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Comparative Biochemistry and Physiology, Part B 141 (2005) 89–94

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Mass spectral analysis of pig (*Sus scrofa*) apo HDL: Identification of pig apoA-II, a dimeric apolipoprotein

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Received 9 November 2004; received in revised form 27 January 2005; accepted 28 January 2005

Abstract

Comparative studies of mammalian high density lipoproteins have clearly indicated that the major apolipoprotein is apoA-I and in some mammals apoA-II is the second major apolipoprotein. However, in pigs, apoA-II has been considered to be either present in trace amounts or absent. Recently, cDNA sequences for pigs A-II have been entered into the database. Translation of these sequences revealed that pig A-II consisted of 77 amino acids and that a cysteine residue was at residue 6. The A-II of three other mammals, chimpanzees, horses and humans, also has a cysteine residue at this position. As a result of a disulfide bond formed between monomers, the A-II in each of these cases circulates as a homodimer. Using electrospray-ionization mass spectrometry (ESI-MS), we obtained molecular mass data demonstrating that dimeric apoA-II is also present in pig plasma. In addition to being the first to report on the presence of apoA-II in pig plasma, we also obtained values for the molecular masses of apoA-I, apoC-III, apoD and serum amyloid A protein.
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Keywords: Dimeric A-II; Serum amyloid A (SAA) protein; ApoA-I; ApoC-III; Propeptide; ProapoA-I

1. Introduction

In response to diets highly enriched in saturated fats and cholesterol pigs will develop atherosclerosis. This has prompted a series of studies which have characterized the physicochemical properties of the two major carriers of porcine plasma cholesterol, the low and high density lipoproteins (LDL and HDL) (Jackson et al., 1973). When analyzed in the analytical ultracentrifuge in a solution having a density of 1.20 g/mL, porcine LDL resolved into two or more distinct components (Janado et al., 1966; Kalab and Martin, 1968). The two major components were designated LDL_1 and LDL_2 (Kalab and Martin, 1968). Jackson et al. (1976) carried out further flotation rate studies in a solution of density 1.060 g/mL and found that LDL_1 and LDL_2 had S_f^0 values of 11.98 and 4.6, respectively. No difference in the protein moiety of these two LDL components was noted. Electrophoretic studies had previously indicated that the protein moiety of pig LDL was large with a molecular weight identical to human apoB, now known as apoB 100 (Chapman and Goldstein, 1976). In contrast to the LDL, pigs HDL were comparatively monodisperse, having a mean diameter between 6.0 and 8.0 nm (Forte et al., 1979). Flotation rate studies indicated that pig HDL were very similar to human HDL_3 (Forte et al., 1979). The peak $F_{1.20}$ value was reported to be 2.83. The major apolipoprotein of pig HDL is apoA-I, and based on several reports, apoA-II is apparently absent (Jackson et al., 1976; Forte et al., 1979). However, using SDS-PAGE, Knipping et al. (1975) were able to detect a faint band with mobility identical to homodimeric human apoA-II.

Like the human apolipoprotein (Brewer et al., 1972), the apoA-II of both chimpanzees (*Pan troglodytes*) (Blaton et al., 1974; Scanu et al., 1974) and horses (*Equus caballus*)

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1096-4959/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.cbpc.2005.01.014

(Puppione et al., 2004) are also able to form homodimers. In each case, a cysteine residue at position 6 enables identical monomers to be joined by a disulfide bond (Puppione et al., 2004). If present in other mammalian plasma, the A-II apolipoproteins, lacking cysteine, are monomeric (Puppione et al., 2004).

Our group became interested in reexamining the existence of pig apoA-II when we discovered that the sequences for the cDNA for this apolipoprotein had recently been deposited in the database (Uenishi et al., 2004). Upon translation, these cDNA were predicted to give rise to a 78 amino acid protein with a cysteine residue at position 6.

