Identification of 4-hydroxy-2-nonenal–histidine adducts that serve as ligands for human lectin-like oxidized LDL receptor-1

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INTRODUCTION

The oxidative modification of LDL (low-density lipoprotein) has been implicated in the pathogenesis of atherosclerosis [1]. The uptake of OxLDL (oxidized LDL) is mediated by scavenger receptors, such as SR-AI/II (class A macrophage scavenger receptor type I/II), SR-BI (class B macrophage scavenger receptor type I), CD36, MARCO (macrophage receptor with a collagenous structure) and macrosinial (CD68) [2]. LOX-1 (lectin-like oxidized LDL receptor-1) is a scavenger receptor that is important for the uptake of OxLDL (oxidized low-density lipoprotein) and contributes to the pathogenesis of atherosclerosis. However, the precise structural motifs of OxLDL that are recognized by LOX-1 are unknown. In the present study, we have identified products of lipid peroxidation of OxLDL that serve as ligands for LOX-1. We used CHO (Chinese-hamster ovary) cells that stably express LOX-1 to evaluate the ability of BSA modified by lipid peroxidation to compete with AcLDL (acetylated low-density lipoprotein). We found that HNE (4-hydroxy-2-nonenal)-modified proteins most potently inhibited the uptake of AcLDL. On the basis of the findings that HNE-modified BSA and oxidation of LDL resulted in the formation of HNE–histidine Michael adducts, we examined whether the HNE–histidine adducts could serve as ligands for LOX-1. The authentic HNE–histidine adduct inhibited the uptake of AcLDL in a dose-dependent manner. Furthermore, we found the interaction of LOX-1 with the HNE–histidine adduct to have a dissociation constant of 1.22 × 10⁻⁸ M using a surface plasmon resonance assay. Finally, we showed that the HNE–histidine adduct stimulated the formation of reactive oxygen species and activated extracellular-signal-regulated kinase 1/2 and NF-κB (nuclear factor κB) in HAECs (human aortic endothelial cells); these signals initiate endothelial dysfunction and lead to atherosclerosis. The present study provides intriguing insights into the molecular details of LOX-1 recognition of OxLDL.

Key words: atherosclerosis, 4-hydroxy-2-nonenal–histidine adduct, 4-hydroxy-2-nonenal-modified protein (HNE-modified protein), lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1), oxidized low-density lipoprotein (OxLDL), scavenger receptor.
events, including the activation of ERK1/2 (extracellular-signal-regulated kinase 1/2) and NF-κB (nuclear factor κB) downstream signalling molecules in the LOX-1 pathway.

EXPERIMENTAL

Materials

DiD (1,1'-dioctadecyl-3,3',3'-tetramethylindocarbocyanine perchlorate) was purchased from Molecular Probes and AcLDL was from Biomedical Technologies. The stock solutions of HNE were prepared following the method of De Montarby et al. [15]. HNE–Nα-acetylhistidine Michael addition adducts were prepared as described previously [16]. The following antibodies were used in the present study: anti-BSA antibody from Antibody Shop, Texas Red-conjugated anti-(mouse IgG) from Vector Laboratories, anti-LOX-1 monoclonal antibody 23C11 from Abcom, anti-ERK1/2 and anti-phospho-ERK1/2 antibodies from Cell Signaling Technology, and anti-β-actin from Sigma. The CHO (Chinese-hamster ovary) cell line was obtained from RIKEN Cell Bank (Tsukuba, Japan). HAECs (human aortic endothelial cells) (passage 3), endothelial cell basal medium (EBM-2) and the EGM-2 BulletKit [2% FBS (fetal bovine serum)] were purchased from Lonza. HAECs from the fourth to the seventh passage were used in these experiments.

Reaction of proteins with aldehydes

BSA (1 mg/ml) was incubated with 1 mM aldehyde in 1 ml of 50 mM sodium phosphate buffer (pH 7.4) at 37°C for 24 h, as described previously [17]. Modification of BSA was verified by detection of protein carbonyls (see Supplementary Figure S1 at http://www.BiochemJ.org/bj/442/bj4420171add.htm).

Modification of human LDL

LDL (1.006 < r < 1.063 g/ml) was isolated from normal human serum by sequential ultracentrifugation and extensively dialysed against PBS containing 0.3 mM EDTA. LDL was sterilized by filtration through a 0.22 μm pore-size filter and stored under argon at 4°C. HNE modification was performed by incubating LDL (1 mg/ml) with 1 mM HNE at 37°C for 24 h; the modified LDL was then dialysed against PBS containing 0.3 mM EDTA. Modification of LDL was verified by ELISA analysis of protein carbonyls (see Supplementary Figure S2 at http://www.BiochemJ.org/bj/442/bj4420171add.htm).

For oxidative modification of LDL by Cu2+, an LDL stock solution was dialysed against 1000 vol. of PBS at 4°C. The oxidation of LDL (1 mg of protein/ml) was initiated by adding CuSO4 (20 μM) and incubating for up to 24 h at 37°C under sterile conditions. Oxidation was terminated by the addition of EDTA (0.2 mM).

Competitive cell uptake assay

DiD-labelled AcLDL was prepared from human LDL as described previously [18]. CFP (cyan fluorescent protein)–LOX-1 CHO cells were grown on coverslips 2 days before the experiments. The competition assay was performed as described previously [19]. The expression level of LOX-1 was determined by measuring the CFP fluorescence intensity using an E4 filter with excitation at 436 nm (7-nm bandpass) and a 470-nm longpass emission filter. The fluorescence intensity of DiD was measured using a Y5 filter with excitation at 620 nm (50-nm bandpass) and a 700-nm (75-nm bandpass) emission filter to assess DiD–AcLDL uptake. Images of more than 50–100 cells from each of three independent experiments were acquired. After subtracting the background emission levels at 436 and 620 nm from the fluorescence images, the fluorescence intensity of each cell was determined from the mean pixel values of the whole cell using MetaMorph software (Universal Imaging). The expression level of LOX-1 was calculated from the CFP intensity, and the level of AcLDL uptake was calculated from the DiD intensity. For each series of experiments, the CFP intensity range for which CFP–LOX-1 expression was proportional to the DiD–AcLDL uptake was determined from more than 50 control cells, and only the cells with fluorescence intensity within that range were used for evaluation (see Supplementary Figure S3 at http://www.BiochemJ.org/bj/442/bj4420171add.htm). The level of AcLDL uptake was averaged for the expression level of LOX-1 and normalized to the no-competitor control.

