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Formation of *N*-formylkynurenine suggests the involvement of apolipoprotein B-100 centered tryptophan radicals in the initiation of LDL lipid peroxidation

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Abstract Tryptophan oxidation products were determined in pronase E digests of apo B of delipidated, native and Cu^{2+} oxidized LDL using a sensitive and specific HPLC method. Oxidized LDL contained *N*-formylkynurenine, kynurenine and tryptamine but no oxindolylalanine and 5-hydroxytryptophan. *N*-Formylkynurenine increased from an initial value of 0.21 to 1.67 mol/mol apo B within 5 h. Apo B of native LDL also contained kynurenine (0.80 mol/mol) and tryptamine (0.13 mol/mol). The results support the assumption that oxidation of Trp residues in apo B is an early event and possibly an elementary reaction involved in initiating LDL oxidation.

Key words: Low density lipoprotein; Tryptophan; *N*-Formylkynurenine; Kynurenine; Tryptamine; Free radicals

1. Introduction

The putative elementary reaction(s) initiating lipid peroxidation in LDL particles are a recurrent theme in the context of the hypothesis that oxidative modification of LDL increases its atherogenic potential [1–3].

In Cu^{2+} dependent LDL oxidation lipid peroxidation proceeds in three characteristic consecutive time periods denoted lag, propagation and decomposition phases [2]. It is generally believed that oxidation commences with the lipid molecules and then gradually spreads over to the protein moiety apo B [4]. Such a scenario appears to be supported by the observation that fragmentation of apo B, loss of lysine ε amino groups and covalent binding of lipid oxidation products to amino acid residues is not an early event occurring during the lag phase, but is associated with propagation and decomposition [2,3]. Recently, however, we have obtained evidence that a relatively large proportion of the 37 Trp residues of apo B is degraded during the lag phase by a lipid peroxidation independent process [5]. The remaining Trp are then lost during propagation presumably by interaction with oxidizing lipids [5–7]. Apo B is an exceptionally large protein consisting of 4536 amino acids [8] and conceivably the loss of a few Trp residues causes only subtle changes of LDL, such as for example decrease of the Trp fluorescence at 330 nm, but no other marked alterations such as are characteristic for oxidized LDL.

About 8–9 Trp residues of apo B are accessible to Cu^{2+} and may be available for one-electron reduction of Cu^{2+} resulting in an apo B centered Trp radical: $Trp+Cu^{2+} \rightarrow Trp^{\bullet}+Cu^{+}$ [5]. That one-electron redox reactions with concomitant formation of Trp radicals can indeed be mediated by certain protein-Trp residues has recently been demonstrated for metmyoglobin [9]. The consequence of generation of Trp radicals is an oxidative degradation of Trp, via intermediate Trp-hydroperoxides, to *N*-formylkynurenine (NFK) and kynurenine. NFK and kynurenine were demonstrated in solutions of free Trp irradiated by UV-B [10] or ionizing radiation [11] and in several proteins exposed to oxygen radicals [12,13].

In this investigation we show that NFK is formed during Cu^{2+} mediated LDL oxidation. The results support the assumption that oxidation of Trp residues in apo B is a crucial elementary reaction in initiating LDL oxidation.

2. Materials and methods

NFK and oxindolylalanine were synthesized as previously described [16]. D,L-Kynurenine was supplied by Sigma, 5-hydroxy-tryptophan by Merck and tryptamine by Aldrich. All other chemicals and reagents were of analytical grade and obtained from Merck or Sigma.

2.1. Preparation and oxidation of LDL

LDL was prepared by ultracentrifugation from pooled (13 donors) human EDTA plasma and oxidized with Cu^{2+} as previously described [5,14,15] using larger volumes of 40–60 ml. LDL was used within 1 day of preparation. Briefly, the LDL solution (0.1 mg protein/ml, equal to 0.2 μM LDL) in PBS pH 7.4 was incubated with 3.3 μM $CuSO_4$ at 37°C for 27 h in the dark in a 500 ml glass beaker. The LDL used in this study had a normal composition, the mean ± SD of three independent analyses was: 31.6 ± 0.26% total cholesterol, 21.4 ± 0.21% protein and 10.56 ± 0.84 and 0.81 ± 0.18 nmol/mg protein of α-tocopherol and γ-tocopherol respectively. Cholesterol was estimated with the CHOD-PAP method (Boehringer-Mannheim, Germany) and protein with the BCA method using bovine serum albumin as a standard (Pierce, USA), α- and γ-tocopherol were determined by HPLC [5].

