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Iron-catalyzed oxidation of Trp residues in low-density lipoprotein

Hsin-Hung Chen[1], Ching-Yi Chen[2], Lu-Ping Chow[3], Chu-Huang Chen[1,4,5], Yuan-Teh Lee[2,5], Charles V. Smith[6] and Chao-Yuh Yang[1,4,5,*]

[1] Section of Atherosclerosis and Lipoprotein Research, Department of Medicine, Baylor College of Medicine, Houston, TX 77030, USA
[2] Department of Internal Medicine, National Taiwan University Hospital, Taipei 100, Taiwan
[3] Graduate Institute of Biochemistry and Molecular Biology, Medicine College, National Taiwan University, Taipei 100, Taiwan
[4] Vascular and Medicinal Research, Texas Heart Institute, Houston, TX 77030, USA
[5] L5 Research Center, China Medical University Hospital, Taichung 40447, Taiwan
[6] Center for Developmental Therapeutics, Seattle Children's Research Institute, Seattle, WA 98101, USA

*Corresponding author
e-mail: chaoyuhyang@gmail.com

Abstract

The mechanisms of oxidation of low-density lipoproteins (LDLs) are not well defined, but epidemiological and experimental studies suggest that iron-catalyzed processes may contribute to atherogenesis. The aim of this study was to test the hypothesis that iron-catalyzed oxidations of LDLs *in vitro* produce diagnostic biomarkers of oxidation of the apolipoprotein that could be applied to studies *in vivo*. LDLs were oxidized in the presence of Fe^{2+}, EDTA, and ascorbic acid for up to 40 h. Following delipidation and trypsin digestion, the peptides were separated by HPLC, with four peaks detected at 365 nm, whereas none were observed in peptides from unoxidized LDLs. The peptides were identified by MALDI-QTOF mass spectrometry as IVQILP(W+4) EQNEQVK, IYSL(W+4)EHSTK, FEGLQE(W+4)EGK, and YH(W+4)EHTGLTLR, with (W+4) rather than the W residues of the unoxidized protein. The mass gains (+4 increase in *m/z* in tryptophan, W) and absorbance at 365 nm indicate kynurenines, which were trypsin-releasable peptides that are on the surface of LDL particles. All four peptides thus characterized share the sequence of WE. The preferential oxidation of W residues in WE sequences suggest contributions from the C-proximate glutamate residues in chelation of the iron species, thereby influencing site selectivities of oxidation. These kynurenine-containing peptides might serve as biomarkers of iron-mediated oxidations *in vivo*.

Keywords: apoB-100; iron-mediated oxidation; kynurenine; low-density lipoprotein (LDL); tryptophan; WE.

Introduction

Cardiovascular diseases comprise the leading cause of death in the United States, as well as in many other countries, and evidence suggests that oxidative modifications of low-density lipoproteins (LDLs) contribute to the initiation and progression of atherosclerosis and the resultant cardiovascular problems (Berliner et al., 1995; Ross, 1999). Although the body of evidence linking oxidation of LDLs with atherosclerosis is substantial, important uncertainties remain, including the identities of the mechanisms responsible for biologically relevant oxidations of LDLs *in vivo*. Oxidations of LDLs *in vitro* have been studied extensively, with the principal experimental models employing redox-active transition metals, copper and iron, and oxidations driven by myeloperoxidase or other peroxidatic enzymes (Lynch and Frei, 1995; Ziouzenkova et al., 1998; Carr et al., 2000). Each of these model systems has been shown to change properties of LDLs in ways that would be likely to contribute to atherosclerosis *in vivo*. Oxidation can cause loss of recognition of LDLs by the LDL receptor, which alters normal distribution and disposition of LDLs. Oxidation of LDLs can also cause gain of recognition of oxidized LDLs (OxLDLs) by scavenger receptors, leading to uptake by macrophages, endothelial cells, and vascular smooth muscle cells by mechanisms that are not subject to feedback inhibition control (Osterud and Bjorklid, 2003; Adachi and Tsujimoto, 2006), which can lead to foam cell formation and progression of atherosclerotic lesions. Furthermore, OxLDLs are cytotoxic to cells in culture, and altered cell functions and/or death *in vivo* could initiate and should accelerate atherogenesis (Balla et al., 1991, 1993; Jeney et al., 2002; Ujhelyi et al., 2006).

Identification of the mechanisms responsible for oxidation of LDLs *in vivo* requires comparisons of modifications introduced by experimental models of oxidations *in vitro* with the respective product profiles observed in LDLs isolated with minimal modifications during separation. Oxidation of LDLs at appreciable rates *in vitro* requires catalysis or stimulation of initiation, and iron- and copper-catalyzed oxidations are the model systems that have been studied most extensively. Iron species are more abundant biologically than are copper species, and epidemiological evidence (Sullivan, 1981; Salonen et al., 1992; Sempos and Looker, 2001) from cohort, case-control, and cross-sectional studies suggest correlations between iron status and risk of cardiovascular disease, although other studies report no correlation (Corti et al.,

1997; Ascherio et al., 2001). Copper-catalyzed oxidations of LDLs have been studied more extensively than have oxidations by iron, perhaps reflecting the more rapid oxidation of LDLs with increases in levels of products that can be measured by methods within the capabilities of most laboratories (Lynch and Frei, 1995; Ziouzenkova et al., 1998; Burkitt, 2001). However, the data available at present do not establish copper-catalyzed oxidations as principal initiators of atherogenesis *in vivo*, either by epidemiological associations or by associations with mechanistically informative biomarkers.

