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COMPARATIVE MOLECULAR PROPERTIES OF SWINE AND HUMAN VERY LOW DENSITY LIPOPROTEINS—APOPROTEINS E AND C

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Abstract—1. By means of 2-dimensional gradient-gel electrophoresis, the very low density lipoproteins (VLDL) apoproteins E and C profiles from human and swine plasma were studied.
2. The molecular properties (isoelectric point and molecular weight) of the VLDL apoproteins and their isoforms were determined and showed many similarities between species.
3. It also appears evident that a previously unrecognized apoprotein (C-III) and several associated isoforms may exist in swine; however, its mobility on 2-dimensional gradient gels is very similar to Apo C-II.

INTRODUCTION

Very low density lipoproteins (VLDL) are synthesized and secreted by the liver, and function primarily to transport endogenous lipid to peripheral tissues (viz., muscle and adipose) for energy utilization and/or storage (Grundy, 1986; Schonfeld, 1983). Very low density lipoproteins are catabolized in peripheral tissues as a result of their interaction with the endothelial-associated lipolytic enzyme lipoprotein lipase (LPL). Very low density lipoprotein-triglyceride hydrolysis by LPL occurs stepwise generating progressively smaller, more dense particles of substantially altered composition called VLDL-remnants or intermediate density lipoproteins (IDL) (Dolphin, 1985). The fate of the VLDL-remnant particle greatly depends on metabolic conditions and species, whereby they are either efficiently cleared by the liver or converted to low density lipoproteins (LDL).

Human VLDL contain several apoproteins (Apo) including Apo B-100, Apo C (C-I, C-II and C-III), Apo E and trace amounts of other apoproteins (A-I, A-IV and D) (Sprecher et al., 1984). The function of Apo B-100 in VLDL is primarily structural while Apo C and Apo E have been shown to play key regulatory roles in VLDL catabolism (Nestel, 1987). The absence of the C apoprotein of swine VLDL has been shown to be Apo C-II, which appears similar in electrophoretic migration (Fidge, 1976), amino acid composition (Knipping et al., 1975) and function (Knipping et al., 1984) to human Apo C-II. Previous studies (Knipping et al., 1975) have also demonstrated other minor C apoproteins in swine VLDL that comigrate with human C apoproteins; however, the identity and function of these minor C apoproteins in swine have yet to be elucidated.

Like the C apoproteins, Apo E is also intimately involved in the regulation of lipoprotein metabolism (Krause, 1985; Mahley, 1983; Mahley, 1988). Apoprotein E primarily functions as the recognition ligand for the LDL Apo B/E receptors in the uptake of VLDL and chylomicron remnants by hepatocytes and numerous other cells (Davignon et al., 1988; Mahley, 1988). Human Apo E occurs in three major isoforms, Apo E2, E3 and E4 and, similar to Apo C-III, Apo E isoforms are separated and identified by C-III are distinguishable by isoelectric focusing (IEF) on acrylamide gels at pH range of 4–6 and comprise approximately 14%, 59% and 27% of plasma Apo C-III, respectively (Breslow, 1987). Two principal regulatory functions have been attributed to Apo C-III. Recent studies have demonstrated that increased levels of Apo C-III inhibit Apo E dependent receptor-mediated hepatic uptake of triglyceride-rich lipoproteins (VLDL and chylomicron remnants) by masking the receptor binding domains of Apo E (Dolphin, 1985; Windler and Havel, 1985). Furthermore, Apo C-III has been purported to impede the activities of LPL (Brown and Baginsky, 1972; Wang et al., 1985) and hepatic triglyceride lipase (HTGL) (Breslow, 1987; Kinnunen and Ehnholm, 1976), resulting in reduced lipolysis of VLDL, and moreso, VLDL-remnants. Therefore, the Apo E/C-III and C-II/C-III ratios of VLDL play a crucial role in the regulation of VLDL catabolism.