Immunofluorescence staining

Cells transiently expressing CFP–LOX-1 were incubated with aldehyde-modified BSA prepared in Ham’s F12 medium without FBS for 15 min at 37°C in a humidified atmosphere containing 5% CO2. Formaldehyde (2% in PBS)-fixed cells were stained for 2 h at room temperature (25°C) with the primary antibody, mouse monoclonal anti-BSA antibody diluted 1:1000 in PBS with 3% fish gelatin (blocking buffer) containing 0.1% Triton X-100. The samples were rinsed with PBS, incubated with blocking buffer, and then exposed to the secondary antibody, Texas Red-conjugated anti-(mouse IgG) (5 μg/ml in blocking buffer containing 0.1% Triton X-100) for 1 h at room temperature. Samples were mounted and imaged using a Leica DM IRE2 microscope as described above. The CFP intensity range from 150 to 250 was used for evaluation in each series of experiments. The number of cells that internalized modified BSA was counted.

HPLC analysis of HNE–histidine adducts

The HNE-modified protein reactions were treated with 340 mM 2-AP (2-aminopyridine) and 16 mM NaCNBH3 for 24 h at 37°C. The reaction mixtures were then extensively dialysed against PBS to remove the majority of the unbound probe. The pyridylaminated samples were hydrolysed in vacuo with 6 M HCl for 24 h at 110°C. The hydrolysates were then concentrated, dissolved in distilled water and analysed by reverse-phase HPLC on a Sunniest C18 column (250 mm length × 4.6 mm internal diameter; ChromaNik). The samples were eluted with a gradient of water with 0.1% trifluoroacetic acid (solvent A) and acetonitrile with 0.1% trifluoroacetic acid (solvent B) (time = 0–40 min, 95–60% solvent A; 40–45 min, 60–0% solvent A) at a flow rate of 0.8 ml/min. The elution profiles were monitored by absorbance at 230 nm and by fluorescence intensity (λex = 315 nm and λem = 380 nm).

Affinity of HNE–LDL for mutant LOX-1

DiD-labelled HNE–LDL was prepared in the same way as for DiD–AcLDL. CHO cells were transfected with a plasmid carrying mutant LOX-1, as described previously [18]. At 48 h post-transfection, cells were incubated with DiD-labelled HNE–LDL, and the LDL-uptake activity was evaluated as for the competitive assay described above.

Plasmid construction

Human LOX-1 cDNA was cloned from HAECs [20]. A DNA fragment encoding the CTLD (C-type lectin-like domain)
Preparation of biotinylated CTLD14

BL21(DE3) (Novagen) cells co-transformed with pET-22b carrying AviTag-CTLD14 and pBirA (Avidity) were cultured in LB (Luria–Bertani) medium containing 50 μg/ml ampicillin and 34 μg/ml chloramphenicol at 37 °C. When the D_{OD} of the culture reached 0.5, 1 mM IPTG (isopropyl β-D-thiogalactopyranoside) and 50 μM biotin were added to induce the expression and biotinylation of CTLD14. After 4 h of incubation, bacterial cells were harvested by centrifugation and resuspended in lysis buffer (10 mM Tris/HCl, pH 7.8, containing 1 mg/ml lysozyme and protease inhibitor cocktail). The cell lysate was incubated for 30 min at 4 °C, sonicated and centrifuged at 10000 g for 15 min to collect the inclusion bodies. The proteins in the inclusion body pellets were refolded using the modified method of Vohra et al. [21]. The sample mass, purity and disulfide bond formation were confirmed by SDS/PAGE, FT-ICR (Fourier-transform ion cyclotron resonance) MS, and MALDI–TOF (matrix-assisted laser-desorption ionization–time-of-flight)-MS (Reflex II, BrukerDaltonik). Protein concentrations were determined using the BCA (bicinchoninic acid) protein assay (Thermo Scientific).

Surface plasmon resonance assay

The surface plasmon resonance assays were performed with a Biacore 2000 apparatus (GE Healthcare). Biotinylated CTLD14 was immobilized on an SA sensor chip (GE Healthcare) via the N-terminal biotin at a density of approximately 5000 (for OxLDL and HNE–LDL) μM) and carrying an NheI restriction site at the 5′-end and both NheI and BamHI restriction sites at the 3′-end was used for in vitro biotinylation; this cassette was cloned into the NdeI and BamHI sites of pET-22b (Novagen). The resulting plasmid adds an N-terminal AviTag and a C-terminal His6 tag to cloned proteins. The pCR2.1 vector bearing CTLD14 was digested with NheI and XhoI, and the fragment encoding CTLD14 was inserted into the AviTag-carrying pET-22b.

Intracellular ROS production assay

The quantities of intracellular ROS were estimated using a fluorescent probe (Enzo Life Science). The specificity of the fluorescent probe was confirmed using the ROS inducer pyocyanine [22]. HAECs grown in EGM-2 BulletKit medium were pre-treated with anti-LOX-1 antibody (2 μg/ml in EBM-2) for 10 min. The cells were then incubated with OxLDL (100 μg/ml), HNE–LDL (100 μg/ml), HNE–histidine adducts (200 μM), histidine (200 μM) or normal (unmodified) LDL (100 μg/ml) for 15 min at 37 °C in a humidified atmosphere containing 5 % CO₂. Cells were detached using trypsin/EDTA solution for HAECs (Lonza). The cells were resuspended in 0.5 ml of ROS detection dye solution and incubated for 30 min at 37 °C in the dark, then harvested by centrifugation and resuspended in PBS. The fluorescence intensity of 5×10^{5} cells at 200 μl/well was detected using a fluorescence plate reader (Wallac Arvo SX, PerkinElmer) with excitation at 485 nm and emission at 535 nm. The results were expressed as the intensity relative to the control (incubated without ligand) from three independent experiments.