2.2. Delipidation and pronase E digestion

LDL samples of 3 ml were withdrawn immediately before addition of Cu^{2+} (0 h) and at different time points during oxidation and transferred into Pyrex tubes containing 2 ml of an ice-cold $CHCl_3$:MeOH mixture (2:1, v/v). After vortexing and centrifugation (3000 × *g*, 10°C, 10 min) the bottom $CHCl_3$ layer was removed with a Pasteur pipette and discarded. The extraction was repeated three times. In the remaining MeOH:water phase most of the precipitated apo B was a smeary film on the wall of the Pyrex tube. The tubes covered with parafilm were placed in an exsiccator and residual $CHCl_3$ was removed by the vacuum of a water suction pump (30 min). Thereafter the parafilm covered tubes were transferred to a chamber of a freeze dryer and the methanol:water mixture was evaporated. To the residue a volume of

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Abbreviations: apo B, apolipoprotein B; LDL, low density lipoprotein; MeCN, acetonitrile; MeOH, methanol; NFK, *N*-formylkynurenine; PBS, phosphate buffered saline; TFA, trifluoroacetic acid, 0.1% in water; Trp, L-tryptophan

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2.9 ml H_2O was added and the tubes were closed with the screw cap and vortexed for 60 s to remove apo B from the tube wall. To each tube 0.1 ml of pronase E solution (3.67 mg/ml in PBS pH 7.4) was added and the tubes were covered again with parafilm. At this stage, the native protein was film like, while heavy oxidized LDL (6–24 h) formed fluffy aggregates. The pronase E mixture was incubated in the dark at 37°C for 27 h. Afterwards the samples in the tubes were frozen and freeze dried. The tubes, closed with a Teflon lined screw cap, were sent to the University of Hamburg for further analysis.

2.3. HPLC analysis of Trp oxidation products

To the freeze dried material in the Pyrex tube was added 0.200 ml TFA (0.1% v/v). The turbid solution was filtered through a 0.2 μm Prep-Disc membrane filter (BioRad) and 20 μl was injected into the HPLC. Separation was performed as previously described [16] with a reversed phase Nucleosil column and a linear gradient starting with 95% TFA+5% MeOH increasing to 86% TFA+14% MeOH within 10 min, and to 46% TFA+14% MeOH+40% MeCN within 30 min. The flow was 1 ml/min and the temperature was 35°C. The effluent was monitored with two detectors in series, the first one was a UV detector set at 260 nm, the second one was a fluorescence detector set at 350 nm emission with 230 nm excitation. Peak quantification was based on peak area relative to peaks of chromatogram of authentic standards separated under identical conditions.

2.4. Measurement of Trp loss

Before addition of Cu^{2+} and at various time points during oxidation samples of the LDL solution were measured in a fluorescence spectrometer (Shimadzu RF 5001) with 282 nm excitation and 331 nm emission as previously described [5]. Percent Trp loss was calculated from the initial emission I_0 (measured before addition of Cu^{2+}) and the emission I at later times according to the percent of intact Trp residues: $100 \times I/I_0$. The absolute Trp loss was calculated assuming that 1 mol apo B (500 kDa) contains 37 mol Trp (100 μg protein/ml = 7.4 μM Trp).

3. Results

Since acid and alkaline hydrolysis destroys Trp and some Trp derivatives, pronase E was used to digest the delipidated apo B of oxidized LDL. Separation of the pronase E digest by