The predominant C apoprotein of swine VLDL has been shown to be Apo C-II, which appears similar in electrophoretic migration (Fidge, 1976), amino acid composition (Knipping et al., 1975) and function (Knipping et al., 1984) to human Apo C-II. Previous studies (Knipping et al., 1975) have also demonstrated other minor C apoproteins in swine VLDL that comigrate with human C apoproteins; however, the identity and function of these minor C apoproteins in swine have yet to be elucidated.

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IEF (Mahley, 1983). Apoprotein E polymorphism is genetically-controlled in humans. It is first synthesized as sialo (fully sialylated) Apo E in the liver and subsequently desialylated in plasma to asialo Apo E (Anson and Breslow, 1982). The extent of desialylation appears to regulate the function of Apo E since not all isoforms of Apo E have the same affinity for the LDL Apo B/E receptor (Mahley, 1983).

Earlier reports demonstrating the absence of Apo C-III isoforms from swine plasma were based primarily on 1-dimensional electrophoretic data. This absence is unusual considering the high degree of conservation of lipoprotein structures and apoprotein sequences among different vertebrates (Chapman, 1980; Lou et al., 1986; Elshourbagy et al., 1986; Barber and Dayhoff, 1977). In addition, previous studies in our laboratory indicate that the gene coding for Apo C-III is closely linked to the genes coding for Apo A-I and A-IV in humans and rats (Haddad et al., 1986). This suggests that the Apo A-I, C-III, A-IV multigene family was established before mammalian radiation, about 80 million years ago (Romer, 1966), and consequently implies that the organization and regulation of these genes are similar in all mammals. Thus, the purpose of this study was to examine and characterize the comparative aspects of the swine and human VLDL-apoprotein profile, especially Apo E and Apo C. We attained a high degree of resolution using 2-dimensional electrophoresis employing exponential gradient gels in the second dimension. This served as a powerful analytical tool in separating low molecular weight isoforms of similar isoelectric point (pI) and molecular weight (mol. wt).

MATERIALS AND METHODS

Animals, human subjects and diets

Swine plasma samples were obtained from nine 1-year-old female Yucatan miniature swines (Sus scrofa) which were fed 800 g per day of a commercially blended diet (Agway Feeds, SYRACUSE, NY) containing 50% of kcal as fat, 50 mg/kg cholesterol and 3000 kcal/kg. Human plasma samples were obtained from free-living normal subjects consuming a typical American diet of approximately 42% of kcal as fat. Animals used in this study were maintained in accordance with the Public Health Service Policy on the Care and Use of Laboratory Animals. Protocols for animal and human use were reviewed, approved, and monitored by the University's Animal Care and Use Committee.

Blood specimens

Following an overnight fast of 16 hr, blood samples for VLDL preparations were collected into EDTA vacutainers (1.5 mg/ml), and immediately placed on ice. Plasma was separated by low speed centrifugation (1500g) at 4°C, followed by the addition of 0.05 M phenylmethylsulfonfluoride (PMSF; Sigma Chemical Company, St. Louis, MO) and 0.05% sodium azide (NaN₃) to inhibit proteolytic and microbial activities.

Preparation of lipoproteins

VLDL were isolated by preparative ultracentrifugation as previously described (Hatch and Lees, 1968) with the following modification to minimize albumin contamination. Whole plasma with a background density of approximately 1.02 g/ml was centrifuged for 16 hr in a Beckman 80 Ti fixed angle rotor at 40,000 rpm (115,000 g) at 4°C. The top 2 ml was collected by aspiration and contained predominantly VLDL, since these samples were shown to contain no detectable LDL. A homogeneous pool of approximately 4 ml of the VLDL-rich supernatant isolated from the initial spin was then adjusted to 1.006 g/ml and overlaid with a 1.006 g/ml NaBr solution to fill the tube, and centrifuged (washed) as above. The top 2 ml were removed by aspiration. The purity of each VLDL preparation was ascertained by both agarose gel (Paragon Electrophoresis System, Beckman Instruments) and 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Weber and Osborne, 1969).

