Different susceptibility to oxidation of proline and arginine residues of apolipoprotein B-100 among subspecies of low density lipoproteins

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Abstract γ-Glutamyl semialdehyde is a primary oxidation product of apolipoprotein (apo) B-100 proline (Pro) and arginine (Arg) side chain residues. By reduction γ-glutamyl semialdehyde forms 5-hydroxy-2-aminovaleric acid (HAVA). Here we describe the application of sensitive and specific HAVA measurement to characterize the formation of γ-glutamyl semialdehyde in several domains of apoB-100 in LDL_1 (S_f 7–12) and LDL_2 (S_f 0–7) subfractions subjected to oxidative damage in the presence of iron in vitro. Results suggest that susceptibility of apoB-100 Pro and Arg residues toward oxygen radicals drastically changes along the lipoprotein metabolic cascade.

Key words: Low density lipoprotein; Apolipoprotein B-100; Protein oxidation; Atherogenesis

1. Introduction

Oxidative modification of low density lipoprotein (LDL) apolipoprotein B-100 (apoB-100) by reactive oxygen species is regarded as a crucial event in atherogenesis [1,2]. ApoB-100 modification, e.g. binding of lipid peroxidation products or direct oxidation of amino acid side chain residues, is thought to finally result in the formation of new epitopes that are specifically recognized by scavenger receptors [2–5]. However, the nature of such new epitopes is still a matter of debate [6,7]. Recently, the oxidation of LDL apoB-100 Pro and Arg residues primarily to γ-glutamyl semialdehyde, which by reduction forms 5-hydroxy-2-aminovaleric acid (HAVA), has been measured in vitro and in circulating LDL in vivo [8,9]. Studies in normolipidemic and hypercholesterolemic subjects revealed that an entity of small, more dense, α-tocopherol-poor LDL particles (LDL_2, Svedberg units (S_f) 0–7) is particularly prone to direct apoB-100 oxidation in vivo [9]. The longer the residence time of these particles the more Pro and Arg residues of apoB-100 become modified [9]. These studies lead to the hypothesis that an alteration of apoB-100 conformation during the conversion of large, buoyant LDL_1 to small, more dense LDL_2 particles along the lipoprotein metabolic cascade should contribute to increased accessibility of apoB-100 toward oxidative modification. To prove this hypothesis, the present study uses specific and sensitive gas chromatography-mass spectrometry (GC-MS) methodology to measure HAVA formation in different domains of apoB-100 of LDL_1 (S_f 7–12) and LDL_2 (S_f 0–7) subjected to oxidative damage in the presence of iron in vitro.

2. Materials and methods

2.1. Chemicals

Ethyl chloroformate was obtained from Fluka (Buchs, Switzerland). Human thrombin (2910 NIH U/mg protein), protein L-amino acids, L-norleucine (internal standard), bovine hemin chloride, and non-specific protease type XIV (from *Streptomyces griseus*) were purchased from Sigma (St. Louis, MO, USA). All other reagents were purchased from Sigma and Bio-Rad (Richmond, CA, USA).

2.2. Isolation and in vitro oxidation of human LDL subfractions

Blood samples were obtained from 10 healthy, normolipidemic male volunteers (23–28 years old) after overnight fasting and collected into polypropylene tubes containing EDTA at a final concentration of 0.1%. The blood was centrifuged at 4°C ($2000\times g$ for 10 min) to separate cells from plasma. Blood plasma and LDL were all processed in subdued light to prevent the photooxidation of LDL. All buffers and solutions were degassed and stored under argon. Buoyant LDL_1 (S_f 7–12) and small, dense LDL_2 (S_f 0–7) were isolated from plasma by a combination of both cumulative and sequential density gradient ultracentrifugation techniques as previously described [10,11]. LDL apoB-100 was measured by immunoelectrophoresis using 'ready-to-use' agarose gels (Sebia, Issy-les-Moulineaux, France). Total LDL cholesterol was determined enzymatically using CHOD-PAP test kits (Roche, Mannheim, Germany). LDL α-tocopherol content was measured by high-performance liquid chromatography as described elsewhere and is expressed as mol/mol apoB-100 [12]. Immediately before oxidation of LDL. EDTA and salt from the density gradient were removed using a size exclusion column (Econo-Pac 10DG, Bio-Rad) and 10 mM Tris, 0.01% NaN_3, pH 8.0, as the eluent. For oxidation, aliquots of native LDL_1 and LDL_2 (100 μg apoB-100/ml) were incubated with hemin/H_2O_2 (1 μM bovine hemin chloride and 10 μM H_2O_2) at 37°C for 40 h in the dark [8,13]. LDL samples of 1 ml were withdrawn from the oxidation system immediately before adding the oxidants (time 0 h) and at various time points during oxidation.