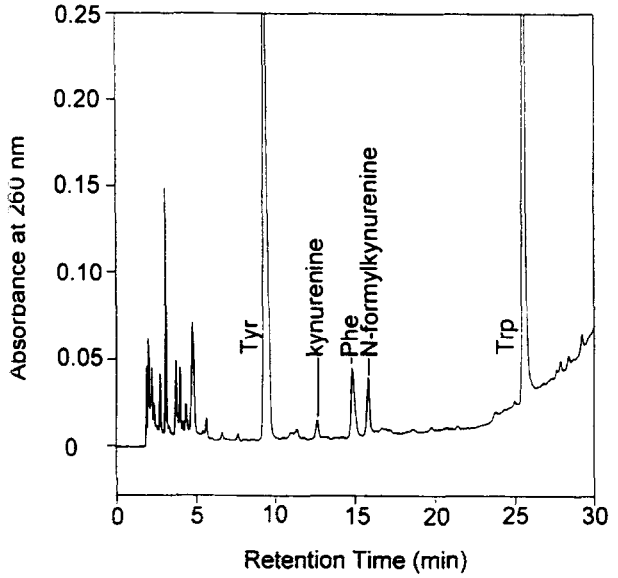

Fig. 1. HPLC separation with 260 nm UV detection of a pronase E digest of apo B of oxidized LDL. 3 ml LDL (0.1 mg protein/ml) oxidized for 5 h with 3.3 μM $CuSO_4$ was delipidated, digested by pronase E and finally dissolved in 0.2 ml TFA. The injected solution (20 μl) had a concentration equivalent to 3 μM apo B.

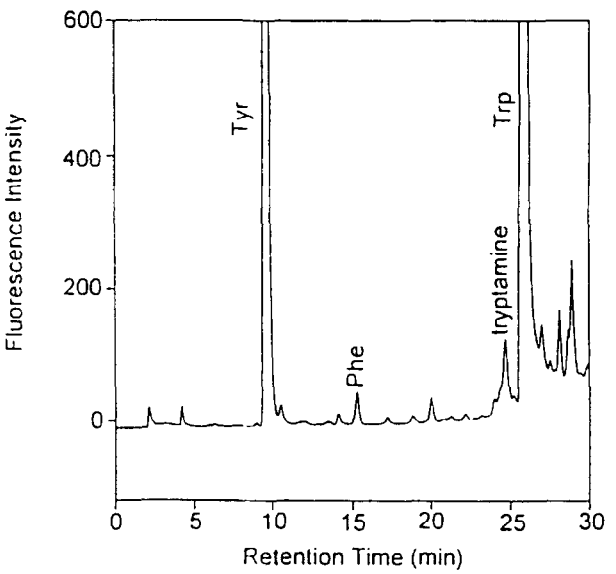

Fig. 2. HPLC separation with 350 nm fluorescence detection (excitation 230 nm) of a pronase E digest of apo B of oxidized LDL. The separation is the same as in Fig. 1, but recording was performed with the fluorescence detector. The units of fluorescence are arbitrary units.

HPLC revealed a number of peaks detectable by UV absorption at 260 nm (Fig. 1) or fluorescence at 350 nm (Fig. 2). The peaks which were identified in the chromatogram are the aromatic amino acids Trp, Tyr and Phe and three Trp derivatives, i.e. tryptamine, kynurenine, and NFK. Preliminary peak identification was based on comparison of retention times with a mixture of authentic standards separated under identical conditions. In the next step, the apo B digest was spiked with standards and separated to assess that the peak in question co-elutes with the respective standard. Further evidence for peak identity is the spectral features. Consistent with the authentic standards, the peaks of Trp, Tyr, Phe and tryptamine showed 260 nm UV absorption and 350 nm fluorescence emission. The quantum yield expressed as ratio of peak area fluorescence to peak area UV absorbance was in full agreement with the standards. Tryptamine has a high quantum yield, this explains the relatively strong peak in fluorescence detection (Fig. 2) compared to the very weak peak in UV detection (Fig. 1). Kynurenine and NFK exhibit 260 nm UV absorption but no 350 nm fluorescence emission at an excitation wavelength of 230 nm and, consistent with this property, the peaks were present in the chromatogram with 260 nm UV detection, but absent in the chromatogram with 350 nm fluorescence emission detection. This also excludes that the kynurenine or NFK peak could result from Trp, Tyr or Phe containing peptides, as such peptides would give fluorescence detectable peaks.The chromatogram did not show peaks characteristic of oxindolylalanine or 5-hydroxytryptophan, two other compounds which have been reported to be formed in the course of Trp oxidation [16].