Iron in mammalian cells and organisms is found mostly in hemoglobin, myoglobin, and other heme- and iron-sulfur proteins. In these and other forms of biological iron, access of redox-active reactants is regulated tightly, which limits and directs the catalysis of redox reactions by iron chelates. Ferritin-transferrin and similar systems regulate intercellular and intracellular transport of iron. Ferritin serves to store much of the iron not in active use in enzymes (Theil, 1983), thereby limiting production of free radical species and derived or related oxidants (Wood, 2004). Unregulated release of iron from these binding and regulatory proteins can result in stimulation of the rates of relatively nonspecific oxidation reactions. When iron escapes these control processes, redox-active iron chelates can generate free radicals via Haber-Weiss-Fenton reactions (Haber and Weiss, 1934; McCord and Day, 1978). Studies by Balla and colleagues suggest that heme released from hemoglobin or myoglobin may accelerate atherosclerosis by catalyzing redox reactions in the lipophilic domains of LDLs, with release of heme iron to catalyze yet other oxidations in more polar domains, as well as by increasing susceptibilities of endothelial cells to oxidant exposures as essential components of the effects (Balla et al., 1991, 1993; Jeney et al., 2002; Grinshtein et al. 2003).

Among the many difficulties in assessing the manner and extent to which oxidative modifications of LDLs contribute to atherogenesis *in vivo* has been the reliance upon nonspecific methods for studies of LDL oxidation and upon similar nonspecific concepts for interpretation of the results of the studies. Measurements of thiobarbituric acid reactive substances and/or of absorbance at 234 nm, attributed to formation of conjugated diene chromophores, while convenient and yielding certain advantages, offer little in the way of information about the specific products formed and the specific mechanisms by which the oxidations of LDLs are mediated.

The wide range of specific products formed in oxidations of LDLs and the wide dynamic range in which those products accumulate dictate that studies of oxidation of LDLs and other biological systems have been, of necessity, focused on products or classes of products that are limited in scope. Peroxidation of lipids in LDLs may be critical to biological effects, but the products of oxidation of LDL lipids are formed predominantly in the propagation phase of lipid peroxidation, and these reactions are not diagnostic of mechanisms of initiation (Smith, 1991). In addition, the products formed in greatest abundance might not necessarily dominate the biological effects mediated by OxLDLs, and the products of oxidation that are identified are seldom related quantitatively to the fraction of precursor molecules oxidized or otherwise altered (Esterbauer et al., 1990; Lenz et al., 1990).

Oxidations of the apolipoprotein are not as dominated by nonspecific radical chain propagation reactions and therefore are more likely to be characteristic of individual mechanisms (Davies et al., 1999). Furthermore, the specific positions of the altered residues in the primary peptide sequence still offer greater possibilities for reaction specificity (Yang et al., 1997, 1999a,b, 2001). Characterization of products at this level is a challenging problem in bioanalytical chemistry, but such studies are needed to delineate specific mechanisms of the oxidation of LDLs. Furthermore, the advances gained in elucidation of the specific products of oxidative modifications of LDL protein *in vitro* and *in vivo* will greatly enhance efforts to characterize even more complex problems in studies of mechanisms of oxidation of cells and tissues.

Oxidatively modified amino acid residues have been observed in proteins exposed to a variety of redox-active iron oxidation systems associated with Fenton chemistry. Using Fe^{2+}/EDTA/ascorbate and Fe^{2+}/DTPA/H_2O_2 systems, Cohen et al. (1998) confirmed the formation of dihydroxyphenylalanine from tyrosine residues in protein. With Fe^{2+}/EDTA/ascorbate and hemin/H_2O_2 systems, arginine and proline were found to be susceptible to form γ-glutamyl semialdehyde (Pietzsch, 2000; Pietzsch and Julius, 2001; Pietzsch and Bergmann, 2003). With Fe^{3+}/ascorbate oxidation, lysine was modified to adipic semialdehyde (Ayala and Cutler, 1996) or carboxymethyllysine (Requena and Stadtman, 1999; Requena et al., 2001).