Sample preparation

Isolated VLDL were dialyzed for 18 hr at 4°C against 41 of 10 mM ammonium bicarbonate, containing 0.05% NaN₃. Aliquots of the VLDL preparations were then flash frozen in liquid nitrogen, lyophilized and immediately delipidated in 5 ml of fresh chloroform-methanol 2:1 (v/v) at 4°C as previously described (Orodvas, 1987). The resulting apo-VLDL was dried under a stream of nitrogen and stored at -70°C until further analysis.

Analytical 1-dimensional isoelectric focusing (IEF)

The delipidated apo-VLDL was resolubilized in 150 μl of 0.01 M Tris-HCl (pH 8.2), containing 8 M urea and 30 mM dithiothreitol (Maguire et al., 1984). The sample was allowed to stand at room temperature for 30 min with gentle vortexing to ensure complete solubilization. Protein content of the resolubilized apo-VLDL was determined by the method of Sedmak and Grossberg (1977). First dimension separation of VLDL apoproteins was carried out in 7.5% polyacrylamide IEF tube gels (3 x 125 mm) or vertical minigels (7 x 8 cm) as described by O'Farrell (1975). Gels contained a mixture of ampholytes (Bio-Rad) of pH 4-6 and 5-7 in a ratio of 4:1 (v/v). Apoprotein-VLDL samples containing 40 µg total protein were applied to each gel. A typical IEF run consisted of six tube gels for each sample, two of which were fixed for 6 hr at room temperature in 12% trichloroacetic acid (TCA), stained for 7 days in 10% acetic acid (Reiner et al., 1975). Of the remaining four gels, two were equilibrated for 2-dimensional SDS PAGE (see below) and frozen at -70°C, and two were used to determine the pH gradient within the gel.

Determination of isoelectric points

Isoelectric points for VLDL apoproteins were determined according to O'Farrell (1975). Four millimeter sections of the gels were soaked in 1.0 ml of degassed distilled water for 1 hr, followed immediately by a pH measurement. Broad range (pI 4.6-9.6) prestained IEF standards (Bio-Rad) were used to confirm pH gradients and to aid in correcting for the increase in gel length caused by fixing and staining.

2-Dimensional gel electrophoresis

Following IEF, tube gels for 2-dimensional slab gel electrophoresis were equilibrated for 2 hr at 37°C in 20 mM Tris-HCl (pH 6.8) containing 1.5% SDS, 2 mM EDTA, 80 mM dithiothreitol and 10% sucrose (Fairbanks, 1971) and either run immediately or stored at ~70°C in the equilibration solution. The second dimension was run in an SDS-polyacrylamide exponential gradient gel (10-22%), and fixed-stained and destained as previously described (O'Farrell, 1975). Following destaining, second dimension slab gels were scanned with an LKB Model 2222-010 ultracan-XL laser scanner densitometer. The molecular weights of Apo C and Apo E were calculated by their relative mobilities (R₀) in the gel as compared to standards of known molecular weight (Bio-Rad) (Hames, 1981).
RESULTS

Analytical 1-dimensional isoelectric focusing

Separation of human and swine VLDL apoproteins (40 μg total protein) by IEF, on either minigels or tube gels, yielded similar results (gels not shown). The pH gradient utilized (pH 4–6) was optimal for resolving VLDL Apo E and Apo C on the same gel. Numerous apoproteins of similar pI, especially from swine VLDL, did not resolve in the first dimension, creating difficulty in identifying various apoprotein isoforms.