The chromatogram of a pronase E digest of native apo B (data not shown) exhibited the expected peaks of Trp, Tyr and Phe. The UV detection showed also minor peaks with the retention time of kynurenine and NFK. As these peaks were present in six samples prepared independently from native LDL, it is evident that small amounts of these Trp

Table 1
The content of Trp oxidation products in apo B of native and Cu^{2+} oxidized LDL

	Recovery (%)	mol/mol apo B		
		0 h	5 h	24 h
N-Formylkynurenine	87.7 ± 5.4	0.21 ± 0.10	1.76 ± 0.34	1.19 ± 0.11
Kynurenine	102.7 ± 4.9	0.80 ± 0.52	0.63 ± 0.42	0.16 ± 0.18
Tryptamine	115.8 ± 7.9	0.13 ± 0.20	0.08 ± 0.07	0.03 ± 0.05
5-Hydroxy-tryptophan	101.5 ± 5.1	n.d.	n.d.	n.d.
Oxindolylalanine	70.9 ± 10.4	n.d.	n.d.	n.d.

Pronase E digests of apo B of native LDL (n = 6), 5 h oxidized LDL (n = 3) and 24 h oxidized LDL (n = 3) were analyzed by HPLC as described in the text. The recovery (mean ± SD, n = 3) was determined with a standard mixture treated with pronase E in the same way as apo B. n.d., not detectable, detection limits were 0.016 and ~0.1 mol/mol apo B for 5-hydroxy-tryptophan and oxindolylalanine respectively.

oxidation products are already contained in the apo B of native LDL. Tryptamine was at the detection limit in the digest of native LDL, some chromatograms showed a small peak, while in others the peak was not present. Expressed on a molar basis the mean content of native apo B was 0.21, 0.80, 0.13 mol NFK, kynurenine and tryptamine respectively (Table 1).

As a control, pronase E blanks were also analyzed by HPLC (data not shown); a sample of pronase E incubated for 27 h without apo B showed the peaks of Trp, Tyr and Phe. This indicates that the protease tends to self-digestion, if incubated for a longer period of time. It should be noted in this context that a 27 h pronase E digestion of oxidized apo B was chosen because the yield of NFK, assessed by peak area, became more or less constant and maximal after this time. Besides the Trp, Tyr and Phe peaks, the pronase E blank showed several minor peaks exhibiting both UV absorption and fluorescence. These minor peaks almost certainly result from aromatic amino acid containing peptides. With the exception of the two start peaks, the pronase E blank did not show the characteristic peak pattern with the retention time of 2–5 min seen in oxidized LDL and native LDL. This demonstrates that these peaks are derived from substances released from the apo B. We have, however, made no attempt at identification. The chromatogram of pronase E blanks did not show the peaks of NFK, kynurenine and tryptamine.

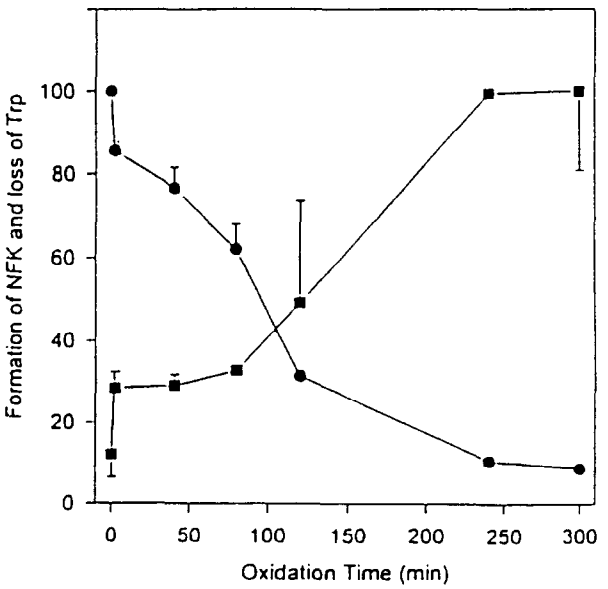

Fig. 3. Time course of loss of Trp residues and formation of NFK residues in apo B during oxidation of LDL. LDL (0.1 mg protein/ml = 0.2 μM) was oxidized with 3.3 μM $CuSO_4$. Samples were withdrawn at the indicated time points and Trp (●) and NFK (■) were determined as described in Section 2. To show the temporal relationship between Trp loss and NFK formation changes relative to the highest values (=100%) were plotted. The highest value of Trp and NFK were 37 and 1.54 mol/mol apo B. The bars are the standard deviations resulting from at least three oxidations. Where no bars are shown, SD was smaller than the symbol.

