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# Analysis of 6-hydroxy-2-aminocaproic acid (HACA) as a specific marker of protein oxidation: The use of *N(O,S)*-ethoxycarbonyl trifluoroethyl ester derivatives and gas chromatography/mass spectrometry

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**Summary.** An alteration of low density lipoprotein (LDL) apolipoprotein (apo) B-100 structure by direct oxidative modification is an important mechanism involved in atherogenesis. There is difficulty in quantifying this type of modification because a lack of specific assays. The use of *N(O,S)*-ethoxycarbonyl trifluoroethyl amino acid esters for a rapid and sensitive determination of 6-hydroxy-2-aminocaproic acid (HACA), a highly specific marker of metal catalyzed protein oxidation, by using standard gas chromatography/electron impact mass spectrometry, is discussed. The derivatives are formed by the unlabored reaction of amino acids with ethylchloroformate plus trifluoroethanol plus pyridine. Femtomole levels of HACA can be reproducible measured in different LDL preparations subjected to oxidative damage in the presence of iron or copper. HACA determination compares well with the measurement of carbonyl groups that are generally accepted as a nonspecific index of protein oxidation. Thus, the method could prove to be a sensitive assay for studying specific apoB-100 modification.

**Keywords:** Amino acids – Hemin – Low density lipoprotein – Protein oxidations – Gas chromatography/mass spectrometry

**Abbreviations:** *Apo* apolipoprotein; *HACA* 6-hydroxy-2-aminocaproic acid; *LDL* low density lipoprotein

## 1. Introduction

2-Aminoadipic-6-semialdehyde is supposed to be a primary product of transition metal catalyzed oxidation of protein lysine side chain residues (Requena et al., 2001; Akagawa et al., 2002). By reduction 2-aminoadipic-6-semialdehyde forms 6-hydroxy-2-aminocaproic acid (HACA) (Requena et al., 2001). For other proteins than apolipoproteins, the formation of HACA has been measured *in vitro* and *in vivo* (Shah et al., 1992; Requena et al., 2001; Brand et al., 2002; Pamplona et al., 2002; Sorensen et al., 2003). These studies also revealed 2-aminoadipic-6-semialdehyde, besides glutamic semialdehyde, to be a major constituent of the total protein carbonyl content in various model proteins subjected to metal catalyzed oxidation *in vitro* as well as in rat liver samples *ex vivo* (Requena et al., 2001). Oxidative modification of proteins can interfere with critical cellular functions and affect enzyme function and cellular signaling, and is widely regarded as a crucial event in the pathogenesis of various diseases ranging from atherosclerosis to rheumatoid arthritis and cancer (Heinecke, 2002; Stadtman, 2002). In this line, oxidative modification of apoB-100, the major protein of LDL, is thought to finally result in the formation of new epitopes that are specifically recognized by scavenger receptors followed by an excessive uptake and accumulation of LDL particles in macrophages and vascular smooth muscle cells that can lead to foam cell formation (Berliner and Heinecke, 1996). The latter appears to be the earliest morphologic substrate of atherogenesis (Ross, 1999). However, the nature of specific modifications of protein amino acid side chain residues, thus altering their cellular functions, is a current matter of debate (Heinecke, 2002; Stadtman, 2002). In this respect, the value of HACA as a specific marker of metal catalyzed oxidation of apoB-100 has not been established. The mature apoB-100 ($M_r$ 516,000; without carbohydrate content) consists of a single polypeptide chain of 4536 amino acids, and there is only one copy of the protein on each LDL particle (Scott, 1989). ApoB-100 contains 351 lysine residues that are supposed to be partially susceptible to direct oxidative damage. The objective of this report is the investigation of the feasibility

of the use of *N(O,S)*-ethoxycarbonyl trifluoroethyl ester (ECEE-$F_3$) derivatives and gas chromatography/mass spectrometry (GC/MS) to quantify low-abundance 2-aminoadipic-6-semialdehyde residues in isolated apoB-100. The analysis of these fluorinated derivatives for quantitative analysis of amino acids by chemical ionization mass spectrometry has been described by Moini and colleagues (Vatankhah and Moini, 1994; Cao and Moini, 1997). More recently, the use of ECEE-$F_3$ derivatives for analysis of protein amino acids by standard electron impact ionization (EI) mass spectrometry has been described (Pietzsch et al., 1997). The present work refers to previously published data on the quantitative analysis of specific oxidation products formed in apoB-100 by using *N(O)*-ethoxycarbonyl ethyl esters (ECEE) derivatives (Pietzsch, 2000; Pietzsch and Julius, 2001). Because ECEE-$F_3$ derivatives provide a higher sensitivity and specificity when compared with their non-fluorinated counterparts, this investigation focuses on the mass spectral behavior of the ECEE-$F_3$ derivative of HACA. The derivatives studied yielded diagnostically useful fragment ions that were found to be of value for use in HACA quantification by mass spectrometry. The approach is currently being applied to studies dealing with mechanisms of protein oxidation *in vitro* and *in vivo*.