2.3. Digestion of LDL apoB-100 with thrombin and separation of thrombolytic peptides

The method of Cardin and coworkers was used to digest apoB-100 of LDL_1 and LDL_2 with thrombin [14]. In brief, after oxidation aliquots of LDL_1 and LDL_2 samples (0.5 ml) were incubated with 5 μg of thrombin (100 μg/ml) at 25°C for 3 h. Undigested and digested LDL samples were transferred into vials containing 2 ml of an ice-cold chloroform:methanol mixture (2:1, v/v). After vortexing and centrifugation ($4000\times g$, 4°C, 10 min) the bottom chloroform layer was removed and discarded. The extraction was repeated three times. In the remaining aqueous phase most of precipitated apoB-100 and apoB-100 digestion products, respectively, were pelleted. The tubes were placed in an exsiccator and residual chloroform was removed

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Abbreviations: apoB-100, apolipoprotein B-100; HAVA, 5-hydroxy-2-aminovaleric acid; LDL, low density lipoprotein

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by the vacuum of a water suction pump (20 min). Thereafter, the tubes were transferred to a freeze dryer and the aqueous phase was evaporated. The formation of HAVA requires the reduction of the polypeptides with sodium borohydride [8,15]. Therefore, to the residue a volume of 10 μl 1 M Tris–HCl (pH 8.5), 100 μl water, and 30 μl of 0.1 M sodium borohydride in 0.1 N NaOH was added and the tubes were vortexed. After incubation at 37°C for 30 min, 1 ml of 10% trichloroacetic acid (TCA) was added. Samples were centrifuged and washed twice with 10% TCA. Samples containing undigested apoB-100 were dried again and 2 ml water was added. Digested LDL samples were incubated for 30 min at 95°C in 200 μl of buffer (8% SDS, 5 mM EDTA, 0.25 M boric acid, 40 mM dithiothreitol, and 0.005% bromophenol blue in 10 mM Tris; pH 6.8). Thrombolytic fragments of both LDL_1 and LDL_2 apoB-100 were then separated by preparative SDS–PAGE (5–15%) using a Tris-glycine buffer system [16]. The gels were stained with Coomassie brilliant blue R250 to visualize the apoB-100 digestion products and scanned in an integrating densitometer to estimate pool sizes of thrombolytic fragments. Immunoblotting (touch blotting) served for definite identification of apolipoproteins [11]. The stained bands were excised, homogenized and 2 ml water was added. Then, to each tube (digested and undigested samples) 0.3 ml of bacterial protease type XIV solution (3.8 mg/ml in phosphate-buffered saline, pH 7.4) was added and the tubes were covered with parafilm. The mixture was incubated in the dark at 37°C for 24 h. Thereafter, the tubes were frozen and freeze-dried.

2.4. Determination of HAVA

The free amino acids were isolated from apoB-100 or thrombolytic fragment hydrolysates, derivatized to their *N*(*O*)-ethoxycarbonyl ethyl ester derivatives, and analyzed by electron-impact ionization GC-MS following the protocol as described elsewhere [8]. HAVA content in all samples is expressed as mol/mol apoB-100 (intraassay CV, $<4.5\%$; interassay CV, $<6.1\%$). A shortcoming of this GC-MS method is the inability to directly determine Arg, owing to the underivatized imino group of the guanidine moiety of Arg that is not eluted under the conditions employed. Therefore, for the present study the loss of apoB-100 Arg side chain residues during iron-catalyzed oxidation was determined by derivatization to *N*(*O*)-heptafluorobutyryl isobutyl esters and GC-methane-negative ion chemical ionization MS as previously published [11].

2.5. Statistical analysis

Descriptive data were expressed as arithmetic means ± S.D. Statistical analyses (Mann–Whitney tests) were calculated using the SPSS 9.0 software package.