To determine stability and recovery, known amounts of the authentic standards were incubated with pronase E for 27 h at 37°C. The incubation mixture was then worked up exactly as the apo B digest and the standards were quantitated by HPLC. The recoveries obtained from at least three independent experiments are shown in Table 1. For kynurenine and NFK the recoveries were 103 and 88% respectively, indicating that these compounds are quite stable and do not decompose to a significant extent during the pronase E digestion period.

To investigate the time course of the conversion of Trp residues in apo B to NFK and kynurenine during the oxidation of LDL, digests of oxidizing LDL samples (0.2 μM LDL) were analyzed at different time points. In parallel the loss of Trp was measured by fluorescence spectroscopy [5] as previously described (Fig. 3). Trp decreased within 5 h from an initial value of 37 to 1.8 mol/mol apo B and in parallel NFK increased from an initial value of 0.21 ± 0.10 mol/mol (n = 6) to 1.76 ± 0.34 mol/mol (n = 3) (Table 1). Remarkable is the rapid change at the outset of oxidation, where within a few minutes of oxidation ~0.3 mol NFK/mol apo B was formed while 5 mol Trp/mol apo B was lost (Fig. 3). After 5 h of oxidation NFK slowly decreased and at 24 h the mean content was 1.19 mol/mol apo B. The kynurenine and tryptamine contents varied strongly between the analyzed LDL samples. There was a trend to a decrease during oxidation, which became statistically significant ($P \leq 0.05$) after 24 h oxidation only for tryptamine.

4. Discussion

During the course of LDL oxidation apo B is extensively modified by fragmentation of the polypeptide chain, covalent binding of aldehydic lipid peroxidation products and loss of amino acids by unknown processes [2–4]. The 37 Trp residues of apo B are particularly sensitive and more or less completely destroyed, if LDL is exposed to prooxidants such as cupric ions [5–7,17], hypochlorous acid [18], or myeloperoxidase [19]. We have previously proposed that Trp residues play a key role in the Cu^{2+} dependent initiation of lipid peroxidation in the LDL particle [5]. In support of this hypothesis are the observations that Cu^{2+} ions do bind in close vicinity of Trp residues of apo B, that Trp destruction is not inhibitable by α-tocopherol and that Trp destruction is an early event

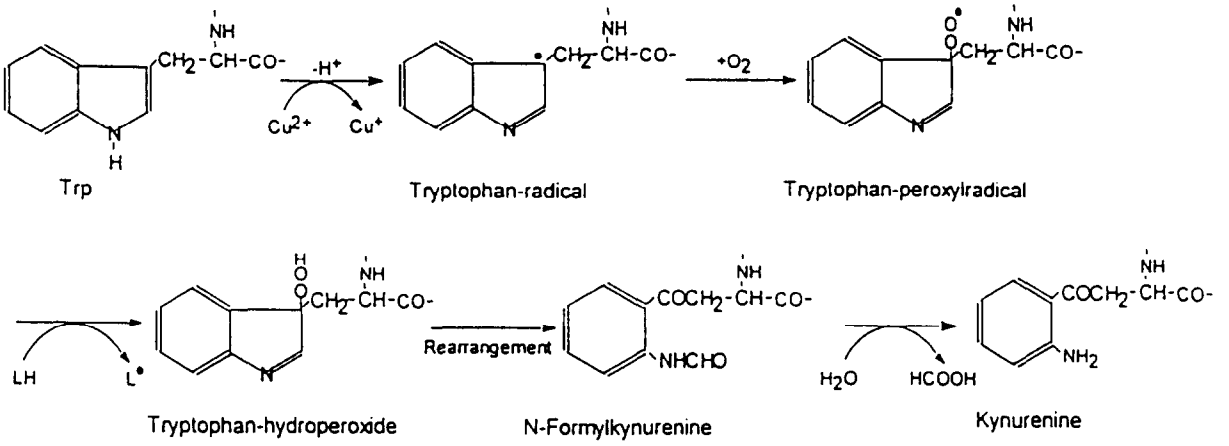

Fig. 4. Proposed mechanism of Cu^{2+} catalyzed formation of NFK and kynurenine in apo B. A one-electron transfer from Trp to Cu^{2+} generates an apo B centered Trp radical, which readily reacts with molecular oxygen. The Trp peroxyl radical abstracts a hydrogen atom presumably from a polyunsaturated lipid (LH) and an apo B containing a Trp hydroperoxide is generated. Rearrangement of the hydroperoxides forms apo B bound NFK. Deformylation of NFK to kynurenine is possible, but apparently does not take place during Cu^{2+} LDL oxidation (see Table 1). Both Cu^{+} and the carbon centered lipid radical L• are candidates for initiating lipid peroxidation.