## 2. Material and methods

### 2.1. Chemicals

Ethyl chloroformate was obtained from Fluka (Buchs, Switzerland). L-Amino acids, L-norleucine (internal standard), 2,2,2-trifluoroethanol, bovine hemin chloride, and nonspecific protease Type XIV (from *Streptomyces griseus*), were purchased from Sigma (St Louis, MO, USA). *N,N*-bis(2-hydroxybenzyl)ethylenediamine-*N,N*-diacetic acid (HBED) was purchased from Dojindo (Gaithersburg, MD, USA). All other chemicals were obtained from Sigma and BioRad (Richmond, CA, USA).

### 2.2. Synthesis of 6-hydroxy-2-aminocaproic acid (HACA) standard

D,L-HACA was synthesized as standard substance by hydrolysis of 5-$\delta$-hydroxybutylhydantoin according to the method of Gaudry (Gaudry, 1948).

### 2.3. Lipoprotein isolation and oxidation

Native, albumin-free low density lipoprotein (LDL, density 1.006–1.063 g/mL) was isolated from plasma of 20 healthy, normolipidemic male volunteers by very fast ultracentrifugation as previously described (Pietzsch, 2000). LDL apoB-100 was measured by immunoelectrophoresis using 'ready-to-use' agarose gels (Sebia, Issy-les-Moulineaux, France). 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 phosphate-buffered saline (PBS, 10 mM sodium phosphate, 150 mM sodium chloride, pH 7.4) as the eluent. For oxidation, aliquots of native LDL (600 $\mu$g apoB-100/mL, equal to 1.2 $\mu$M LDL) were incubated with various well characterized oxidation systems: *i*) 10 $\mu$M bovine hemin chloride and 100 $\mu$M $H_2O_2$, *ii*) 60 $\mu$M $FeSO_4$, 100 $\mu$M EDTA, and 1 mM ascorbate, *iii*) 60 $\mu$M $FeCl_3$ and 1mM ascorbate, and *iv*) 30 $\mu$M $CuSO_4$ and 100 $\mu$M $H_2O_2$ at 37°C for 40 hours in the dark (Matthews et al., 1997; Cohen et al., 1998; Kopprasch et al., 1998; Pietzsch, 2000). For the hemin containing oxidation system control experiments were performed using the potent ferric ion chelator HBED (100 $\mu$M). LDL samples (2 mL) were withdrawn from the oxidation systems and delipidated (Pietzsch, 2000). After the addition of 100 nmol L-norleucine, apoB-100 was enzymatically hydrolyzed as previously described (Pietzsch, 2000).

### 2.4. Derivatization and GC/MS analysis

Free amino acids were isolated from protein hydrolysates by cation exchange chromatography using Dowex AG-50W-X8 ($H^+$, 100–200 mesh) resin. The purified and dried protein amino acids were derivatized to their ECEE-$F_3$ derivatives and analyzed following the protocol described previously by us (Pietzsch et al., 1997). In brief, amino acids in residues (not more than 50 $\mu$g in total) were treated with 100 $\mu$l of trifluoroethanol:pyridine in a volume ratio 80:20. Then 10 $\mu$l of ethyl chloroformate were added and mixed by shaking the tube gently for 30 seconds. The derivatives were extracted with 150 $\mu$l of chloroform containing 2% ethyl chloroformate. An aliquot was taken from the chloroform layer and injected. The analysis was conducted using a model 5890II GC/5989A MS-Engine equipped with a 25 m × 0.20 mm HP-5 capillary column (5% diphenyl-95% dimethylpolysiloxane, 0.33 $\mu$m; Hewlett-Packard, Palo Alto, CA, USA). The GC conditions were as follows: carrier gas, helium; column head pressure, 70 kPa; injector temperature, 250°C; oven temperature gradient program, 110°C increased to 320°C at 25°C/min; interface temperature 250°C. EI-MS conditions were as follows: source temperature, 250°C; analyzer temperature, 120°C; electron energy, 70 eV. All samples were run in triplicate. Diagnostically useful ions were monitored in SIM mode under the control of the HP-ChemStation data system. Data acquisition was delayed until 2 min after injection. Diagnostically useful ions for HACA (at $m/z$ 174 and $m/z$ 212), lysine (at $m/z$ 245 and $m/z$ 156), and norleucine (at $m/z$ 158 and $m/z$ 196) were monitored in the selected ion monitoring (SIM) mode with a dwell time of 25 or 50 ms for each ion under the control of the HP-ChemStation data system. For quantification of HACA, calibration curves were obtained by plotting the peak area ratio for the characteristic $m/z$ 174 (HACA) – $m/z$ 158 (norleucine) ion pair vs. the mass ratio of HACA and norleucine. The HACA content in all samples is expressed as mol/mol apoB-100.