Native proteins and peptides derived from unmodified proteins essentially exhibit no innate absorbance at 365 nm and no reactivity with 2,4-dintrophenylhydrazine (DNPH), whereas the modifications induced by some types of oxidation impart reactivity with DNPH, which can be used to produce derivatives that exhibit detectable absorbance at 365 nm. In earlier studies, we observed that the oxidation of LDLs in the presence of copper *in vitro* (Yang et al., 1999b) resulted in formation of peptides that absorbed at 365 nm, without reaction with DNPH. The transformations were characterized as oxidations of tryptophan (Trp) residues on the particle surface to the corresponding kynurenines. By contrast, in oxidation of LDLs by HOCl or by myeloperoxidase *in vitro* (Yang et al., 1997, 1999a, 2001), we observed no detectable formation of kynurenines, as evidenced primarily by the absence of detectable peaks observed at 365 nm, in samples not treated with DNPH. In the present study, we investigated the oxidation of LDLs by a Fe^{2+}/EDTA/ascorbate system in phosphate-buffered saline (PBS), to test the hypothesis that iron-catalyzed oxidation of LDLs *in vitro* would provide potentially diagnostic biomarkers of modification of the apolipoprotein.

Results

For the present study of LDL oxidation *in vitro*, we used the L1 fractions separated by UnoQ chromatography, because

this fraction, which typically represents 90% of the total LDLs isolated from healthy subjects, is indicated from our other studies, to date, to contain the lowest concentrations of oxidation or other modifications (Yang et al., 2003). HPLC analyses of tryptic peptides for native L1 did not show any peaks at 365 nm at signal-to-noise ratios exceeding three (Figure 1). Peptides from Fe^{2+}-oxidized LDLs, without treatment with DNPH, monitored at 365 nm, showed four peaks that were clearly detectable, indicating the possible formation of kynurenine-containing peptides. The four specific peaks, labeled A, B, C, and D, exhibited retention times of 27.4, 28.8, 30.0, and 32.8 min, respectively, as depicted in Figure 1. Peptides from Fe^{2+}-oxidized LDLs treated with DNPH prior to delipidation and trypsin digestion did not yield identifiable peaks, although nonspecific patterns of increased absorbance at 365 nm were observed between 15 and 45 min, when most of peptides observed at 210 nm eluted.

SDS-PAGE analyses of aliquots of the LDL fractions taken during incubation with the iron oxidation system showed that the band of intact apoB-100 largely disappeared by 1 h, and by 8 h only relatively homogeneous smears were observed (Figure 2). No prominent bands indicative of selective cleavage of the apoprotein and no high-molecular-weight covalent aggregates were observed at any time examined. Efforts to relate HPLC peak areas of fragments A–D to molar yields, using external standardization with peak areas from authentic kynurenine and estimates of moles of apoB-100 based on mg of protein from Lowry or more precise means of protein quantitation, indicate that more specific methods for these analyses will be needed.

Peaks A, B, C, and D were purified by sequential HPLC separations, using three different buffer systems, as described previously (Yang et al., 1999b). The chromatograms for purification of peak D are demonstrated in Figure 3, in which the absorbance of peak D at 365 nm is displayed with the corresponding peptide absorbance profile at 210 nm (gray

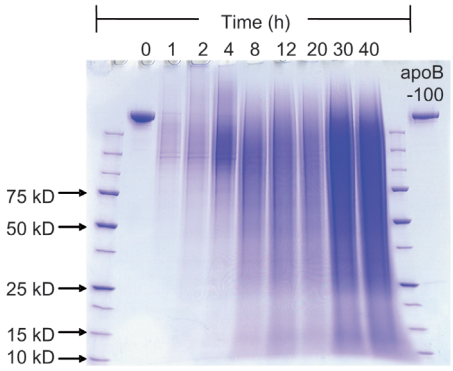

Figure 2 SDS-PAGE analysis of the time course of Fe^{2+}-catalyzed oxidation of LDLs.
The purified L1 fraction of LDLs (2 mg/ml of apoB-100) was incubated in the presence of Fe^{2+}/EDTA/ascorbate at 37°C in the dark. Aliquots were removed and delipidated at the times indicated, with sample preparation, loading, and detection as described in the Methods section. Lanes 2–10 were samples obtained at reaction time points from 0 through 40 h, as indicated. Lanes 1 and 11 present MW standards, and lane 12 is apoB-100.

curve with scale modified to facilitate comparison). Peak D was first pooled from manual collections from 10 injections (total of 20 mg of LDL protein) with the ammonium acetate buffer system. This material was purified further in the second HPLC separation with the phosphate buffer mobile phase (Figure 3B). Peak D was collected manually and separated with the trifluoroacetic acid (TFA) buffer, giving a substantially purified peptide more amenable to characterization by mass spectral analyses (Figure 3C).