3. Results

For the present study large, buoyant LDL_1 (total cholesterol, 2.37 ± 0.34 mM; apoB-100, 0.59 ± 0.11 g/l; α-tocopherol, 5.14 ± 1.47 mol/mol apoB-100) and small, more dense LDL_2 (0.38 ± 0.09 mM, $P<0.01$; 0.12 ± 0.02 g/l, $P<0.01$; 1.34 ± 0.32 mol/mol apoB-100, $P<0.01$) from 10 normolipidemic donors were subjected to iron-catalyzed oxidation using hemin/H_2O_2 in vitro. To investigate the time course of the conversion of Pro and Arg side chain residues in apoB-100 to γ-glutamyl semialdehyde during oxidation of LDL subfractions, HAVA levels were measured at different time points (Fig. 1). HAVA content in LDL_1 apoB-100 increased within 40 h from an initial value of 0.005 ± 0.001 to 13.95 ± 0.91 mol/mol apoB-100 and in LDL_2 apoB-100 from 0.020 ± 0.003 to 20.37 ± 1.05 mol/mol apoB-100, respectively. For native LDL ($t=0$ h) the HAVA content in LDL_2 was higher when compared with LDL_1 (0.62:10 000 vs. 0.16:10 000 Pro/Arg; $P<0.01$). Furthermore, the initial slope and the extent of HAVA formation during oxidation were significantly higher in LDL_2 apoB-100 when compared with LDL_1 (Fig. 1). In parallel, the loss of apoB-100 Pro and Arg side chain residues was measured during oxidation of LDL subfractions. Pro residues in LDL_1 apoB-100 decreased within 40 h from 170 ± 3 to 158 ± 4 mol/mol apoB-100 ($P<0.05$) and in LDL_2 apoB-100 from 170 ± 3 to 156 ± 3 mol/mol apoB-100 ($P<0.05$), respectively. Arg residues in LDL_1 apoB-100 decreased from 148 ± 3 to 140 ± 2 mol/mol apoB-100 ($P<0.05$) and in LDL_2 apoB-100 from 148 ± 3 to 131 ± 3 mol/mol apoB-100 ($P<0.01$), respectively. To further explore the distribution of modified Pro and Arg residues along the apoB-100 molecule, the approach of limited proteolysis with thrombin was used to break oxidized apoB-100 of LDL subfractions into definite fragments [14,17]. The mature apoB-100 is a large protein consisting of a single polypeptide chain of 4536 amino acids, and there is one copy of the protein on each LDL particle. ApoB-100 has a molecular weight (M_r) of ≈ 550 000 (with carbohydrate content), and contains 170 proline residues and 148 arginine residues [17]. Under the proteolytic conditions employed apoB-100 cleavage of both LDL_1 and LDL_2 particles gives the two fragments T_1 and T_2, corresponding to residues 1–3249 ($M_r \approx 380\,000$) and 3250–4536 ($M_r \approx 170\,000$) of the apoB-100 molecule, respectively [14]. Only trace amounts ($<3\%$ of total apoB-100 mass) of T_3 and T_4 fragments, corresponding to residues 1298–3249 and 1–1297, have been found that were not subjected to further analysis. A fragmentation of apoB-100 only due to hemin-mediated oxidation of LDL before thrombin cleavage of apoB-100 has not been observed [18].

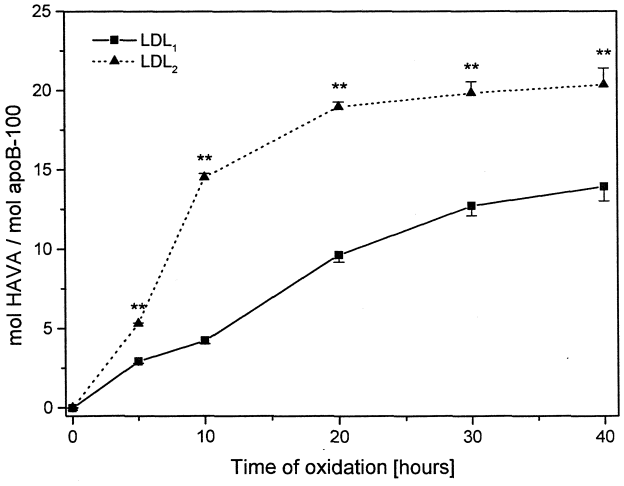

Fig. 1. Kinetics of formation of HAVA in LDL_1 (squares) and LDL_2 (triangles) apoB-100 oxidized by hemin/H_2O_2. Data are means ± S.D. of 10 different samples. Asterisks indicate statistical difference between LDL subfractions during oxidation ($P<0.01$, Mann–Whitney test). Initial differences ($t=0$ h) are given in Section 3.