Using ESI-MS we recently have reported on the molecular weights of horse monomeric and dimeric apoA-II (Puppione et al., 2004). Analyses were done before and after reduction with DTT. We have used the same protocols to determine the molecular masses of the apolipoproteins of pig HDL (Whitelegge et al., 1998, 1999). In the absence of a reducing agent, we were able to detect apolipoproteins having the molecular masses corresponding to apolipoproteins A-I, C-III, D and the serum amyloid A protein (SAA); however, the response for dimeric apoA-II was extremely low. Following reduction with DTT a molecular mass consistent with the value predicted for the 77 amino acid apoA-II was observed. In addition, we have analyzed by MSMS the enzymatic fragments derived from apoA-II. The molecular masses of the tryptic fragments were consistent with the calculated values based on the predicted sequences. In addition to being the first to report on the masses of the various porcine apo HDL, our study is also the first to demonstrate conclusively the presence of apoA-II on pig HDL.

2. Methods

2.1. Source of pig blood

A pool of fresh blood collected in containers containing EDTA was obtained commercially. A 5% solution of NaN_3 was added subsequently to the recovered plasma (final concentration 0.05%).

2.2. Ultracentrifugal isolation of high density lipoproteins

All ultracentrifugal runs were done using a Ti 70.1 rotor spun in a Beckman Optima-LE 80 K ultracentrifuge at 50,000 rpm (171,000 × *g* at r av) at 20 °C. Densities were adjusted as described previously (Schumaker and Puppione, 1986). Initially 4 tubes containing 8.5 mL of plasma adjusted to a density of 1.063 g/mL were centrifuged for 25 h. After removing the top 4 mL, the infranatants were pooled and centrifuged again for 24 h. Afterwards the top 3 mL were removed and the density of infranatants were adjusted to a density of 1.210 g/mL upon the addition of 3 mL of a NaBr/NaCl solution (density = 1.4890 g/mL). Following 32 h of centrifugation, the HDL were recovered in the top 1 mL. All salt solutions used for ultracentrifugation contained 0.04% Na_2 EDTA and 0.05% NaN_3.

2.3. Determination of apolipoprotein mass by size exclusion chromatography-mass spectrometry SEC-MS

The protocol of Whitelegge et al. (1999) were used to separate the apolipoproteins by SEC prior to analysis in the mass spectrometer. Dialyzed or undialyzed HDL fractions were acidified by mixing 10 μl with 90 μl of 90% formic acid immediately prior to SEC-MS. For samples run under reduced conditions, 5 μl 1.0 M DTT were added to the sample and allowed to incubate for 10 min before the addition of 85 μl of 90% formic acid. SEC-MS was performed in $CHCl_3$/MeOH/1% aqueous formic acid (4/4/1; v/v/v) using a Super SW 2000 column (4.6 × 300 mm, Tosoh Bioscience, Montgomeryville, PA) at 250 μl /min and 40 °C. Prior to interacting with the electrospray–ionization source, the column effluent was monitored with a UV detector set at 280 nm. Mass spectrometry (ESI-MS) was performed using a triple quadrupole instrument (API III, Applied Biosystems) tuned and calibrated as described (Whitelegge et al., 1998). Data were processed using MacSpec 3.3, Hypermass and BioMultiview 1.3.1 software (Applied Biosystems).

2.4. Separation by reverse phase column chromatography

Partial delipidation was performed by mixing 100 μl of the HDL fraction with 1 ml of 80% acetone. Precipitated apolipoproteins were dissolved in 100 μl of 90% formic acid and loaded on a reverse phase column (PLRP/S 5 μm, 300 Å, 2 × 150 mm, Polymer Labs, Amherst, MA). The eluting solvent was a mixture of 0.1% TFA in water and 0.05% TFA in a 1:1 mixture of acetonitrile/isopropanol in a gradient as described (Whitelegge et al., 2002). Column temperature was 40 °C and the flow rate was 100 μl/min. ESI-MS was performed as described above.

2.5. Tryptic digestion of apolipoproteins

Prior to tryptic digestion, apolipoproteins were first reduced and alkylated. Aliquots of 10 μl from fractions were incubated with 15 μl of 100 mM ammonium bicarbonate and 2 μl of 1.0 M DTT for 1 h at 37 °C. A second hour incubation after the addition of 5 μl 1.0 M iodoacetamide followed. The digestion with trypsin was at 37 °C for 3 h. The samples were then stored in a freezer at −80 °C.

2.6. μLC-MSMS (micro-liquid chromatography with tandem mass spectrometry)

Samples were analyzed by μLC-MSMS with data-dependent acquisition (LCQ-DECA, ThermoFinnigan, San

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