### 2.5. Spectrophotometric determination of total carbonyl group content

Total carbonyl group content in apoB-100 was determined by spectrophotometric measurement of their 2,4-dinitrophenylhydrazone derivatives according to the method of Levine and coworkers (Levine et al., 1990). The total carbonyl group content is expressed as mol/mol apoB-100.

## 3. Results

All mass spectral analyses were performed in the EI mode. Initial studies of the ECEE-$F_3$ derivatives of protein amino acids by GC/MS showed them to be well separated under the conditions employed; retention times were very reproducible (Pietzsch et al., 1997). The results of a representative GC/MS analysis of a hydrolysate of oxidized apoB-100 is shown in Fig. 1. The HACA derivative is cleanly separated with a retention time of 5.21 min. For comparison, the retention time of lysine and the hydroxy amino acids threonine, serine, and hydroxyproline were

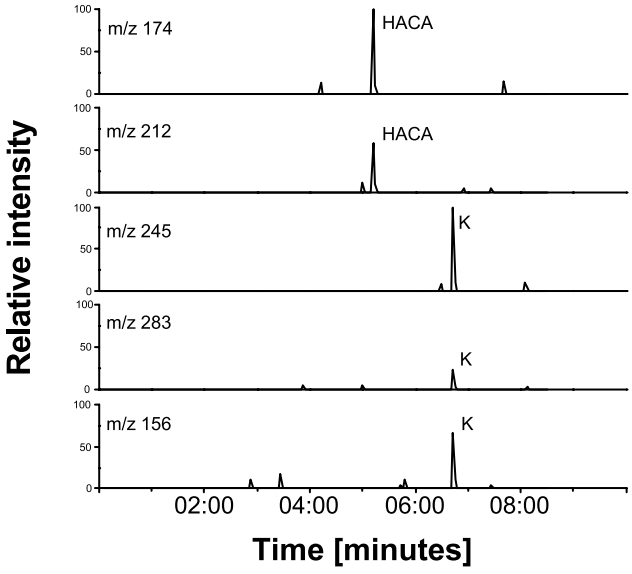


**Fig. 1.** Representative mass chromatograms of $[M\text{-}89]^{+\bullet}$ and $[M\text{-}127]^{+}$ ions obtained from the GC/EI-MS analysis of ECEE-$F_3$ derivatives of an apoB-100 hydrolysate. The figure shows the selected mass traces for HACA (at $m/z$ 174 and $m/z$ 212) and L-lysine (K, at $m/z$ 245 and $m/z$ 283). Furthermore, the selected mass trace for L-lysine at $m/z$ 156 ($[M\text{-}127\text{-}89]^{+}$) is shown. The latter results from the formation of a stable cyclic immonium ion after consecutive loss of $^{\bullet}CO_2CH_2CF_3$ and $NH_2CO_2Et$ from the ionized molecule. The dwell time on each ion was either 25 or 50 ms

6.74, 3.16, 3.41, and 4.84 min, respectively. No interference was observed in the analysis of HACA from the more common protein amino acids or matrix components normally present in human LDL samples. Identification of HACA was carried out by establishing that the retention time of the selected characteristic ions of its ECEE-$F_3$ derivative was identical to those of the derivatized authentic standard substance. The full-scan EI mass spectrum and the proposed fragmentation pattern of the ECEE-$F_3$ derivative of HACA are depicted in Fig. 2. The spectrum of derivatized HACA ($M_r$ 301) followed a fragmentation pattern essentially as previously described for aliphatic and aromatic amino acids (Pietzsch et al., 1997). In brief, the characteristic ions of the HACA ECEE-$F_3$ derivative were a weak molecular ion signal at $m/z$ 301 ($M^{+\bullet}$, mass peak intensity was approximately 7% of the base peak), as well as abundant and highly characteristic ions at $m/z$ 174 (base peak) resulting from the loss of $^{\bullet}CO_2CH_2CF_3$ ($[M\text{-}127]^{+}$) and at $m/z$ 212 resulting from the loss of $NH_2CO_2Et$ ($[M\text{-}89]^{+\bullet}$) from the molecular ions. Two other abundant ions at $m/z$ 129 and $m/z$ 101 are daughter ions from the non-characteristic fragment at $m/z$ 229 that arises from a McLafferty-type rearrangement due to transfer of a hydrogen originating from the labile $\varepsilon$-OH group and a subsequent cyclization reaction. For determination of the HACA derivative the highly characteristic ions at $m/z$ 174 and $m/z$ 212 were monitored. For calibration graphs, increasing amounts of HACA were added to an aqueous standard amino acid mixture containing norleucine as internal standard. Calibration curves were analyzed by unweighted least-squares linear regression