The mass spectrometric analyses were performed with QSTAR, using MALDI ionization and time-of-flight (TOF) detection. With the same method, we previously identified copper-modified Trp exhibiting a +4 increase in *m/z* (W+4)

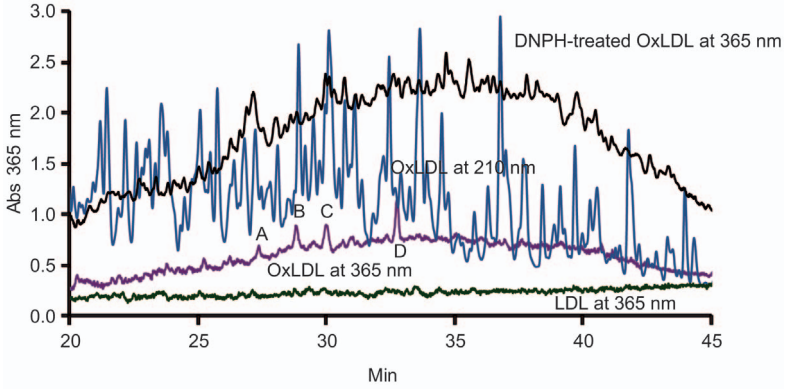

Figure 1 HPLC chromatograms at 365 nm (curves in black) in TFA buffer system for tryptic peptides from native LDLs, Fe^{2+}-oxidized LDLs, and DNPH-treated Fe^{2+}-oxidized LDLs.
Fe^{2+}-oxidized LDLs were obtained by oxidation with the Fe^{2+}/EDTA/ascorbate system (200 μM iron ascorbate, 60 μM EDTA, and 5 mM ascorbic acid) in PBS buffer at 37°C in the dark for up to 40 h, delipidated and trypsinized, as described in the Methods section, to yield distinguishable HPLC peaks A, B, C, and D, as indicated. Treatment of Fe^{2+}-oxidized LDLs with DNPH prior to delipidation and digestion gave the nonspecific chromatographic pattern shown. Peptides from native LDLs did not yield peaks detectable at 365 nm, whether or not treated with DNPH. A chromatogram of tryptic peptides from Fe^{2+}-oxidized LDLs showing detection at 210 nm is presented in the background in gray for comparison.

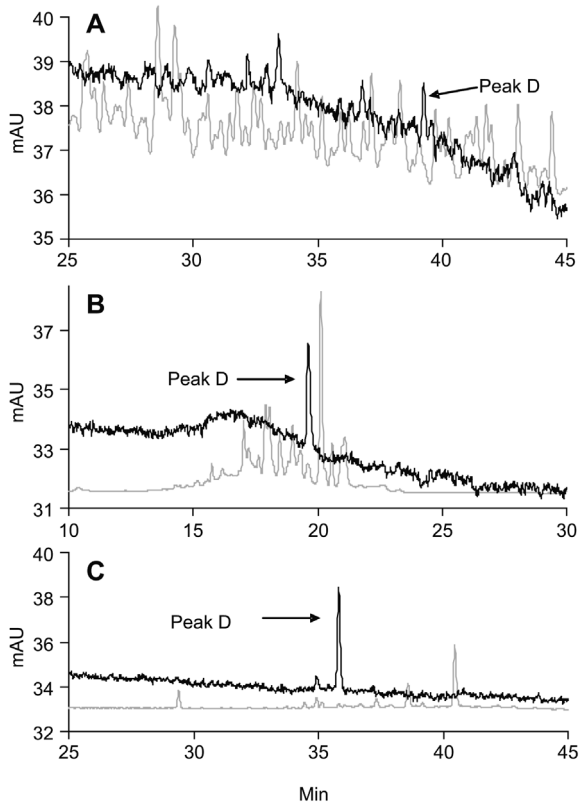

Figure 3 HPLC chromatograms for the purification of peak D with C_{18} reverse phase column utilizing (A) ammonium acetate, (B) 5 mM of phosphate at pH 6.0, and (C) 0.1% TFA buffer systems. Elution profiles were monitored by absorbance at 365 nm (dark chromatograms) and at 210 nm (gray lines), fitted to the scale of that for absorbance at 365 nm, for comparison.

on apoB-100 residues (Yang et al., 1999b). Here, a representative MS/MS spectrum for peak D (Figure 4) indicates a sequence of IVQILP(W+4)EQNEQVK (amino acids 550–563), with oxidation of Trp (W_{556}) to the corresponding (W+4) mass, indicating oxidation of this tryptophan residue to the corresponding kynurenine. Three immonium ions, 86.096, 101.071, and 101.107 of Leu (Ile), Gln, and Lys, respectively, were observed. Both *b* and *y* ion peaks were assigned. The corresponding ion masses for the unmodified peptide IVQILPWEQNEQVK were observed from CAD fragments before *b*6 and *y*7 ions, whereas those after *b*7 and *y*8 gave ions showing mass/charge (*m/z*) ratios that were 4 Da greater than for the unmodified sequence, consistent with modification of Trp to kynurenine. In addition to peak D, the sequences and the amino acid (AA) positions for the other three peaks that exhibited detectable absorbances at 365 nm from Fe^{2+}-catalyzed oxidation of LDLs *in vitro* were similarly identified and determined, and are listed in Table 1, with their corresponding theoretical ion masses $[M'+H]^+$ for native peptides and experimental ion masses $[M+H]^+$ for the peptides isolated from oxidized LDLs. The other peptides thus identified are: A, FEGLQE(W+4)EGK (AA: 3937–3946) of 1226.58; B, YH(W+4)EHTGLTLR (AA: 4061–4071) of 1416.82; and C, IYSL(W+4)EHSTK (AA: 3532–3541) of 1267.72. These peptides were indicated by our previous studies of limited trypsin digestion of intact LDL particles to be located in trypsin-releasable peptides, which we reason are located in hydrophilic domains of apoB-100 on the surface of LDLs (Yang et al., 1989). Each of the four peptides characterized as undergoing iron-catalyzed oxidation of the W residue to the corresponding kynurenine has a glutamate immediately C-terminal to the W (WE).