Western blotting

HAECs were grown in 100-mm-diameter dishes (EGM-2 BulletKit) until almost confluent. The cells were washed with EBM-2 and incubated with OxLDL (100 μg/ml), HNE– LDL (100 μg/ml), HNE–histidine adducts (200 μM), histidine (200 μM) or normal LDL (100 μg/ml) at 37 °C in a humidified atmosphere containing 5 % CO₂, for the indicated times. The cells were washed with ice-cold TBS (Tris-buffered saline: 20 mM Tris/HCl and 150 mM NaCl, pH 7.6) and then treated with 0.5 ml of lysis buffer (TBS containing 1 % Triton X-100 and protease inhibitor cocktail) on ice. The collected lysate was sonicated on ice
Effects of aldehyde-modified proteins on LOX-1

(A) Cells transiently expressing CFP–LOX-1 were incubated with aldehyde-modified BSA as described in the Experimental section. A-1: cells incubated with native BSA. A-2: cells incubated with 2-nonenal-modified BSA. A-3: cells incubated with HNE-modified BSA. A-4: cells incubated with CRA-modified BSA. The expression level of CFP–LOX-1 was highly variable. The uptake of aldehyde-modified BSA was observed in cells expressing CFP–LOX-1 (arrows in A-2 and A-3). Left-hand panels: phase-contrast; central panels: CFP–LOX-1 (excitation at 436 nm); right-hand panels: Texas Red (excitation at 620 nm). The intensity of the Texas Red fluorescence directly reflected the amount of aldehyde-modified BSA internalized by the cells. (B) Images of more than 100 cells were acquired from three independent experiments. The number of cells that internalized modified BSA was evaluated as described in the Experimental section. Results are the mean ± S.D. ratios of cells that internalized modified BSA to total cells. *P < 0.05 compared with native BSA. BSA was modified by the following aldehydes: ACR, CRA, 2-pentenal, 2-hexenal, 2-heptenal, 2-octenal, 2-nonenal, MDA, HNE, ONE or 4-oxo-hexenal (OHE).

Uptake of HNE-modified LDL by LOX-1

(A) Inhibition of LOX-1 DiD–AcLDL uptake by HNE-modified LDL. CFP–LOX-1-expressing CHO cells were pre-incubated with normal LDL or HNE-LDL (4 μg/ml) for 10 min at 37°C in a humidified atmosphere with 5% CO₂ and then incubated with DiD–AcLDL (0.4 μg/coverslip) for another 15 min. The cells were then fixed with 2% formaldehyde and observed as described in the Experimental section. Results are mean ± S.D. percentages of uptake compared with that observed in the absence of LDL or HNE–LDL (control). (B) Effect of amino acid substitutions in LOX-1 on HNE-modified LDL uptake. CHO cells on coverslips were transfected with a plasmid carrying mutant LOX-1. At 48 h post-transfection, the cells were incubated with DiD-labelled HNE–LDL, and the uptake activity was evaluated as described in the Experimental section. *P < 0.05 compared with control.

The clarified lysates were pre-cleared by incubation with Protein G–agarose beads (20 μl of 50% slurry) at 4°C for 60 min and then centrifuged at 14 000 g for 10 min. A total of 250 μl of pre-cleared lysate containing 120 μg of protein was treated according to the “Immunoprecipitation Protocol for Analysis by Western Immunoblotting” (Cell Signaling Technology). The samples (15 μl/lane) were resolved by SDS/PAGE (12% gels) under reducing conditions and transferred on to PVDF membranes. The membranes were blocked in 5% (w/v) dried skimmed milk powder, and ERK1/2 activation was detected by anti-ERK1/2 antibody (1:1000) or anti-phospho-ERK1/2 antibody (1:1000) followed by HRP (horseradish peroxidase)-conjugated goat anti-(rabbit IgG) (1:3000, Bio-Rad Laboratories). The signals were detected using an ECL (enhanced chemiluminescence) detection system (GE Healthcare). For the detection of β-actin, pre-cleared lysate (5 μg of protein/lane) was subjected to SDS/PAGE followed by western blotting with anti-β-actin antibody (1:1000 dilution) as performed for the comparative controls. The relative intensities of the bands of interest were analysed using Scion image software, and the intensity of each protein band was normalized to that of β-actin. Because anti-ERK1/2 antibody did not efficiently immunoprecipitate the ERK2, the activation of ERK1/2 was expressed the proportion of phosphorylated ERK1 from three independent experiments.

NF-κB luciferase reporter assay

NF-κB activation was determined by the NF-κB–luciferase (Luc) reporter assay system (Clontech). HAECs were transfected
with the reporter construct pNFκB-Luc, which is designed to measure the binding of transcription factors to the κ enhancer and thereby provide a direct measurement of activation of the NF-κB signal transduction pathway, using Effectene Transfection Reagent (Qiagen) at a DNA/Effectene ratio of 1:10. At 24 h post-transfection, the cells were pre-treated with anti-LOX-1 antibody (2 μg/ml in EBM-2) for 10 min, then incubated with OxLDL, HNE–LDL, HNE–histidine adducts, histidine or normal LDL for 15 min at 37°C in a humidified atmosphere containing 5% CO₂. The cell lysates were prepared according to the Luciferase Assay System (Promega) protocol. A total of 20 μl of cell lysate (containing 8 μg of protein) was mixed with 100 μl of assay reagent (Promega), and the luciferase activity was measured using a plate-reading Luminometer (Wallac Arvo SX, PerkinElmer). The results were expressed as the intensity relative to the control (incubated with EBM-2 only) from three independent experiments.

Statistical analysis

Student’s t test was used for comparison of two data sets; ANOVA was used for multiple data sets. P values < 0.05 were considered statistically significant.