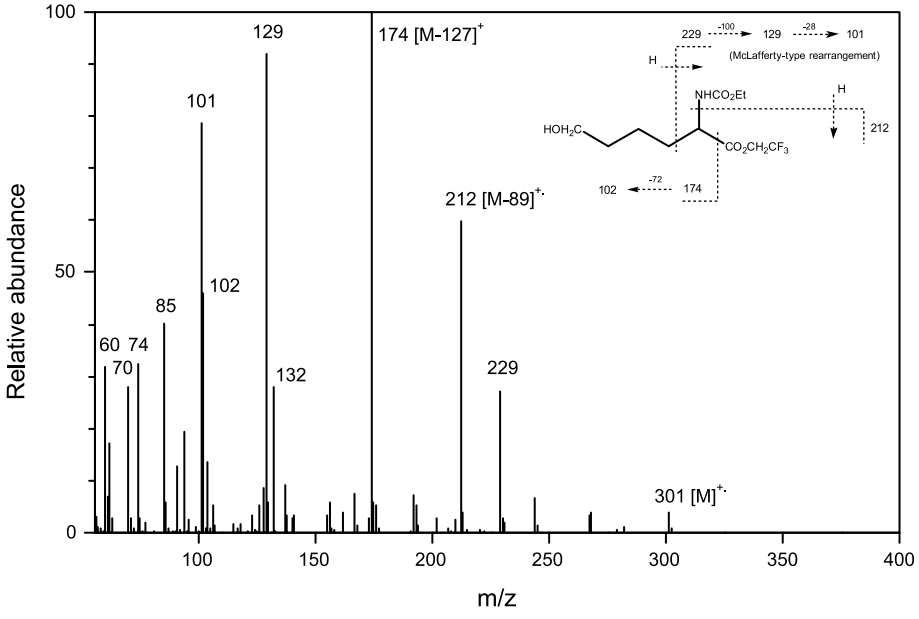


**Fig. 2.** EI ionization (70 eV) mass spectra of the ECEE-$F_3$ derivatives of HACA (top, $M_r$ 301). Spectra show the prominent $[M\text{-}89]^{+\bullet}$ and $[M\text{-}127]^{+}$ ions, due to the losses of $NH_2CO_2Et$ and $^{\bullet}CO_2CH_2CF_3$, respectively, from the ionized molecules. GC/MS conditions are described in the Experimental Section

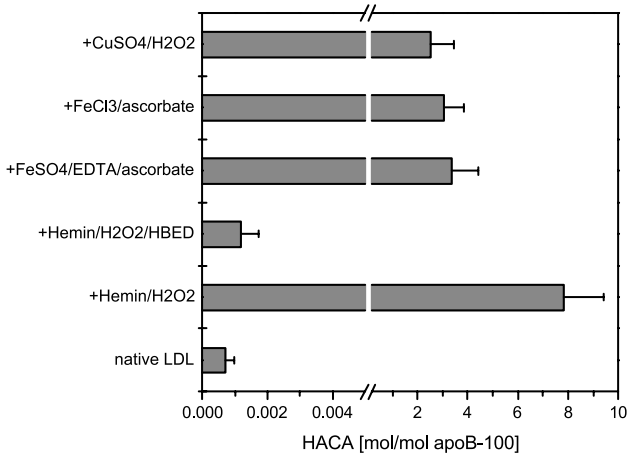


**Fig. 3.** Formation of HACA in human LDL particles exposed to various oxidation systems *in vitro*. The composition of the oxidation systems is described in the Experimental Section. Values are means ± standard deviation of triplicate determinations. Similar results were observed in five independent experiments

analysis and were found to be linear over the range studied (2–600 nM; $R^2 > 0.99$) and of good quality (within-batch precision $< 4.5\%$, between-batch precision $< 6.1\%$). Signal reproducibility was found to be less than 8% variation in the peak areas being observed for the $[M\text{-}NH_2CO_2Et]^{+\bullet}$ and $[M\text{-}CO_2CH_2CF_3]^+$ ions, respectively. The limit of determination of HACA was 1 nM (1 fmol/injection) by this method.