Discussion

Evidence of oxidatively modified amino acid residues in apoB-100 in circulating LDLs and in peptides derived from fragmentation of LDLs has been reported (Bruce et al., 1999), but the approaches employed in most studies, to date, are not designed to distinguish among the principal candidate mechanisms likely to contribute to oxidative modifications of LDLs *in vivo*, thereby contributing to the initiation and progression of atherosclerosis. The formation of *N*-formylkynurenine and kynurenine residues in oxidations of proteins has been characterized immunologically (Ehrenshaft et al., 2009), but the antibody-based methods described thus far do not distinguish positional selectivity of oxidation in the protein sequence, as is needed for the primary purposes of our study, which is to determine whether specific products of protein oxidation can distinguish iron-catalyzed oxidation from other mechanisms. Iron and copper are the two principal biological redox-active metals that have received the most attention as potential contributors to the oxidation of LDLs that is associated with atherosclerosis, and accumulations of both iron and copper have been observed in human atherosclerotic plaques (Stadler et al., 2004). Accumulation in the necrotic core of an atheroma of nondigestable cellular or tissue components would not be surprising, and distinctions between causes and effects in the process of atherosclerosis will need to include such considerations. Stanley et al. (2006) reported that iron concentrations in advanced atherosclerotic lesions correlated with contents of oxidized proteins, as characterized by altered amino acid residues, such as *o*-tyrosine. As with the antibody-driven studies of Ehrenshaft et al. (2009), the products of protein oxidation reported by Stanley et al. (2006) were not positionally specific, as is required for the goals of our study.

The four kynurenine-containing peptides identified in the present study of iron-catalyzed oxidation of LDLs contrast with the nine kynurenine-containing peptides we observed in our earlier studies of LDLs oxidized in the presence of copper (Yang et al., 1999b). Selective oxidation of intrinsically more reactive W residues by iron does not explain the data, however, because only two of the four kynurenines observed in the present study with iron oxidation were among the nine found with copper-catalyzed oxidations. Two of the alterations characterized in the present study of iron-catalyzed oxidation of LDLs, YH(W+4)EHTGLTLR, and FEGLQE-(W+4)EGK, were not identified as products in our previous studies of oxidation with copper. Although other explanations and mechanisms are not precluded by the data available

