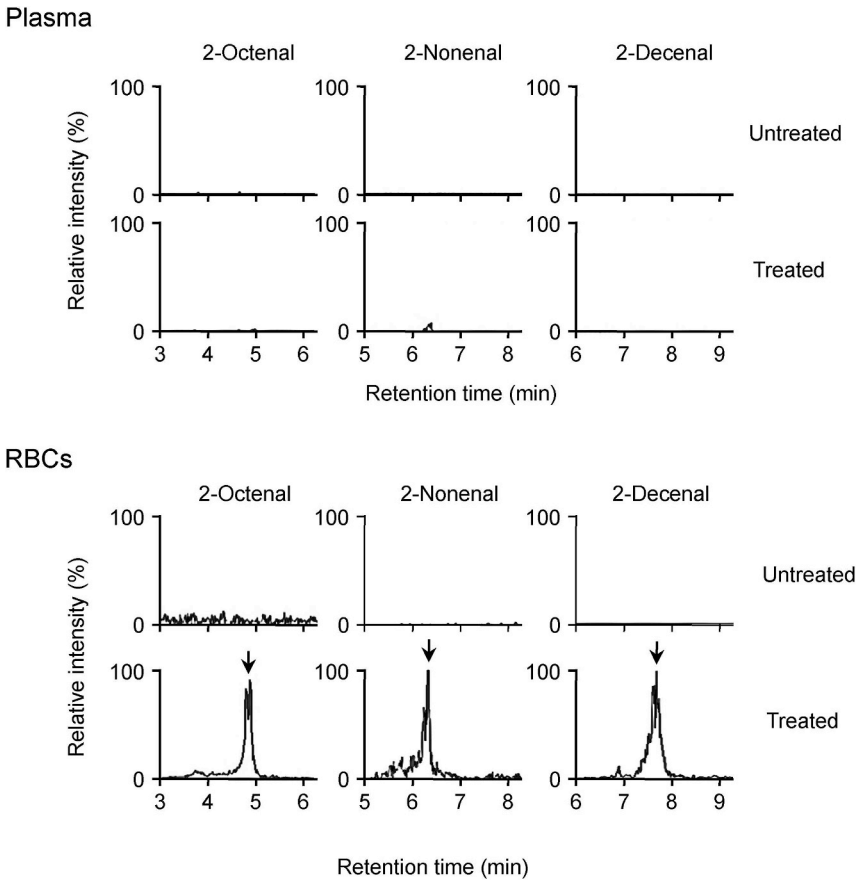

Fig. 6. Formation of alkanoic acid-histidine adducts in RBCs treated with 2-alkenal. Human blood was divided into plasma and RBCs fractions, then treated with the 2-alkenals. Both fractions were analyzed by LC-ESI-MS/MS in the SRM mode following $NaBH_4$ reduction and acid hydrolysis. *Upper*, plasma; *lower,* RBCs.

but can also progress through non-enzymatic mechanisms, such as the reaction between lipid metabolites and nucleophilic amino acids like histidine and lysine [16]. These modifications modulate biological functions such as gene expression, protein activity and stability, intracellular localization of proteins and protein-protein interactions [17–19]. Based on these and the fact that post-translational modifications increase the complexity of the eukaryotic proteome, the identification and comprehensive evaluation of the covalent modifications in proteins are important to gain an understanding of various cellular functions. Taking advantage of the fact that the histidine and lysine adducts produced specific fragment ions that were observed at *m/z* 110 and 84, respectively, we performed a comprehensive analysis of the histidine and lysine adducts using LC-ESI-MS/MS and identified N^{ε}-(8-carboxyoctanyl)lysine as the most abundant lysine adduct in the oxidized LDL [15]. In the present study, to gain further structural insight into the covalent modification of proteins by lipid peroxidation products, we comprehensively analyzed the histidine adducts in the 2-alkenal-treated hemoglobin and unexpectedly identified an adduct specifically generated in the 2-alkenal-modified heme proteins. We also observed that the alkanoic acid-histidine adducts could be formed in (i) the oxygen-binding heme proteins (hemoglobin and myoglobin) treated with 2-alkenal (Fig. 5A), and (ii) the 2-alkenal-treated HSA in the presence of hemin (Fig. 5B). Based on these findings, we propose a possible mechanism for the formation of the alkanoic acid-histidine adducts as follows: (i) The 2-alkenal-histidine Michael adduct is formed upon the reaction of heme proteins with the 2-alkenal. (ii) The Michael adduct is oxidized by Fe^{3+} to form an acyl radical, then the acyl radical reacts with molecular oxygen to afford a peracid radical. (iii) When the peracid radical is reduced by Fe^{2+} in the presence of H^+, a peracid is generated. (iv) The alkanoic acid-histidine adduct is formed *via* the reaction between the peracid and aldehyde (Michael adduct) or *via* a Fe^{3+}-catalyzed conversion of the peracid to the carboxylic acid (alkanoic acid-histidine adduct). This iron ion and oxygen-dependent mechanism is also suggested by our preliminary data that His59 in the α chain of hemoglobin, which is involved in the molecular oxygen binding with an iron ion in heme [20,21] (Fig. S1), has been identified as one of the target histidine residues in hemoglobin (Yoshitake, Shibata, Shimayama, & Uchida, unpublished observation). These findings also speculated that hemoglobin modification by 2-alkenals may contribute to the oxidative-stress-dependent dysfunction of hemoglobin.