In LDL_1-T_1 the HAVA content increased within 40 h from an initial value of 0.002 ± 0.001 to 9.88 ± 0.74 mol/mol apoB-100 and in LDL_1-T_2 from 0.002 ± 0.001 to 3.64 ± 0.31 mol/mol apoB-100, respectively. In LDL_2-T_1 the HAVA content increased within 40 h from an initial value of 0.013 ± 0.004 to 12.24 ± 0.91 mol/mol apoB-100 and in LDL_2-T_2 from 0.005 ± 0.001 to 7.01 ± 0.52 mol/mol apoB-100, respectively (Figs. 2 and 3). The mean recovery of HAVA in T_1 and T_2 fragments of LDL subfractions compared to undigested apoB-100 was 95.2 ± 3.2%. For both T_1 and T_2 fragments the slope and the extent of HAVA formation in LDL_2 were significantly higher when compared with LDL_1 (Figs. 2 and 3).

From the apoB-100 amino acid sequence of Knott and co-

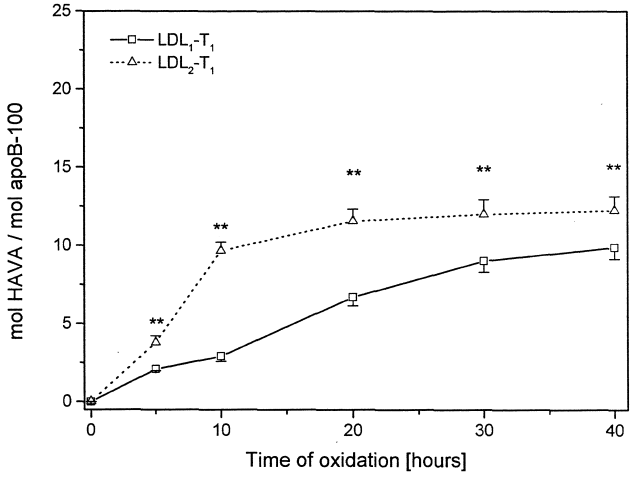

Fig. 2. Kinetics of formation of HAVA in LDL_1-T_1 (squares) and LDL_2-T_1 (triangles). Data are means ± S.D. of 10 different samples. Asterisks indicate statistical difference between LDL subfractions during oxidation ($P < 0.01$, Mann–Whitney test).

workers, the total number of Pro and Arg residues in T_1 and T_2 is 234 (73.6% of total Pro/Arg) and 84 (26.4%), respectively [17]. Pro and Arg residues are distributed in a similar ratio in T_1 (Pro:Arg = 1.13) and T_2 (1.21). For both LDL_1 and LDL_2, the increment in HAVA formation in T_1 fragments was significantly higher when compared with T_2 fragments (Figs. 2 and 3). On the assumption that apoB-100 is completely cleaved by thrombin into T_1 and T_2 fragments and HAVA, Pro, and Arg were completely released from digestion products by enzymatic hydrolysis, respectively, and considering a mean recovery of 95%, after 40 h LDL_1-T_1 contained 72.9% and LDL_1-T_2 contained 27.1% of total HAVA formed. This is nearly proportional to the occurrence of Pro/Arg residues in each fragment. Of note, in LDL_2 the distribution of HAVA lost this proportionality and significantly changed to 64.7% in LDL_2-T_1 and 35.3% in LDL_2-T_2 ($P < 0.05$), respectively.

4. Discussion

Oxidation of LDL apoB-100 in the presence of iron as an oxidant is an experimental model that may have pathophysiological relevance [19,20]. Apparently, redox-active transition metals (e.g. Fe^{2+}/Fe^{3+}) bind to discrete metal binding sites of apoB-100 and form centers for repeated radical production. The exact number of such binding sites is not known, and values ranging from three to about 12 have been reported by others [5,21,22]. γ-Glutamyl semialdehyde is a primary product of iron-mediated oxidation of both Pro and Arg side chain residues and may arise directly via oxidation of the former, or via initial hydrogen abstraction at carbon six, and subsequent loss of the guanidine group of Arg in the presence of oxygen [15,23,24]. By reduction with sodium borohydride, γ-glutamyl semialdehyde forms HAVA that recently has been shown to be a specific marker for human LDL apoB-100 oxidation in vitro and in vivo [8,9,15].