Figure 3 shows the formation of HACA in human LDL particles exposed to the various oxidation systems employed. The HACA content in LDL apoB-100 is normalized to the content of LDL apoB-100. The level of HACA strongly increased from an initial value of $7.1 \times 10^{-4}$ mol/mol apoB-100 (0.02/10,000 lysine residues) found in native LDL obtained from normolipidemic, young male volunteers, to $7.82 \pm 1.62$ mol/mol apoB-100 (223/10,000 lysine residues, $P < 0.001$) and $3.38 \pm 1.06$ mol/mol apoB-100 (96/10,000 lysine residues, $P < 0.001$), respectively, on exposing LDL to two well characterized oxidation systems containing either hemin/$H_2O_2$ or $FeSO_4$/EDTA/ascorbate (Pietzsch, 2000). The HACA level also rose to $3.04 \pm 0.82$ mol/mol apoB-100 (86/10,000 lysine residues, $P < 0.001$) and $2.53 \pm 0.94$ mol/mol apoB-100 (72/10,000 lysine residues, $P < 0.001$) on exposing LDL to $FeCl_3$/ascorbate and $CuSO_4/H_2O_2$, respectively. In contrast, no HACA formation could be observed on adding the redox-inert iron chelator HBED to the hemin/$H_2O_2$ system when compared with native LDL. As a blank, HACA levels have been measured in LDL samples without sodium borohydride reduction. In these samples, no significant differences in HACA concentration between native and oxidized LDL could be observed ($0.0007 \pm 0.0002$ vs. $0.0009 \pm 0.0004$ mol/mol apoB-100, $P = 0.254$; Mann-Whitney test). In parallel, we measured the increment in total carbonyl group content as a well accepted, but non-specific marker of protein oxidation. Exposure of human LDL to the four oxidation systems employed resulted in similar formation of carbonyl groups (mean carbonyl group content after 40 hours: $12.98 \pm 2.69$ mol/mol apoB-100) that was strongly correlated with HACA formation for each experiment ($r = 0.940$, $P < 0.001$ for hemin/$H_2O_2$; $r = 0.877$, $P < 0.001$ for $FeSO_4$/EDTA/ascorbate; $r = 0.850$, $P < 0.001$ for $FeCl_3$/ascorbate $r = 0.750$, $P < 0.001$ for $CuSO_4/H_2O_2$). These results confirm data reported by others on the extent and the specificity of HACA formation *in vitro* (Requena et al., 2001). The increment in HACA residues in LDL apoB-100 was accompanied by a parallel loss of lysine side chain residues. Lysine residues decreased by approximately 3% from $351 \pm 4$ to $340 \pm 3$ mol/mol apoB-100 ($P < 0.05$) for the hemin/$H_2O_2$ system. For the other oxidation systems a decrease in apoB-100 lysine residue content by approximately 1% to $347 \pm 4$ mol/mol apoB-100 ($P < 0.01$) for $FeSO_4$/EDTA/ascorbate, to $347 \pm 6$ mol/mol apoB-100 ($P < 0.01$) for $FeCl_3$/ascorbate, and to $348 \pm 6$ mol/mol apoB-100 ($P < 0.01$) for $CuSO_4/H_2O_2$ could be observed. Thus, on the assumption that HACA and lysine were completely released from apoB-100 by enzymatic hydrolysis and considering a mean recovery of 95%, and further assuming that HACA is formed only by oxidation of lysine at least 70% of oxidized lysine side chain residues were converted to HACA on exposing LDL to the oxidation systems employed.

## 4. Discussion

2-Aminoadipic-6-semialdehyde is a primary product of metal catalyzed oxidation of protein lysine side chain residues and may arise via initial hydrogen abstraction at carbon six and formation of a reactive lysyl radical. The latter is a target for hydroxylation and subsequent loss of the $\varepsilon$-amino group. By reduction with sodium borohydride, 2-aminoadipic-6-semialdehyde forms HACA (Requena et al., 2001). A possible reaction scheme for the formation 2-aminoadipic-6-semialdehyde and its reaction product HACA is depicted in Fig. 4. The present work first reports on experiments to use HACA as a specific marker of direct modification of apoB-100 in native and oxidized human LDL samples. The physiological level of HACA found in native circulating LDL obtained from normolipidemic, young male volunteers was $7.1 \times 10^{-4}$ mol/mol apoB-100 (0.02/10,000 lysine residues).