2-Dimensional slab gel electrophoresis

After separation by IEF, VLDL apoproteins were electrophoresed on SDS slab gels providing a 2-dimensional profile of the apoproteins. The exponential polyacrylamide gradient (10–22%) produced distinct resolution of Apo E and Apo C isoforms in both human and swine samples. Figure 1 shows a representative 2-dimensional slab gel of human VLDL apoproteins. The complex patterns of Apo C and Apo E are the result of both charge and size differences of the various isoforms of each family of apoproteins. Figure 2 shows a representative 2-dimensional gel of swine VLDL apoproteins. As mentioned above, numerous apoproteins appeared in the 2-dimensional patterns which were not resolved in the first dimension IEF gels. This phenomenon was primarily the result of overlapping apoproteins of similar pI which resolve in the second dimension based on their molecular weight.

Molecular properties of human and swine VLDL apoproteins

Numerous similarities were detected between the human and swine Apo C and Apo E profiles. Since little information exists on these apoproteins in swine, especially 2-dimensional electrophoretic analyses, and a nomenclature convention does not exist for the isoforms of swine apoproteins, a schematic representation of the 2-dimensional gels of swine and human VLDL apoproteins was constructed for comparative purposes (Fig. 3). Several isoforms of swine Apo E, and especially Apo C, show a striking resemblance to the 2-dimensional migration patterns of these apoproteins in humans. In fact, Table 1 shows that several swine VLDL apoproteins (viz., Apo C-II, C-IIIa, C-IIIb, C-IIIc, and E-3) closely resemble the pI and molecular weights of human VLDL apoproteins as determined by this study.

DISCUSSION

Molecular properties of swine Apo E and C

Earlier investigations (Fidge, 1973) of swine VLDL apoproteins clearly demonstrated that even though Apo C-II was the predominant C apoprotein, other small molecular weight peptides of the C family were present. Further studies (Fidge, 1976; Knipping et al., 1975) showed that at least two more C apoproteins that co-migrated with human C apoproteins in 10% PAGE were apparent. In an attempt to identify these co-migrating apoproteins, Knipping et al. (1984) isolated seemingly homogenous apoprotein C fractions by chromotofocusing. Their findings, as determined by the ability of each fraction to activate LPL and cross-react with anti-Apo C-II, purported that the co-migrating peptides were, in fact, isoforms of Apo C-II (Apo C-II,) and not Apo C-III. Since Apo C-II polymorphs capable of activating LPL have been demonstrated in humans (Jackson and Holdsworth, 1986), Knipping’s (1984) assumptions appear valid. Preliminary 1-dimension IEF data from this study initially corroborated these previous findings, especially when human Apo-VLDL was co-electrophoresed with swine. However, when swine
Apo-VLDL was subjected to 2-dimensional electrophoresis, a more complex protein pattern emerged. Numerous proteins which appeared to represent a single apoprotein by IEF, separated into two distinct proteins in the second dimension. Swine apoproteins which correspond to human C-II₀ and C-III₁ have nearly identical pIs and resolve only after SDS-PAGE (Figs 2, 3). Although the previously cited studies did recognize minor C peptides, these investigations may have overlooked several apoproteins. Firstly, 10% polyacrylamide gels do not yield optimal resolution of similar low molecular weight proteins (Haines, 1981). Linear or exponential gradient gels containing polyacrylamide concentrations of 20-30% have been shown to optimize the separation of such proteins. Secondly, although IEF is a useful tool to separate divergent proteins, those of very similar pIs may not resolve. Thus, 2-dimensional separation following IEF becomes a most powerful qualitative and quantitative tool for separating closely related proteins (O'Farrell, 1975). Furthermore, chromatofocused Apo C-II fractions contaminated with Apo C-III will still activate LPL and cross-react with anti-Apo C-II (Knipping, 1984). Therefore, it appears evident from this study (Fig. 2), that several previously unrecognized isoforms of Apo C may exist in swine which are only clearly distinguishable by 2-dimensional electrophoresis.

Fig. 2. Representative 2-dimensional SDS-PAGE slab gel of swine apo-VLDL. The swine C apoproteins, located in the lower left quadrant, correspond to human C apoproteins as follows: 1 to C-III₀; 2 to C-II₀; 3 to C-III₁; 4 to C-II₁; and 5 to C-III₂. The swine E apoproteins are located in the upper right quadrant.