The present work first reports on experiments using HAVA measurement and limited proteolysis of apoB-100 to further understand differences in accessibility of definite domains of apoB-100 of human LDL subfractions to oxidative attack. The data indicate that during the metabolic conversion of LDL particles from large, buoyant LDL_1 into small, more dense LDL_2 the susceptibility of apoB-100 to iron-catalyzed oxidation clearly increases. As lipoprotein kinetic studies supposed the majority of LDL_1 is a precursor of LDL_2. During intravascular processing by concerted action of lipoprotein and hepatic lipases, lipid transfer proteins, and receptor-mediated clearance the large, buoyant LDL_1 particles become smaller and more dense [11,25]. This process is accompanied by changes in LDL particle composition and apoB-100 conformation [21,26]. In normolipidemic subjects LDL_2 contains less cholesterol and α-tocopherol molecules per particle than LDL_1. Cholesterol-depleted LDL_2 has been shown to be more prone toward lipid peroxidation in vitro than LDL_1 [21]. In these studies, only the amount of free cholesterol per particle was associated with LDL oxidizability [21]. Recently, an in vivo study in subjects with normolipidemic and hypercholesterolemic subjects showed lower LDL α-tocopherol content to be weakly associated with a higher degree of apoB-100 oxidation in LDL_2 [9]. Under hypertriglyceridemic conditions, pool sizes of small, dense LDL increase, and additionally, the particles become enriched in triglycerides and free fatty acids. The latter also is supposed to favor the susceptibility of LDL_2 toward lipid peroxidation [26]. Furthermore, compared to large LDL_1, small, more dense LDL_2 particles have a different apoB-100 overall structure [26,27].

In this context, the present data showed Pro/Arg side chain residues in LDL_2 apoB-100 to be more sensitive to form γ-glutamyl semialdehyde and HAVA, respectively, than in LDL_1 apoB-100. In LDL_2, there seems to exist a preference for modification of Arg when compared with LDL_1. In addition, the domain of apoB-100 covered by the T_2 fragment becomes to a significantly higher extent susceptible to oxidation during metabolic conversion of LDL when compared with T_1. The latter clearly indicates that changes in three-dimensional structure and folding of apoB-100 within the lipoprotein particles during their metabolic conversion contribute to the accessibility of apoB-100 to oxidative attack. Both T_1 and T_2 fragments contain Pro-rich clusters and Arg-rich stretches of basic residues that are supposed to be involved in receptor binding of LDL [17,28,29]. Furthermore,

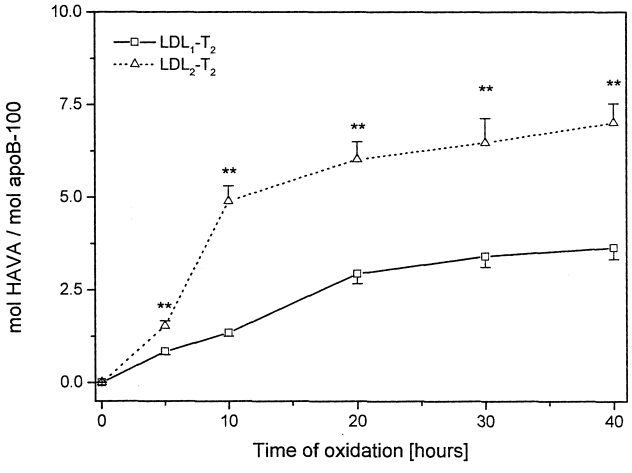

Fig. 3. Kinetics of formation of HAVA in LDL_1-T_2 (squares) and LDL_2-T_2 (triangles). Data are means ± S.D. of 10 different samples. Asterisks indicate statistical difference between LDL subfractions during oxidation ($P < 0.01$, Mann–Whitney test).

the T_2 fragments comprise the putative LDL receptor binding region (residues 3373–3393) that alone contains three Arg residues [17,28,29]. Thus, a higher extent of oxidative Pro/Arg modification in small, more dense LDL_2, and particularly, in the T_2 fragment, is suggested to have consequences for the metabolic fate of these lipoprotein particles. In conclusion, the present experimental findings support former findings on pronounced LDL_2 oxidation in hyperlipidemic subjects in vivo and provide further explanation for increased atherogenicity of small, more dense LDL particles. The experiments support the hypothesis that a pathway involving a ferric/ferryl cycle of the heme iron and hydroperoxides may be of pathological significance [19,30]. However, additional work is needed to understand the specific consequences of γ-glutamyl semialdehyde formation for the metabolic fate of apoB-containing lipoproteins in vivo.

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