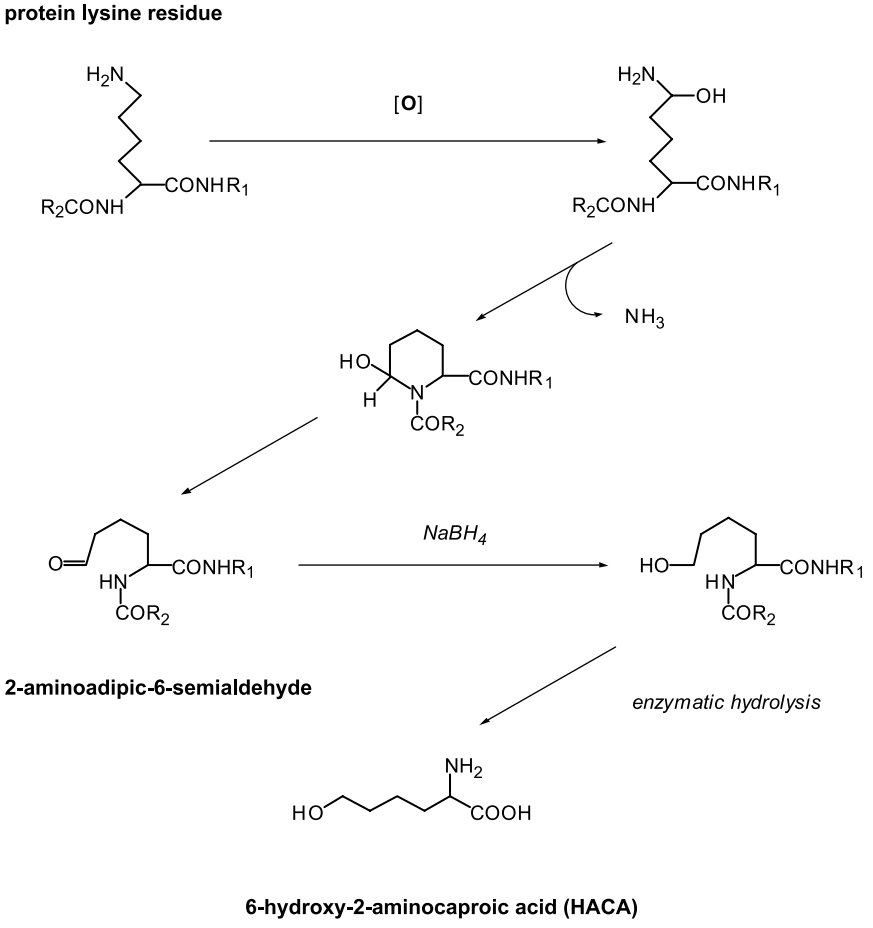


**Fig. 4.** Proposed reaction scheme for the formation of 2-aminoadipic-6-semialdehyde and its reaction product HACA according to Requena et al. (Requena et al., 2001)

The present data show that lysine side chain residues of apoB-100 are highly sensitive to form 2-aminoadipic-6-semialdehyde and HACA, respectively, if LDL is exposed to low concentrations of iron or copper as pro-oxidants by using established oxidation systems *in vitro*. Under the conditions employed, the increment in HACA level is strongly associated with the increment in total carbonyl group content of apoB-100. The latter is commonly used as a non-specific marker for oxidation of the protein moiety of LDL (Sattler et al., 1998). The present results indicate, that beside proline and arginine residues oxidative damage of lysine should directly contribute to carbonyl group formation in oxidized LDL (Sattler et al., 1998; Pietzsch, 2000; Requena, 2001). The overall yield of HACA that has been found in apoB-100 in this experiment is remarkably high and indicates that lysine is a good target for oxidative attack in the presence of low concentrations of iron or copper in different oxidation systems. Of note, using the hemin/$H_2O_2$ oxidation system the extent of HACA formation is more than twofold higher when compared with the $FeSO_4$/EDTA/ascorbate system ($P<0.01$), the $FeCl_3$/ascorbate system ($P<0.01$), and the $CuSO_4/H_2O_2$ system ($P<0.01$, Mann-Whitney test). As supported by various experiments, the hydrophobic part of the amphiphilic hemin ($Fe^{3+}$-protoporphyrin IX) molecule intercalates within phospholipids and free cholesterol in the surface monolayer of the LDL particle (Kongshaug and Moan, 1995; Camejo et al., 1998). In addition, the two ionized propyl carboxyl groups of hemin are supposed to be in close contact with positively charged surface amino acid side chains of the apoB-100. In consequence of this, hemin becomes a potent agent to specifically modify the basic amino acids lysine and arginine, respectively, following a pathway involving a perferryl cycle (via [$Fe^{IV}$=O] and [$Fe^{V}$–OH]) of the heme iron and hydroperoxides (Ziouzenkova et al., 1999; Balla et al., 2000; Pietzsch and Julius, 2001). This model system strongly depends on the presence of the complexed ferric iron as chelating of $Fe^{3+}$ by redox-inert HBED completely inhibits the direct oxidation of apoB-100 lysine residues. Because the availability of free copper or iron *in vivo* is under tight control, oxidation of LDL apoB-100 in the presence of hemin as a pro-oxidant is an experimental model that is considered to be of high