RESULTS

Inhibition of AcLDL uptake by aldehyde-modified BSA

During oxidation, LDL apolipoprotein is modified by lipid peroxidation products [23]. The decomposition products most likely to be responsible for this modification are aldehydes, particularly the 2-unsaturated aldehydes. To determine whether the structural requirements for recognition by LOX-1 can be fulfilled by modified proteins other than LDL, we modified BSA with lipid peroxidation-derived reactive aldehydes. The modification of BSA was verified by protein carbonyl analysis (see Supplementary Figure S1). We compared the inhibition by modified and native albumin of uptake of modified LDL by CHO cells stably expressing CFP-tagged LOX-1 (CFP–LOX-1 CHO) [24]. To avoid ambiguity due to variations in the extent of LDL oxidation, AcLDL, which has an affinity for LOX-1 comparable with that of OxLDL [25], was used as an alternative ligand. We also chose to use DiD (λex = 644 nm and λem = 665 nm) instead of DiI (1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate), the more commonly used lipophilic dye, because the background fluorescence and autofluorescence of cells are lower at the optimal wavelengths for DiD (see Supplementary Figure S5 at http://www.BiochemJ.org/bj/442/bj4420171add.htm). No uptake of DiD–AcLDL by native CHO cells was detected, and the uptake of DiD–AcLDL by CFP–LOX-1-expressing CHO cells was inhibited completely by pre-incubation with LOX-1-neutralizing antibody (see Supplementary Figure S6 at http://www.BiochemJ.org/bj/442/bj4420171add.htm). The amounts of incorporated DiD–AcLDL directly reflected the binding activity of LOX-1 (see Supplementary Figure S3). The stable cell line expressed a moderate amount of LOX-1, and DiD–AcLDL fluorescence was observed in the lysosomes after 15 min of incubation (Figure 1A, A-1). The presence of modified proteins (e.g. HNE-modified BSA; Figure 1A, A-3) inhibited DiD–AcLDL uptake. Quantitative evaluation of the fluorescence intensity by region-measurement analysis [19] revealed that proteins modified by reactive aldehydes, such as HNE and ONE (4-oxo-2-nonenal), inhibited the uptake of AcLDL (Figure 1B). Although the BSA-modification ratio was not identical for each aldehyde (see Supplementary Figure S1), HNE- and ONE-modified BSA still produced greater inhibition than BSA modified by other aldehydes when the level of inhibition was averaged per protein carbonyl (see Supplementary Figure S7 at http://www.BiochemJ.org/bj/442/bj4420171add.htm).

Uptake of aldehyde-modified BSAs through LOX-1

To explore further the role of aldehyde-modified proteins as LOX-1 ligands, we investigated whether the modification of BSA by reactive aldehydes accelerated the uptake of the modified proteins in cells overexpressing LOX-1. There was no uptake of native BSA (Figure 2A, A-1), whereas the aldehyde-modified proteins, such as HNE and 2-nonenal, exhibited increased uptake (Figures 2A, A-2 and 2A, A-3). The fluorescence intensity, which represents the amount of internalized aldehyde-modified proteins, correlated with the LOX-1 expression level (see Supplementary Figure S8 at http://www.BiochemJ.org/bj/442/bj4420171add.htm). This correlation suggests strongly that the
uptake of modified proteins depends on LOX-1. Quantitative evaluation of the percentage of cells that internalized the modified BSA revealed that proteins modified by HNE and ONE, which efficiently inhibited the uptake of AcLDL, were also well incorporated (Figure 2B). CRA (croton aldehyde)-BSA, which showed no inhibitory effect on the uptake of AcLDL (Figure 1B), was only marginally incorporated into the cells (Figures 2A, A-4, and 2B). Although ACR (acrolein)-lysine, MDA (malondialdehyde)-lysine and HNE-histidine adducts have all been reported in OxLDL in arterial lesions [26], the results described above indicate that HNE has the strongest affinity for LOX-1. To gain insight into the recognition of LOX-1 by aldehyde-modified proteins, we focused on HNE, one of the major products generated during the peroxidation of \( \omega - 6 \) polyunsaturated fatty acids, such as linoleic acid and arachidonic acid [13,14]. HNE-LDL significantly inhibited AcLDL uptake, whereas the native LDL had no such effect (Figure 3A). The linear arrangement of the basic residues (namely, Arg\(^{229} \), Arg\(^{231} \) and Arg\(^{248} \)) on the surface of the dimer is necessary for OxLDL binding [18]. We therefore evaluated the uptake of DiD-labelled HNE-LDL by LOX-1 basic residue mutants. The substitution of asparagine for Arg\(^{229} \), Arg\(^{231} \) and Arg\(^{248} \) significantly decreased the uptake of HNE-LDL (Figure 3B). Moreover, substitution of alanine for Trp\(^{150} \), which is necessary for maintaining the size of the cavity in the dimer interface of LOX-1 [18], ceased HNE-LDL uptake (Figure 3B). These results were consistent with those from a previous study on OxLDL and mutant LOX-1 [18].

Identification of HNE-histidine adducts as LOX-1 ligands

We next sought to identify a covalent adduct that functions as a LOX-1 ligand. HNE specifically reacts with nucleophilic amino acids, such as cysteine, histidine and lysine, to form stable cyclic hemiacetal adducts [14].

To identify the LOX-1 ligand generated by HNE modification of BSA, we used the reductive amination method to analyse the Michael addition-type HNE adducts in the HNE-modified BSA [27]. Figure 4 demonstrates the time-dependent formation of HNE-histidine adducts in protein treated with 1 mM HNE at 37°C. The concentration reached 9.5 adducts per protein molecule after 12 h of incubation. At this concentration, the adducts constituted approximately 56% of the histidine residues in the BSA. Moreover, the HNE-histidine adduct was also the major product of HNE modification of OxLDL (Figure 4B). HNE-histidine adducts contain chiral centres and therefore comprise a racemic mixture of (4R)- and (4S)-isomers (Figure 4A, lower panel, *). We also analysed the Michael addition-type HNE adducts in OxLDL using the reductive amination method [27] followed by LC (liquid chromatography)-ESI (electrospray ionization)-MS/MS (tandem MS) analysis (see Supplementary Figure S9 at http://www.BiochemJ.org/bj/442/bj442017add.htm). Six HNE-histidine adducts were detected per OxLDL molecule, whereas no HNE-histidine adducts were detected in normal LDL.