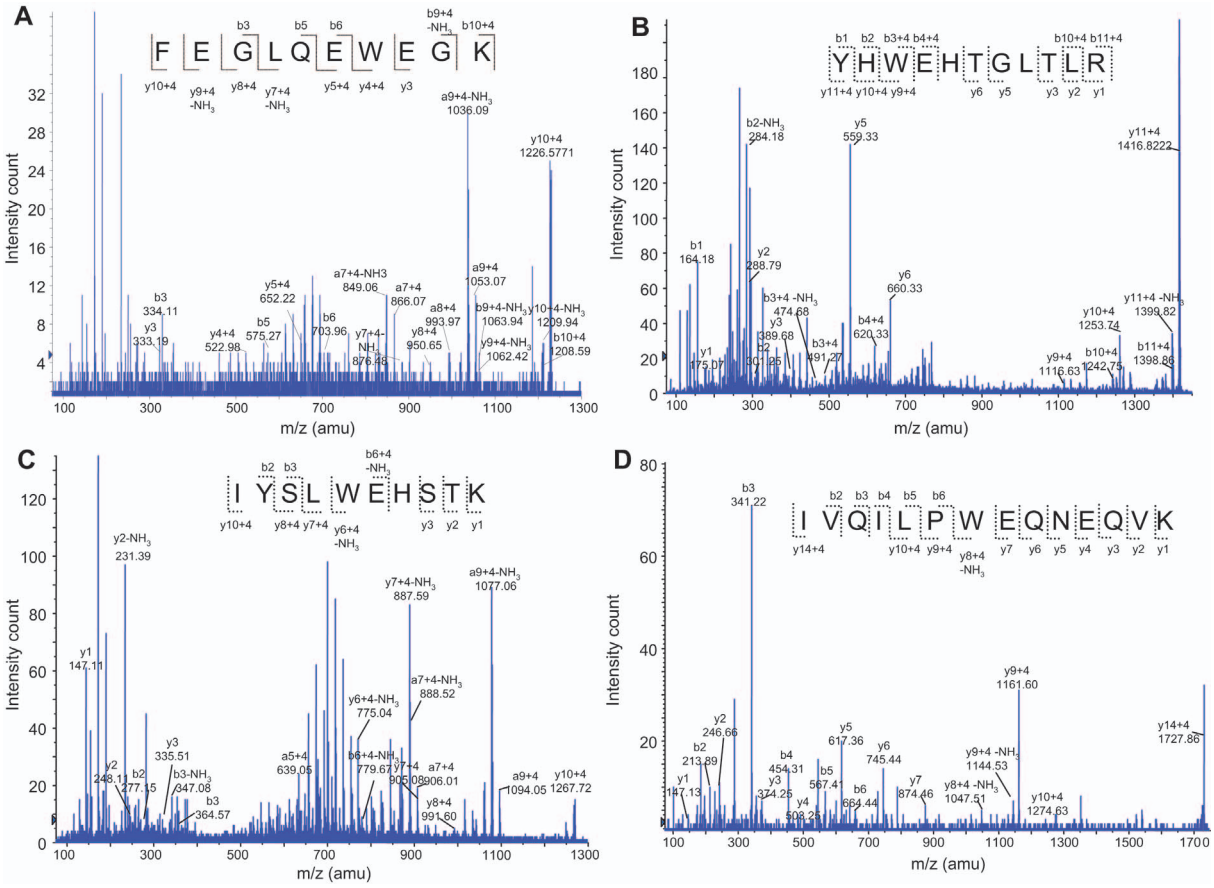

Figure 4 Tandem mass spectra for peak peptides isolated from HPLC peaks A–D, as indicated.
The peptides assigned to the spectra are indicated and were identified on the bases of fragment ions and the known amino acid sequence of apoB-100. The ions reflecting increases in *m/z* of 4 Da from the ions predicted for the respective fragments from the unoxidized sequence are indicated, uniformly indicating oxidation of the W to the corresponding kynurenine. See also Table 1 for sequence position of the peptides identified.

Table 1 Tryptic peptides of apoB-100 containing kynurenines converted from the corresponding Trp by iron-mediated oxidation.

Peptide	Position on apoB-100	Cu	Fe-ascorbate	$[M+H]^+$ *m/z*	$[M'+H]^+$ *m/z*
VA(W+4)HYDEEK	1138–1146	x			
SEILAH(W+4)SPAK	1108–1118	x			
IYSL(W+4)EHSTK	3532–3541	x	C	1263.64	1267.72
(W+4)NFYYSPQSSPDK	3995–4007	x			
NLQNNAE(W+4)VYQGAIR	4080–4094	x			
IVQILP(W+4)EQNEQVK	550–563	x	D	1723.94	1727.93
NLTDFAEQYSIQD(W+4)AK	2533–2548	x			
VN(W+4)EEEAASGLLTSLK	4029–4044	x			
TIDQMLNSELQ(W+4)PVPDIYLR	2648–2667	x			
FEGLQE(W+4)EGK	3937–3946		A	1222.57	1226.58
YH(W+4)EHTGLTLR	4061–4071		B	1412.71	1416.82

The tryptic peptides identified from oxidation of LDLs by Cu-catalyzed oxidation (Yang et al., 1999b) and with iron-ascorbate in the present study. The letters listed with iron-catalyzed products correspond to the HPLC peaks identified in Figure 1. Peptides A and B in the present study were not identified in the experiments with Cu-catalyzed oxidation of LDLs.

at this time, a working hypothesis is that the respective product profiles reflect differences in sites of chelation of the respective transition metals and/or redox activities of the chelates thus formed.

Structural studies of LDLs indicate that although particle sizes can vary, the particles exhibit substantial organization of the apolipoprotein and probably a considerable degree of ordering of the lipid phase (Orlova et al., 1999; van Antwerpen et al., 1999). The products of copper oxidation that we observed in our previous studies and the peptides characterized in the present study of iron-catalyzed oxidations are in peptides that are released by limited trypsin treatment of intact LDL particles, giving what we term trypsin-releasable peptides. The results of our study are therefore consistent with the working hypothesis that the W residues subject to oxidation in the presence of $Cu^{+/2+}$ or $Fe^{2+/3+}$ are on the surface of the LDL particles, accessible to the aqueous phase that is more readily accessible to polar salts and hydrophilic chelates of these redox-active transition metals. The 20% decrease in fluorescence emission that Giessauf et al. (1995) observed and interpreted as indicating that eight to nine of the 37 W residues in apoB-100 are accessible to the aqueous phase for binding Cu^{2+} ions and catalysis of oxidation is consistent with our experiments with copper oxidation. In addition, Giessauf et al. (1995) concluded that a limited number of copper ions bound to LDLs in the vicinity of W residues and that oxidation of these residues in the presence of copper was an initial event in oxidation of LDLs, not a consequence of lipid peroxidation.

The fact that the four peptides found to be oxidized in the iron-catalyzed reactions in the present study all contain W residues that are immediately (N-terminal) to glutamate (E) is intriguing. Of the 37 W residues in apoB-100, seven contain the WE sequence, far in excess of what would be expected from random distribution of amino acids in the primary sequence. Nevertheless, the preferential oxidations by iron of the W in the WE sequences suggest that the adjacent glutamate may help determine the sites of oxidation, presumably by participating in chelation of $Fe^{2+/3+}$, thereby directing or favoring oxidation of the proximate W.