pathophysiological relevance (Miller and Shaklai, 1999; Lynch et al., 2000; Jeney et al., 2002).

In conclusion, we have developed and validated a sensitive GC/MS method to assay the formation of HACA in oxidized apoB-100 that is useful when performing studies that aim at mechanisms of protein and lipoprotein oxidation in various disorders. The key steps of this methodology were: *i*) the use of very fast ultracentrifugation (VFU) for isolation of native LDL free of contaminating albumin; *ii*) enzymatic hydrolysis of apoB-100 using nonspecific protease Type XIV to prevent decomposition of HACA during hydrolysis; *iii*) an uniquely rapid derivatization of all protein amino acids (except arginine, owing to the underivatized imino group of its guanidine moiety) completing sample preparation for GC within a few minutes in aqueous solution at room temperature; and *iv*) the use of standard EI mode that is easy to maintain and reproduce compared with chemical ionization mode. Finally, the GC/MS method presented here allows $>100$ samples to be processed completely within 1 day. The latter would be of importance in clinical experiments where large numbers of samples must be handled daily.

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## References

Akagawa M, Sasaki T, Suyama K (2002) Oxidative deamination of lysine residue in plasma protein of diabetic rats. Novel mechanism via the Maillard reaction. Eur J Biochem 269: 5451–5458

Balla J, Balla G, Jeney V, Kakuk G, Jacob HS, Vercellotti GM (2000) Ferriporphyrins and endothelium: a 2-edged sword-promotion of oxidation and induction of cytoprotectants. Blood 95: 3442–3450

Berliner JA, Heinecke JW (1996) The role of oxidized lipoproteins in atherogenesis. Free Radic Biol Med 20: 707–727

Brand MD, Pamplona R, Portero-Otin M, Requena JR, Roebuck SJ, Buckingham JA, Clapham JC, Cadenas S (2002) Oxidative damage and phospholipid fatty acyl composition in skeletal muscle mitochondria from mice underexpressing or overexpressing uncoupling protein 3. Biochem J 368: 597–603

Camejo G, Halberg C, Manschik-Lundin A, Hurt-Camejo E, Rosengren B, Olsson H, Hansson GI, Forsberg GB, Ylhen B (1998) Hemin binding and oxidation of lipoproteins in serum: mechanisms and effect on the interaction of LDL with human macrophages. J Lipid Res 39: 755–766

Cao P, Moini M (1997) Quantitative analysis of fluorinated ethylchloroformate derivatives of protein amino acids and hydrolysis products of small peptides using chemical ionization- mass spectrometry. J Chromatogr A 759: 111–117

Cohen G, Yakushin S, Dembiec-Cohen D (1998) Protein L-dopa as an index of hydroxyl radical attack on protein tyrosine. Anal Biochem 263: 232–239

Gaudry R (1948) The synthesis of D,L-$\alpha$-amino-$\varepsilon$-hydroxycaproic acid and a new synthesis of D,L-lysine. Can J Res 26 Sec. B: 387–392

Heinecke JW (2002) Oxidized amino acids: culprits in human atherosclerosis and indicators of oxidative stress. Free Radic Biol Med 32: 1090–1101

Huang ZH, Wang J, Gage DA, Watson JT, Sweeley CC, Husek P (1993) Characterization of N-ethoxycarbonyl ethyl esters of amino acids by mass spectrometry. J Chromatogr 635: 271–281