To evaluate the LOX-1 ligand activity of the HNE-histidine adducts, we prepared HNE-N\(^{2} \)-acetylhistidine adducts and tested their effect on the uptake of modified LDL by CFP-LOX-1-expressing CHO cells. As shown in Figure 5(A), the HNE-histidine adducts inhibited the uptake of DiD-AcLDL in a dose-dependent manner. The inhibition was measurable at concentrations less than 10 \( \mu \)M, and 60% inhibition was achieved at a concentration of 100 \( \mu \)M. In contrast, unmodified \( N^{2} \)-acetylhistidine had no effect on the uptake of DiD-AcLDL even at concentrations greater than 200 \( \mu \)M (Figure 5A). In a
parallel set of experiments, the HNE–N\textsuperscript{ω}-acetylcysteine adducts were analysed and found to have no detectable LOX-1 ligand activity (Figures 5B and 5C). These data suggest that the HNE–histidine adducts could serve as LOX-1 ligands and account for the receptor-mediated uptake of OxLDL and HNE-modified proteins. In addition, the finding that the LOX-1-dependent uptake of DiD–AcLDL was inhibited by HNE–histidine, but not by HNE–cysteine, suggests that a basic ring structure (imidazole) within the HNE–histidine adduct is involved in its recognition by LOX-1.

**Molecular interaction of CTLD14 with the HNE–histidine adducts**

We prepared soluble CTLD14, the domain of LOX-1 responsible for ligand recognition [18], and used surface plasmon resonance (Biacore) to study the molecular interaction of LOX-1 with HNE–histidine adducts. The recombinant CTLD14 was purified as a dimer, which is the native form of LOX-1 on the cell surface [28]. The CTLD14 was almost 100% biotinylated near the N-terminus in the bacterial expression system and could therefore be immobilized on an SA sensorchip without any additional treatment. We performed a single-cycle kinetic analysis [29], as it was very difficult to regenerate CTLD14, a necessary process for the usual multi-cycle kinetic analysis, without loss of activity. First, we prepared fully oxidized LDL [30] and confirmed the binding of OxLDL to CTLD14 (Figure 6A). The $K_a$ value of 1.38 × 10\textsuperscript{-10} M was similar to that obtained for the binding of OxLDL to CTLD14 prepared for crystallization [18]. Therefore the CTLD14 expressed as inclusion bodies in the present study was refolded correctly and retained its ligand-binding activity.

We next examined the binding of HNE–LDL to CTLD14. Sensorgrams (Figure 6B) showed an increase in RU, indicative of HNE–LDL binding (association), and a slow decrease in the response consistent with a loss of mass from washout (dissociation) after each injection (arrows). We analysed the sensorgrams by fitting them to a simple 1:1 Langmuir model. The $K_d$ value was calculated to be 3.96 × 10\textsuperscript{-10} M; the association rate constant, $k_a$, was 1.8 × 10\textsuperscript{4} M\textsuperscript{-1}s\textsuperscript{-1} (S.E.M. 455 M\textsuperscript{-1}s\textsuperscript{-1}), and the dissociation rate constant, $k_d$, was 7.13 × 10\textsuperscript{-8} s\textsuperscript{-1} (S.E.M. 2.16 × 10\textsuperscript{-7} s\textsuperscript{-1}); these values were similar to those of OxLDL. The $\chi^2$ value of 1.51 indicated an adequate fit of the data to the model (a value less than 10 indicates a good fit and a value less than 2 indicates an ideal fit). Furthermore, the S.E.M. was less than 10%. These parameters ($\chi^2$ and S.E.M.) indicated that the data were reliable. However, the very low dissociation rate constant made it very difficult to perform valuable kinetic analyses. Prolonging the dissociation time did not improve the kinetics. This very slow dissociation was observed for both OxLDL and HNE–LDL. Finally, we analysed the interaction between HNE–histidine adducts and CTLD14 (Figure 6C). The $K_d$ value was 1.22 × 10\textsuperscript{-8} M [$k_a$ was 7.4 × 10\textsuperscript{4} M\textsuperscript{-1}s\textsuperscript{-1} (S.E.M. 5.24 × 10\textsuperscript{4} M\textsuperscript{-1}s\textsuperscript{-1}) and the $k_d$ was 9.03 × 10\textsuperscript{-4}s\textsuperscript{-1} (S.E.M. 1.91 × 10\textsuperscript{-3}s\textsuperscript{-1})]. In this case, the $\chi^2$ value, which reflects the closeness of the least-squares fitting, was 0.269. Although the data fitting was not perfect at all points, the statistical parameters indicated that the data were reliable.

**LOX-1-dependent activation of the MAPK (mitogen-activated protein kinase) signalling pathway by HNE–histidine adducts**

The activation of LOX-1 by OxLDL can stimulate the formation of ROS and initiate a cascade of redox-sensitive signalling events, including the activation of the MAPK pathway. To gain an insight into the LOX-1 ligand function of HNE–histidine adducts, we examined whether HNE–histidine adducts could stimulate ROS production via LOX-1. HAECs were incubated with OxLDL (100 μg/ml), HNE–LDL (100 μg/ml), HNE–N\textsuperscript{ω}-acetylhistidine (200 μM), histidine (200 μM) or LDL (100 μg/ml) for 15 min, and the ROS formation was then evaluated. As shown in Figure 7(A), HNE–LDL and HNE–histidine adducts induced ROS formation. To test whether this ROS formation was specifically induced by LOX-1, we assessed the effect of anti-LOX-1 neutralizing antibody, which blocks the LOX-1-mediated uptake of OxLDL (see Supplementary Figure S6) on ROS formation. Pre-incubating the HAECs with this antibody markedly reduced the ROS formation (Figure 7A). Meanwhile, no reduction in ROS formation was detected in cells incubated with histidine and very little in cells incubated with normal LDL.