(MW, molecular weight in kilodaltons; IEF, isoelectric focusing; SDS, SDS-PAGE.)

Fig. 3. Schematic representation of the 2-dimensional SDS-PAGE patterns of typical swine and normal human (Zannis and Breslow, 1985) VLDL apoproteins. (MW, molecular weight in kilodaltons; pl, isoelectric point.)
Table 1. Comparative molecular properties of swine and human VLDL—apoproteins E and C

<table>
<thead>
<tr>
<th>Human VLDL apoprotein</th>
<th>Swine designation†</th>
<th>Swine Mol. wt (kd)</th>
<th>Human* Mol. wt (kd)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-I</td>
<td>ND†</td>
<td>—</td>
<td>7.5</td>
</tr>
<tr>
<td>C-II</td>
<td>2 (C-II)</td>
<td>4.88§</td>
<td>8.7</td>
</tr>
<tr>
<td>C-III</td>
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<td>8.3</td>
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<tr>
<td>C-III</td>
<td>3</td>
<td>4.88</td>
<td>8.8</td>
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<tr>
<td>C-III</td>
<td>5</td>
<td>4.72</td>
<td>8.6</td>
</tr>
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<td>8.7</td>
</tr>
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<td>6.02</td>
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<tr>
<td>E-3</td>
<td>E-2</td>
<td>5.77</td>
<td>5.89</td>
</tr>
<tr>
<td>E-3</td>
<td>E-3</td>
<td>—</td>
<td>5.78</td>
</tr>
<tr>
<td>E-3</td>
<td>E-3</td>
<td>—</td>
<td>5.68</td>
</tr>
<tr>
<td>ED-4</td>
<td>ND</td>
<td>—</td>
<td>6.18</td>
</tr>
</tbody>
</table>

*Zannis and Breslow (1985).
†Not detected.
§Reported pl of swine Apo C-II is 4.82 by Jackson et al. (1977).
‡Reported mol. wt of swine Apo E is 37,000 by Weisgraber et al. (1980).

Results obtained from this study indicate a striking qualitative resemblance between the 2-dimensional gels of swine and human Apo C and E (Fig. 3). The molecular properties of swine Apo C-II determined by this study are remarkably similar to those reported in humans (Table 1) (Zannis and Breslow, 1985) which is not unexpected given the sequence homology of their amino acid compositions (Knipping et al., 1975). Furthermore, human Apo E-3, C-III0, C-III1, and C-III2, migrate in similar patterns to swine Apo E and C isoforms isolated in this study.

Figure 2 offers evidence to support the concept that sialylated forms of both Apo C-II and C-III exist in swine. We propose the pattern of proteins designated as 1, 3 and 5 (see Fig. 2) represents the parent peptide Apo C-III1, and two isoforms with one and two sialic acid residues, respectively. The proteins differ in pl and mol. wt equivalent to the addition of sialic acid residues and have the same relationship in the gel as human C-III0, C-III1 and C-III2 (Figs 1, 3). A similar protein exists for apoproteins designated 2 and 4 (see Fig. 2) which differ in pl and mol. wt by the equivalent of one sialic acid residue. These two proteins have the same electrophoretic relationship as human apoproteins C-III0 and C-III1 (Fig. 1). The striking resemblance of their molecular properties (Table 1) infers that these proteins are, in fact, homologous to human isoforms. However, further qualification, as could be demonstrated by immunospecificity to anti-Apo C-III, is required.

In conclusion, our data indicate that apoprotein C-III is present in swine plasma; however, the mobility on IEF and SDS-PAGE is very similar to the other member of the family, Apo C-II. We also determined that only 2-dimensional electrophoresis is able to partially resolve these two apoproteins in the swine. These results are more in agreement with the concept, based on previous evolutionary data, that Apo C-III is present in all mammals and is probably closely linked to the Apo A-I and Apo A-IV genes.

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