RBCs transport respiratory gases between the lungs and tissues *via* hemoglobin, which constitutes about 90% of the dry weight of the RBCs. Various previous studies showed the chemical modifications of hemoglobin with endogenous and exogenous electrophiles [22,23]. Hemoglobin is readily covalently modified by reduced glucose at the N-terminal valine of the beta chain to form glycated hemoglobin (hemoglobin A_{1c}) [24]. In the clinical scenes, the hemoglobin A_{1c} is a useful indicator for the long-term average glucose level and the risk of diabetic complications [25]. In addition, Stevens and his co-workers reported that acetaldehyde, a reactive metabolite of ethanol, reacts with valine, lysine and tyrosine residues of hemoglobin to form acetaldehyde-hemoglobin adducts. They also demonstrated that the amount of acetaldehyde-hemoglobin adducts from alcoholic patients was significantly elevated compared to normal subjects [26]. Moreover, Carlsson et al. established the novel Edman degradation-based method for the LC-MS/MS analysis of N-terminal valine adducts in hemoglobin detached as fluorescein thiohydantoin derivatives. Using this methods, they detected some known adducts, such as the methyl adduct and methyl vinyl ketone adduct, and many unknown adducts in RBCs from smokers [27]. In the present study, we detected CHH in the H_2O_2-

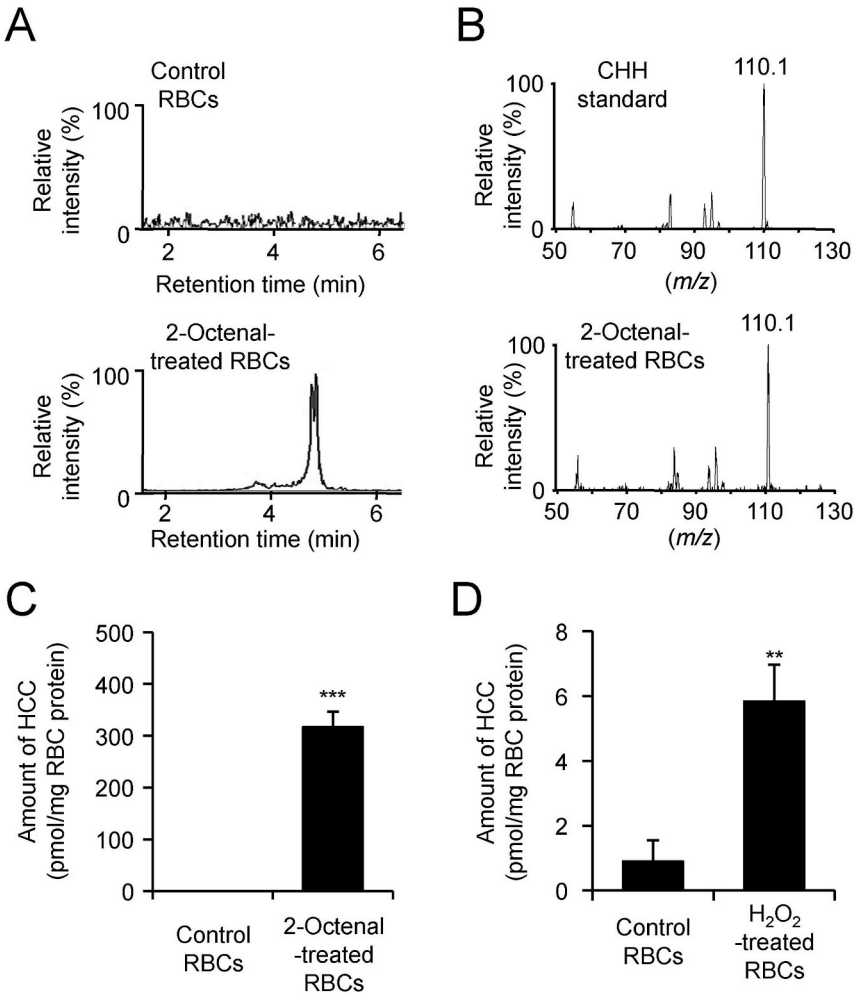

Fig. 7. Quantitative analysis of CHH formed in 2-octenal-treated and H_2O_2-treated RBCs. (A) The total ion monitoring chromatogram (SRM mode (m/z, 298 > 110)) and (B) product ion scanning (m/z of precursor ion, 298) of the reaction mixture of the RBCs with 2-octenal were performed. The amounts of CHH in the 2-octenal-treated (C) or H_2O_2-treated (D) RBCs were quantified by the stable isotope dilution method. Experiment was repeated for at least three different preparations. Data was expressed as means ± S.D. Statistically significant differences were determined using the unpaired t-test. (**, $p < 0.01$; ***, $p < 0.005$).

treated RBCs in the presence of nitrite, which can convert hemoglobin to methemoglobin (Fig. 7D). On the other hand, CHH was not detected in the H_2O_2-treated RBCs in the absence of nitrite (Yoshitake, Shibata, Shimayama, & Uchida, unpublished observation), suggesting that CHH could be preferentially formed in methemoglobin. These results suggest a possibility that the alkanoic acid-histidine adduct could be a candidate biomarker for methemoglobinemia, a condition caused by elevated levels of methemoglobin in the blood [28].

In summary, we identified the alkanoic acid-histidine adducts as novel adducts specifically generated in the 2-alkenal-modified hemoglobin. The adducts were suggested to be formed by the oxidation of 2-alkenal-histidine Michael adducts by the iron ion and oxygen. In addition, the alkanoic acid-histidine adducts were also detected in the RBCs treated with H_2O_2. These findings suggest the connection between the heme-dependent modification of proteins by 2-alkenals and human pathogenesis. Further studies are required to understand the biological consequences of the production of alkanoic acid-histidine adducts.

Conflicts of interest

The authors declare no conflicts of interest in this work.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.redox.2019.101115.

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