Next, we evaluated the activation of the ERK1/2 component of the MAPK pathway. Both HNE–LDL and HNE–histidine adducts stimulated the phosphorylation of ERK1/2 in a time-dependent manner (Figures 7B and 7C). The phosphorylation reached its maximum level 10–15 min after the initiation of treatment with OxLDL, HNE–LDL or HNE–histidine, and this response persisted for 30 min. No activation was observed following the addition of histidine, and the time course of activation by LDL was completely different from that produced by another ligand. Finally, we investigated the effect of LOX-1 ligand binding on NF-κB activation. The HNE–histidine adducts induced the activation of NF-κB, and this activation was attenuated by pre-treatment with anti-LOX-1 antibody (Figure 7D). Although native LDL also weakly induced NF-κB activation, this activation was not
attenuated by anti-LOX-1 antibody. Therefore it is likely that the binding of HNE–histidine adducts to LOX-1 activates its downstream signal transduction mechanism.

**DISCUSSION**

A large number of oxygenated species have been identified in non-enzymatically oxidized unsaturated fatty acids. The primary and secondary non-volatile products include hydroperoxides, epoxides, dihydroperoxides and hydroperoxy cyclic peroxides. Many of these products can undergo decomposition reactions to yield a large number of other products, including aldehydes. The formation of lipid peroxidation product–protein adducts in vascular lesions, such as atherosclerotic lesions, is a common phenomenon in most, if not all, types of vascular damage associated with oxidative stress. The possibility that the aldehydic molecules generated by lipid peroxidation are involved in the pathogenesis of atherosclerosis is suggested by the facts that (i) the level of reactive aldehydes in the plasma increases in relation to extensive aortic atherosclerosis, (ii) high concentrations of aldehydes are generated during the oxidation of LDL phospholipids, and (iii) the structural and functional changes associated with the in vitro oxidation of LDL can also be produced by the direct interaction of LDL with aldehydes.

Scavenger receptors bind to aldehyde-modified proteins [16,31]. These receptors are thought to provide a mechanism for the clearance of these modified proteins from the circulation by a number of cell types. Indeed, a previous study showed that endothelial cells can bind and degrade aldehyde-modified proteins [32]. In the present study, we found that extensive derivatization of BSA with aldehydes that are major fatty acid oxidation products resulted in significant BSA uptake via the LOX-1 pathway (Figure 1). Modification of the protein with HNE, a peroxidation product that is relatively abundant in OxLDL, also led to recognition by the receptor (Figure 2). The recognition of the lipid peroxidation product-modified albumin by LOX-1 (Figure 2) suggests that the formation of aldehyde–protein adducts generates the critical structural unit that is recognized by LOX-1. In addition, the substitution of the crucial OxLDL-binding amino acids in LOX-1 severely reduced HNE–LDL binding (Figure 3B). These results indicate that HNE–LDL and OxLDL recognize the same binding site.

Thiol-derived Michael adducts were initially considered to be the products of HNE [13]. However, studies have found that HNE can also form Michael adducts with the imidazole moieties of histidine residues. The modification of histidine by HNE consists of (i) the formation of Schiff base adducts between the imidazole moiety of histidine and the aldehyde group of HNE, (ii) the possibility that the chemical reaction can also form Michael adducts with the imidazole moiety of the primary product may react with the 4-hydroxy–2-alkenal–histidine Michael adducts, which possess a free aldehyde group, undergo further cyclization to form cyclic hemiacetal derivatives [33]. Because the o xo–cyclo equilibrium favours hemiacetal formation, the free aldehyde moiety of the primary product may react with the 4-hydroxy group to form the hemiacetal derivative [13]. On the basis of the finding that these HNE–histidine Michael adducts can be formed as the major products of HNE-modification of BSA and OxLDL (Figure 4), we examined whether the HNE–histidine adducts

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**Figure 7** Activation of the LOX-1 signalling pathway in HAECs by HNE adducts

(A) Relative RDS formation. HAECs were treated as described in the Experimental section, and the fluorescence intensity of 5 × 10⁵ cells was measured using a fluorescence plate reader. The results were expressed as the intensity relative to that of the control (treated with EBM-2 only) from three independent experiments. (B) HAECs were treated with OxLDL (100 μg/ml), HNE–LDL (100 μg/ml), HNE–histidine (200 μM), histidine (200 μM) or LDL (100 μg/ml) for the indicated times. The cell lysate (containing 120 μg of protein) were immunoprecipitated with either anti-ERK1/2 or anti-phospho-ERK1/2 (p-ERK1/2) antibody, followed by Western blotting analysis with anti-ERK1/2 or anti-p-ERK1/2 as described in the Experimental section. Panels from left to right are cells incubated with OxLDL, HNE–LDL, HNE–histidine adducts, histidine and LDL. (C) Activation of ERK1/2. Activation of ERK 1/2 was expressed as the ratio of phosphorylated ERK1 to ERK1, because the anti-ERK1/2 antibody did not efficiently immunoprecipitate ERK2. Results are means ± S.D. *P < 0.05 compared with no anti-LOX-1 antibody treatment. (D) NF-κB activation. HAECs were transfected with reporter plasmid (pNF-κB-Luc) as described in the Experimental section. Transiently transfected HAECs were treated with OxLDL, HNE–LDL, HNE–histidine adducts, histidine or LDL. The luciferase activity was measured with a luminometer, and the results were expressed as the intensity relative to that of the control. Control, cells incubated with EBM-2 without any ligand; Ab (−), cells without anti-LOX-1 antibody pre-treatment; Ab (+), cells pre-treated with anti-LOX-1 antibody; OxLDL, cells treated with OxLDL (100 μg/ml); HNE–LDL, cells treated with HNE–LDL (100 μg/ml); HNE-His, cells treated with HNE–histidine adducts (200 μM); His, cells treated with histidine (200 μM); and LDL, cells treated with LDL (100 μg/ml). Results are means ± S.D. *P < 0.05 compared with no anti-LOX-1 antibody treatment. In the case of HNE–LDL, P = 0.07.
could serve as ligands for LOX-1. To this end, we expressed and purified soluble recombinant CTLD14, a ligand-recognition domain of LOX-1, to examine the binding of the HNE–histidine adducts to the receptor. Kinetic analyses performed with the Biacore SPR system revealed that the HNE–histidine adducts bind more weakly to CTLD14 (9.03 × 10⁻⁴ M) than did OxLDL (Kₐ = 1.38 × 10⁻¹⁰ M). However, the Kₛ value for the binding of HNE–LDL to CTLD14 was the same as that for OxLDL. The weaker binding, together with the finding that the HNE–histidine adducts inhibited 60% of DiD–AclDL binding (Figure 5), indicates that multiple minor chemically analogous adducts in OxLDL drive its binding to LOX-1. Moreover, other aldehyde modifications (e.g. those shown in Figure 1) simultaneously contribute to binding.