Jeney V, Balla J, Yachie A, Varga Z, Vercellotti GM, Eaton JW, Balla G (2002) Pro-oxidant and cytotoxic effects of circulating heme. Blood 100: 879–887

Kongshaug M, Moan J (1995) Separation of lipoproteins, albumin and gamma-globulin by single-step ultracentrifugation of human serum. Application. I: Binding of hematoporphyrin to human serum and to albumin. Int J Biochem Cell Biol 27: 371–384

Kopprasch S, Leonhardt W, Pietzsch J, Kühne H (1998) Hypochlorite-modified low-density lipoprotein stimulates human polymorphonuclear leukocytes for enhanced production of reactive oxygen metabolites, enzyme secretion, and adhesion to endothelial cells. Atherosclerosis 136: 315–324

Levine RL, Garland D, Oliver CN, Amici A, Climent I, Lenz AG, Ahn BW, Shaltiel S, Stadtman ER (1990) Determination of carbonyl content in oxidatively modified proteins. Methods Enzymol 186: 464–478

Lynch SM, Campione AL, Moore MK (2000) Plasma thiols inhibit hemin-dependent oxidation of human low-density lipoprotein. Biochim Biophys Acta 1485: 11–22

Matthews AJ, Vercellotti GM, Menchaca HJ, Bloch PH, Michalek VN, Marker PH, Murar J, Buchwald H (1997) Iron and atherosclerosis: inhibition by the iron chelator deferiprone (L1). J Surg Res 73: 35–40

Miller YI, Shaklai N (1999) Kinetics of hemin distribution in plasma reveals its role in lipoprotein oxidation. Biochim Biophys Acta 1454: 153–164

Pamplona R, Portero-Otin M, Requena JR, Gredilla R, Barja G (2002) Oxidative, glycoxidative and lipoxidative damage to rat heart mitochondrial proteins is lower after 4 months of caloric restriction than in age-matched controls. Mech Ageing Dev 123: 1437–1446

Pietzsch J (2000) Measurement of 5-hydroxy-2-aminovaleric acid as a specific marker of iron-mediated oxidation of proline and arginine side-chain residues of low-density lipoprotein apolipoprotein B-100. Biochem Biophys Res Commun 270: 852–857

Pietzsch J, Julius U (2001) Different susceptibility to oxidation of proline and arginine residues of apolipoprotein B-100 among subspecies of low density lipoproteins. FEBS Lett 491: 123–126

Pietzsch J, Julius U, Hanefeld M (1997) Stable isotope ratio analysis of amino acids: the use of N(O)-ethoxycarbonyl trifluoroethyl ester derivatives and gas chromatography/mass spectrometry. Rapid Commun Mass Spectrom 11: 1835–1838

Requena JR, Chao CC, Levine RL, Stadtman ER (2001) Glutamic and aminoadipic semialdehydes are the main carbonyl products of metal-catalyzed oxidation of proteins. Proc Natl Acad Sci USA 98: 69–74

Ross R (1999) Atherosclerosis – an inflammatory disease. N Engl J Med 340: 115–126

Sattler W, Malle E, Kostner GM (1998) Methodological approaches for assessing lipid and protein oxidation and modification in plasma and isolated lipoproteins. Methods Mol Biol 110: 167–191

Scott J (1989) The molecular and cell biology of apolipoprotein-B. Mol Biol Med 6: 65–80

Shah MA, Bergethon PR, Boak AM, Gallop PM, Kagan HM (1992) Oxidation of peptidyl lysine by copper complexes of pyrroloquinoline quinone and other quinones. A model for oxidative pathochemistry. Biochim Biophys Acta 1159: 311–318

Sorensen M, Daneshvar B, Hansen M, Dragsted LO, Hertel O, Knudsen L, Loft S (2003) Personal PM(2.5) Exposure and Markers of Oxidative Stress in Blood. Environ Health Perspect 111: 161–166

Stadtman ER (2002) Importance of individuality in oxidative stress and aging. Free Radic Biol Med 33: 597–604

Vatankhah M, Moini M (1994) Characterization of fluorinated ethylchloroformate derivatives of protein amino acids using positive and negative chemical ionization gas chromatography/mass spectrometry. Biol Mass Spectrom 23: 277–282

Ziouzenkova O, Asatryan L, Akmal M, Tetta C, Wratten ML, Loseto-Wich G, Jurgens G, Heinecke J, Sevanian A (1999) Oxidative cross-linking of ApoB100 and hemoglobin results in low density lipoprotein modification in blood. Relevance to atherogenesis caused by hemodialysis. J Biol Chem 274: 18916–18924

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