Of the seven WE-containing peptide sequences, LPQQANDYLNSFNWER (2118–2133) and DFSLWEK (3149–3155) were not observed to form the corresponding kynurenine-containing peptides either in our previous studies with copper-catalyzed oxidations or in our present study with iron oxidation. These two peptides are located in the trypsin nonreleasable domains of apoB-100, presumably existing in environments with limited access to the aqueous exterior and less able to form or stabilize a redox-active iron chelate. The seventh WE-containing peptide, VNWEEEAASGLLTSLK (4056–4071), is in a trypsin-releasable domain, but was not observed among the products of oxidation by the Fe^{2+}/EDTA/ascorbic acid system, despite the fact that the oxidation of the W in this peptide was identified in our earlier studies of copper oxidation of LDLs (Yang et al., 1999b). The additional glutamate carboxylates might inhibit localized effects of iron, while not interfering similarly with copper, which has different ligand chelation affinities, but this speculation will need to be tested critically.

The present results are subject to the same issues that limit the interpretations of previous studies, principally the lack of data regarding the extent to which products identified account for decreases in contents of intact starting materials. Such issues are best resolved by increasing specificities of separation and detection. The successive purifications illustrated for peak D in Figure 3 show that the material isolated in the first step of separation by HPLC is far from pure, but this working hypothesis needs additional critical examination. Product yield efforts would be enhanced by the ability to quantitate the contents of precursor molecules, in the present case the contents of intact, unoxidized peptides from which the kynurenine-containing peptides are derived. The principle of percent yield is seldom applied to studies of oxidation of endogenous substrates *in vivo*. Development of methods adequate for meaningful comparisons of product formation with substrate consumption in such oxidative alterations *in vivo* is one of the longer-range goals of our studies of LDL oxidation *in vitro* (Arai et al., 2005).

The rapid loss of protein migrating with the intact apoB-100 evidenced in Figure 2 suggests that fragmentation of the primary peptide chain might be even more characteristic of iron-catalyzed oxidation of LDLs than are the kynurenine-containing modifications we describe. In addition to the time course of the effects observed, the gels are striking for an absence of any obvious selectivity for fragmentation of the primary sequence, as would be expected if chain cleavage were directed by selectivity in chelation of the transition metal catalyst or by any other factors. If oxidative alterations of LDLs in the presence of iron are as rapid and as random as is suggested by Figure 2, the selectivities for formation of the kynurenine-containing peptides we observed are somewhat surprising, but suggest significant preservation of particle structure during iron-catalyzed oxidation of the intact particles.

The iron-catalyzed oxidations of W residues in the four peptides from apoB-100 to the corresponding kynurenines reflect the element of accessibility to the aqueous exterior of the particles, as previously observed for copper-catalyzed oxidation of LDLs. The present data indicate additional selectivities expressed in iron-catalyzed oxidation of LDLs, and the fact that the products identified represent oxidation of four of the five W residues in apoB-100 found in trypsin-releasable peptides as shown in Figure 5 and located immediately N-terminal to E residues suggest participation of the WE sequence in chelation of $Fe^{2+/3+}$, with resultant localized oxidation of the W. The observed selectivity for oxidation of these specific W residues suggests potential biomarkers for LDL oxidation due to disordered iron regulation that might be adapted for studies of LDL oxidation *in vivo*.

Materials and methods

LDL preparation and fractionation

LDLs were isolated from the plasma of normal subjects by sequential ultracentrifugation between 1.019 and 1.063 g/ml. LDL fractions were separated on a UnoQ12 strong ion exchange column (BioRad,