In contrast, the formation of the OxLDL–LOX-1 complex increases intracellular ROS formation and activates NF-κB [34], resulting in the subsequent expression of genes related to endothelial dysfunction and injury [6,7]. LOX-1-mediated NF-κB activation by OxLDL is crucial for increasing the expression of the adhesion molecules, such as E- and P-selectins, ICAM-1 (intercellular adhesion molecule-1), VCAM-1 (vascular cell adhesion molecule-1) and MCP-1 that cause pro-inflammatory changes to the vessel wall [7]. Treating HAECs with HNE–histidine adducts indeed stimulates the formation of ROS and initiates a cascade of redox-sensitive signalling events including phosphorylation of ERK1/2 and activation of NF-κB (Figure 7). In addition, the activation of NF-κB was attenuated by LOX-1-neutralizing antibody. Whereas normal LDL also stimulated NF-κB activation, this stimulation was not attenuated by LOX-1-neutralizing antibody. These data suggest that the formation of HNE–histidine adducts may participate in LOX-1-mediated recognition of OxLDL and endothelial dysfunction. We therefore suggest that OxLDL with a high HNE–histidine adduct content can serve as a ligand to activate the LOX-1 signalling pathways.

In conclusion, we identified HNE–histidine adducts, which are lipid peroxidation-specific modifications of OxLDL molecules, as LOX-1 ligands. We found that the HNE–histidine adducts have a significant affinity for LOX-1. Moreover, we demonstrated that the HNE–histidine adducts stimulate ROS formation and activate ERK1/2 and NF-κB, downstream signalling molecules in the LOX-1 pathway, in HAECs. The present study thus provides intriguing new insights into the molecular details of the ligand recognition of OxLDL by this scavenger receptor.

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SUPPLEMENTARY ONLINE DATA
Identification of 4-hydroxy-2-nonenal–histidine adducts that serve as ligands for human lectin-like oxidized LDL receptor-1

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Figure S1  Protein carbonyl analysis of aldehyde-modified BSA
Aldehyde modification was performed by incubating BSA (1.0 mg/ml) with 1 mM aldehyde in 10 ml of 50 mM sodium phosphate buffer (pH 7.4) at 37 °C for 24 h. An aliquot (0.5 ml) of each protein sample was treated with an equal volume of 0.1 % DNPH (2,4-dinitrophenylhydrazine) in 2 M HCl and incubated for 1 h at room temperature. This mixture was treated with 0.5 ml of 20 % trichloroacetic acid (w/v, final concentration) and the precipitate was collected by centrifugation and extracted three times with ethanol/ethyl acetate (1:1, v/v). The protein sample was then dissolved in 2 ml of 8 M guanidinium chloride/13 mM EDTA/133 mM Tris/HCl solution (pH 7.4), and the UV absorbance was measured at 365 nm. Results are expressed as moles of DNPH incorporated/protein (mol/mol) based on an average absorptivity of 21.0 mM−1·cm−1.

Results are means ± S.D. Aldehydes used are ACR, CRA, 2-pentenal, 2-hexenal, 2-heptenal, 2-octenal, 2-nonenal, MDA, HNE, ONE and 4-oxo-hexenal (OHE).

Figure S2  ELISA analysis of protein carbonyls
LDL (0.1 mg/ml) was incubated with 0.1 mM HNE in PBS for 12 h at 37 °C. The HNE-treated LDL was diluted in PBS to 0.01 mg/ml and treated with 1 mM biotin hydrazide (Sigma) for 30 min at room temperature. The biotinylation efficiency was measured by ELISA analysis using HRP (horseradish peroxidase)-conjugated streptavidin. A 100 μl aliquot of the biotin-labelled LDL was added to each well of a 96-well ELISA plate and incubated for 1 h at 37 °C. Each well was incubated with 200 μl of 4 % Block Ace (Yukijirushi) in TBS-T for 1 h at 37 °C to block the unsaturated plastic surface. The supernatants were discarded, the wells were washed three times with TBS-T, and 100 μl of a 1:1000 dilution of HRP-conjugated streptavidin in TBS-T was added to each well. The plates were washed 3 times with TBS-T, and 100 μl of a 1:1000 dilution of HRP-conjugated streptavidin in TBS-T was added to each well. The plates were then incubated for 1 h at 37 °C. The enzyme-linked antibody bound to the wells was revealed by adding 100 μl of o-phenylenediamine (0.5 mg/ml) in 0.1 mM citrate/phosphate buffer (pH 5.0) containing 0.03 % hydrogen peroxide per well. The reaction was terminated by the addition of 50 μl of 1 M sulfuric acid, and the absorbance at 490 nm was read with a micro-ELISA plate reader (Bio-Rad Laboratories). Results are means ± S.D.