which starts immediately after addition of Cu^{2+} to LDL and proceeds to the onset of the lipid peroxidation propagation phase. In this study we have demonstrated that apo B of oxidized LDL contains significant amounts of NFK as well as minor amounts of kynurenine and tryptamine. The former two products, which can be considered markers characteristic of intermediate Trp radicals and Trp hydroperoxides [9,10,12], further support the hypothesis that LDL oxidation starts at the apo B with the involvement of Trp and formation of a protein centered radical.

Regarding the reaction mechanism it is of interest that NFK is a major product formed, when free Trp in aqueous solution is treated with ionizing radiation or irradiated with UV-B light. For ionizing radiation it was proposed [11] that the intermediate is a tryptophan hydroperoxide resulting from initial attack of OH radicals at the indole ring. For photolysis it was assumed [10] that an electron is ejected from Trp leading to a Trp radical, which then reacts with oxygen to a tryptophan hydroperoxide. Both mechanisms assume that the hydroperoxy group is on carbon atom 3 of the indole ring. This is consistent with theoretical calculations showing that the greatest electron density resides on carbon atom 3 [20]. The involvement of OH radicals in formation of NFK during Cu^{2+} dependent LDL oxidation is rather unlikely, because it would require both preformed hydrogen peroxide and cupric ions ($H_2O_2+Cu^{+} \rightarrow OH^{\bullet}+OH^{-}+Cu^{2+}$). A reasonable mechanism would be that Cu^{2+} bound in close vicinity of Trp residues [5] and mediates a site specific redox reaction resulting in a Trp• radical and Cu^{+} (Fig. 4). As free Trp in aqueous solution or Trp residues of albumin are not destroyed by Cu^{2+} [5], it appears that Cu^{2+} needs to be complexed to certain types of Trp residues and likely appropriate other ligands to be able to abstract an electron from Trp. Certain Cu^{2+} amine complexes were reported to be capable of producing organic radicals by one-electron transfer reactions [21]. That protein Trp residues can indeed donate one electron has recently been shown for the metmyoglobin/hydrogen peroxide system where a Trp radical was detected by electron spin resonance spectroscopy [9].

On the assumption that NFK was completely released from apo B by the pronase E treatment and considering the 88% recovery, the NFK content of LDL increased 8-fold during a 5 h incubation period from 0.21 to 1.76 mol/mol apo B (Table 1) and then slowly decreased if oxidation was continued for up to 24 h. This means that 5.2% of the oxidized Trp residues in apo B were converted to NFK within 5 h. Inclusion of the other Trp oxidation products (Table 1) increases identified Trp oxidation products to 7.3%, which is very close to the amount of oxidation products identified for the tripeptide Ala-Trp-Ala [23]. Remarkable is the rapid change at the outset of the oxidation, where within a few minutes apo B lost five molecules of Trp, while 0.3 molecules of NFK were generated. It may be that these Trp residues of apo B ultimately initiate lipid peroxidation. According to the scheme in Fig. 4 the initiating species might be Cu^{+}, which is a strong prooxidant, the tryptophan peroxyl radical or both. This would be consistent with the finding that complexing of Cu^{+} by bathocuproine [22] inhibits Cu^{2+} initiated LDL oxidation. Although this does of course not exclude the involvement of other independent initiation mechanisms, such as for example decomposition of preformed peroxides, it provides evidence for the first time for the possibility that LDL oxidation starts with protein oxidation rather than with lipid oxidation.

Various studies (for review see [2–4]) suggest that isolated native LDL contains a small proportion of minimally oxidized LDL, characterized by an increased number of negative surface charges, slightly increased content of oxysterols, increased immunologically detectable 4-hydroxynonenal and malonaldehyde epitopes and several other features. Since this minimally oxidized LDL has been found even in the most carefully isolated LDL, it is believed that it represents a subfraction of circulating LDL. Our observation that the apo B of isolated native LDL contains small amounts of NFK and kynurenine is consistent with this assumption and demonstrates for the first time that a small proportion of the LDL molecules have oxidized Trp residues in their apo B, which may serve as specific oxidation markers.

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