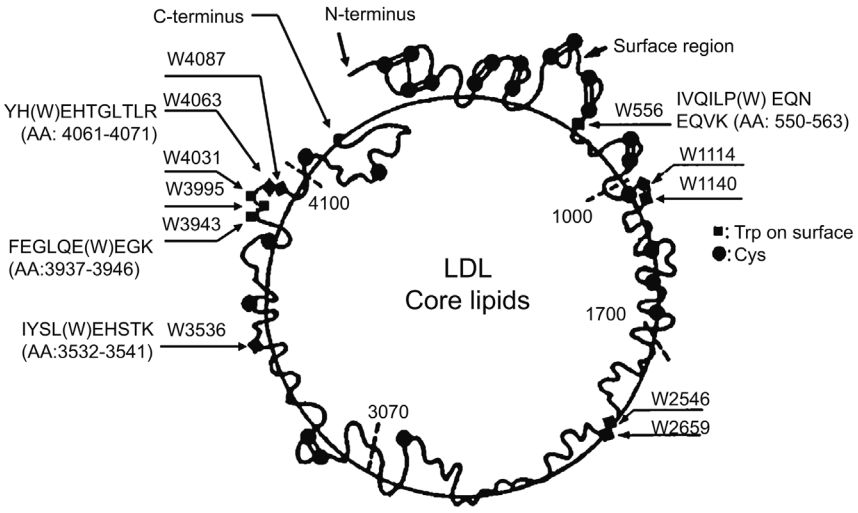

Figure 5 Relative locations of the Trp residues on apoB-100 of LDL oxidized by Fe^{2+}/EDTA/ascorbate.
An illustrative LDL particle is shown, with apoB-100 principally on the surface and penetrating the lipid core. The relative lengths of peptides are not drawn to scale. The surface core orientation presents the concepts of tryptic-releasable and tryptic-nonreleasable motifs, instead of implication of domains on outer surface or in buried core. The identified sequences of peptides are labeled with the amino acid positions of the corresponding Trp residues. Trp residues are indicated by solid squares, and Cys residues by solid circles. In this illustration, double lines are used to indicate disulfide bonds, although disulfide bonds are single, not double, bonds.

Hercules, CA, USA) as described previously (Yang et al., 2003). The isolated fractions were stored at 4°C for further analyses, which were conducted within 2 weeks of isolation.

SDS-PAGE electrophoresis

For SDS-PAGE, 25 μg of LDL subfractions was delipidated with ethanol:ethyl acetate (1:1) and solubilized overnight with 10% SDS (35 μl), followed by the addition of 15 μl of sample buffer. Aliquots of 5 μl of samples were separated on 4–20% SDS gels (InVitrogen Inc., Carlsbad, CA, USA) at room temperature at 30 mA for 1 h.

Iron-mediated oxidation

Oxidations of LDLs at 2 mg/ml in total volumes of 2 ml were carried out in the presence of ferrous-EDTA-ascorbic acid (200 μM iron ascorbate, 60 μM EDTA, and 5 mM ascorbic acid) in PBS buffer at 37°C in the dark for up to 40 h (Pietzsch, 2000). Reactions were terminated at times through 40 h, by addition of 10% TCA, to precipitate lipoproteins, and further delipidation was performed by four washes with 1:1 EtOH:EtOAc.

All LDL samples used for oxidation in varying EDTA concentrations were dialyzed against PBS buffer with three exchanges in the dark to remove EDTA. EDTA was then added to final concentrations of 0, 5, or 60 μM, followed by the addition of ascorbic acid, then Fe^{2+}.

Treatment of LDL with 2,4-dinitrophenylhydrazine

Native or Fe^{2+}-oxidized LDLs (2 mg/ml) were mixed with 10 mM of 2,4-dinitrophenylhydrazine (DNPH) (Sigma, St. Louis, MO, USA) in 2 N HCl in 1:1 ratio (vol/vol) for 1 h at room temperature, with gentle shaking, followed by delipidation, as described previously (Yang et al., 1999a).

Liquid chromatography

The delipidated native LDLs or Fe^{2+}-oxidized LDLs were dried under a gentle stream of dry N_2, and the resulting pellets were digested with trypsin at 1:50 (w/w) for 20 h in 100 mM ammonium bicarbonate. The resulting peptides were separated with C_{18} reversed phase column (4.6×250 mm; polymeric 5 mm; Vydac Inc.) heated to 50°C by ÄKTA™ purifier 10 (Amersham Pharmacia Inc.), using three buffer systems, as described previously (Yang et al., 1997): (1) buffer A: ammonium acetate (25 mM); buffer B: 90% acetonitrile; (2) buffer A: phosphate (5 mM Na_2HPO_4/NaH_2PO_4; pH 6.0); buffer B: 90% acetonitrile and 10% water; (3) buffer A: 0.1% TFA in water; buffer B: 90% acetonitrile with 0.08% TFA. The flow rate was 1.5 ml/min, and effluents were monitored at 210, 280, and 365 nm. The peptides observed at 365 nm were collected and lyophilized before next use.

Peptide analysis by MALDI-QTOF

A QStar (Applied Biosystems, Foster City, CA, USA) hybrid QqTOF mass spectrometer equipped with a MALDI source and a nitrogen laser (337 nm) was used to acquire MALDI-MS and -MS/MS spectra. The instrument was calibrated externally using an acquired MS and MS/MS spectrum of renin peptide at *m/z* 1758.8, where two fragment ions and the parent were used as calibration points. Peptide samples were dissolved in 0.1% TFA in 30% aqueous acetonitrile and mixed with α-cyano-4-hydroxycinnamic acid solution (in 0.1% TFA in 50% acetonitrile), then spotted on conventional MALDI plates. The laser was run at 20 Hz. All mass spectra were acquired in positive ion mode. Dissociations of the protonated molecules were optimized, typically employing energy values of 45–95 eV and a collisionally activated dissociation (CAD) gas pressure of 6. Argon was used as the collision gas. The predominant fragments were then enhanced, using a feature of the Pulsar that decelerates and traps ions prior to accelerating them into the orthogonal time-of-flight region, to minimize ion loss. Raw data

were analyzed using Analyst QS software of the instrument, which was developed in collaboration with MDS Sciex.

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