Figure S3  Correlation between CFP–LOX-1 expression level and DiD–AcLDL uptake
CHO cells expressing CFP–LOX-1 were incubated with DiD–AcLDL (0.4 μg/coverslip) for 15 min at 37 °C in a humidified atmosphere containing 5 % CO2. Next, cells were fixed with 2 % formaldehyde and observed as described in the Experimental section of the main paper. Horizontal axis, fluorescence after excitation at 436 nm, which represents the expression of CFP–LOX-1; vertical axis, fluorescence at 620 nm, which represents AcLDL uptake.

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Figure S4  Sensorgram of binding of serially diluted OxLDL to CTLD14 immobilized on an SA sensorchip

(A) Binding curves of each flow cell. (a) Curve derived from an immobilized CTLD14 flow cell. (b) Curve derived from non-immobilized CTLD14. (c) Curve derived from the injection of buffer only. (B) Sensorgram with the two reference curves (b and c) subtracted from the binding curve (same curve shown in Figure 6A of the main paper). The broken line shows the data-fitting result.

Figure S5  Fluorescence intensity of cells expressing CFP–LOX-1 with no ligand added

CHO cells expressing CFP–LOX-1 were examined by fluorescence microscopy. (A) Phase contrast. (B) Excited at 436 nm (CFP). (C) Excited at 535 nm (suitable for DiI (1,1-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate)). (D) Excited at 620 nm (suitable for DiD). The background fluorescence intensity was higher in (C) than in (D).

Figure S6  DiD–AcLDL uptake by native and CFP–LOX-1-expressing CHO cells

Cells were incubated with DiD–AcLDL (0.4 μg/coverslip) for 15 min at 37 °C in a humidified atmosphere containing 5% CO2. Next, cells were fixed with 2% formaldehyde and observed as described in the Experimental section of the main paper. (A) Native CHO cells; (B) CHO cells stably expressing CFP–LOX-1; (C) cells pre-incubated with anti-LOX-1 neutralizing antibody 23C11. Left-hand panels: phase contrast; central panels: CFP–LOX-1 (excitation at 436 nm); right-hand panels: DiD–AcLDL (excitation at 620 nm).
Figure S7  LOX-1 inhibition of DiD–AcLDL uptake by aldehyde-modified BSA

Cells were treated as described in Figure S1. Results are mean ± S.D. percentages of uptake/carbonyl.

Figure S8  Correlation between CFP–LOX-1 expression level and aldehyde-modified BSA uptake

Cells were incubated with HNE–BSA for 15 min at 37 °C in a humidified atmosphere containing 5% CO₂. Next, cells were stained for immunofluorescence as described in the Experimental section. (A) A-1: native CHO cells; A-2: CHO cells stably expressing CFP–LOX-1. (B) Correlation between CFP–LOX-1 expression level and HNE–BSA uptake. Horizontal axis, fluorescence after excitation at 436 nm, which represents the expression level of CFP–LOX-1; vertical axis, fluorescence at 620 nm, which represents HNE–BSA uptake.
MS analyses were performed using a Waters quadrupole MS/MS Acquity TQD equipped with an ESI probe and used with a Waters UPLC Acquity system. The injected samples (10 μl each) were separated on a Acquity UPLC BEH C18 150 mm length × 2.1 mm internal diameter, 1.7 μm particle size, column at a flow rate of 0.3 ml/min. A discontinuous gradient was used by mixing solvent A (water containing 0.1 % formic acid) with solvent B (acetonitrile containing 0.1 % formic acid) as follows: initial equilibration of column at 2 % solvent B, increasing from 2 to 7 % solvent B from 0 to 4 min, increasing to 50 % solvent B from 4 to 10 min, increasing to 95 % solvent B from 10 to 11 min, and isocratic elution with 95 % solvent B from 11 to 13 min. MS analyses were performed online using ESI–MS/MS in the positive-ion mode with MRM mode (cone potential 15 eV/collision energy 10 eV). The MRM transition monitored was m/z 195.6 → 172.6. The amount of HNE–histidine adducts was quantified by the comparison of the peak areas with those obtained from the calibration curve. (A) CID (collision-induced dissociation) of the [M + H]+ of pyridylaminated HNE–histidine at m/z 195.6 and the proposed structures of individual ions. (B) LC–ESI–MS/MS analysis of authentic pyridylaminated HNE–histidine. The ion current tracing of pyridylaminated HNE–histidine using LC–ESI–MS/MS with MRM is shown. (C) LC–ESI–MS/MS analysis of HNE–histidine generated in the OxLDL. The ion current tracing of pyridylaminated HNE–histidine generated in the OxLDL is shown (left-hand panel). The right-hand panel represents the levels of HNE–histidine adducts in native and oxidized LDL. * P < 0.05 compared with native LDL. The protein samples were pyridylaminated with 0.34 M 2-AP (2-aminopyridine) and 15.75 mM NaCNBH3. After the reaction, the protein was precipitated by the addition of an equal volume of 20 % (w/v) TCA (trichloroacetic acid). The mixtures were incubated for 30 min in an ice bath and centrifuged at 17 000 g for 15 min at 4 °C to collect the pellet of precipitated protein. The pellet was washed twice with 0.5 ml of cold 20 % (w/v) TCA and 0.5 ml of ice-cold acetone and then centrifuged as before. The pellet was allowed to air dry, and the resulting protein was hydrolysed for 24 h at 110°C with 1.5 ml of 6 M HCl. The hydrolysate was evaporated to dryness at 40°C in vacuo followed by reconstitution with 1 ml of distilled water containing 2 % formic acid. After the acid hydrolysis, the samples were partially separated with Oasis MCX cartridges (Waters) as follows. The samples were loaded and the MCX cartridges washed with 10 ml of distilled water containing 0.2 % formic acid and 3 ml of methanol, and then the pyridylaminated–HNE–histidine adducts were eluted with 2 ml of 5 % ammonia in methanol. The samples were then dried, dissolved in distilled water and subjected to LC–ESI–MS/MS analysis. A calibration curve was obtained from the authentic HNE–histidine adducts by the same procedure. Results are means ± S.D.

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