Enzyme-Linked Immunosorbent Assay for Bovine Apolipoprotein A-IV

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ABSTRACT. The present report describes an enzyme-linked immunosorbent assay for bovine apolipoprotein (apo) A-IV. This assay was applied to the determination of its concentration and distribution in sera from cattle. The distribution of apoA-IV in lipoprotein fractions separated by ultracentrifugation was mostly recovered in the non-lipoprotein fractions (d>1.21 g/ml, 90%), but, in the case of gel filtration chromatography, apoA-IV was mainly eluted in HDL and non-lipoprotein fractions. The apoA-IV concentrations during early, mid- and late lactating stages in cows were significantly higher than during the nonlactating stage (p<0.05). From early to late lactating stages, the concentration of apoA-IV was unaltered. After 4 days of fasting, the concentration of plasma apoA-IV had decreased significantly (p<0.05) at days 3 and 4, and was returned to the basal level by 3 days of refeeding. These results suggested that the concentration of apoA-IV is modified by nutritional conditions.

KEY WORDS: apolipoprotein A-IV, bovine, ELISA, lipoprotein.

Apolipoprotein (apo) A-IV was first described by Swaney et al. as a polypeptide with a molecular weight of 46-kDa present in rat high-density lipoproteins (HDL) [22]. An examination of the distribution of apoA-IV mRNA in different tissues of the rat and human showed that apoA-IV mRNA was abundant in both the liver and small intestine of the rat, but abundant only in the small intestine of the human [8]. ApoA-IV is an activator of lecithin-cholesterol acyltransferase [6, 21], cholesteryl ester transfer protein [2], and may also influence activities of lipoprotein lipase [9].

In bovine apoA-IV, Grummer et al. reported a 46-kDa protein in triglyceride-rich lipoproteins, that might be analogous to non-ruminant apoA-IV [10]. Bauchart et al. reported that the bovine apoA-IV-like component (42-kDa) was detected in heavy HDL (d=1.091–1.180 g/ml) [3]. Although the existence of bovine apoA-IV in lipoprotein fractions has already been reported, quantitative studies on the changes in apoA-IV in plasma and lipoprotein fractions are lacking.

In the present study, we attempted to detect bovine apoA-IV in lipoproteins and to determine the N-terminal amino acid sequence. Bovine apoA-IV was purified and an enzyme-linked immunosorbent assay (ELISA) developed. This assay was applied to the determination of its concentration and distribution in sera from cattle.

MATERIALS AND METHODS

Animals: Five Holstein cows were used to evaluate the distribution of apoA-IV among lipoprotein subclasses in bovine plasma. Holstein dairy cows from farms in Hokkaido were studied to determine the concentrations of apoA-IV in their sera. They were allotted to 4 groups: group 1, cows in the nonlactating stage (1 to 30 days before parturition); group 2, cows in early lactation (0 to 90 days after parturition); group 3, cows in midlactation (91 to 200 days after parturition); and group 4, cows in late lactation (201 to 300 days after parturition). Four Holstein calves, 6 to 7 months old, were used to study the effect of fasting. After 4 days of fasting, calves were refed for 3 days (day 7). Blood was collected at 0, 1, 2, 3 and 4 days (fasting stage) and on day 7 (refeeding stage). EDTA was used as an anticoagulant (1 mg/ml). All cattle were apparently healthy, and no signs of pain were evident when blood samples were taken.

Separation of lipoproteins by sequential ultracentrifugation or gel filtration chromatography: VLDL (d<1.006 g/ml), LDL (d<1.063 g/ml), HDL (d<1.21 g/ml) and non-lipoprotein fractions (d>1.21 g/ml) were separated by sequential ultracentrifugation (70.1T rotor, L-60, Beckman Instruments, Inc., Fullerton, CA, U.S.A.) according to the method of Hatch and Lees at 20°C [11]. A Superose 6 column (1 cm × 30 cm, Amersham Biosciences, Upplands, Sweden) was equilibrated with 0.15 M NaCl and 0.01% EDTA (pH 7.2) at 4°C. Plasma (0.5 ml) was applied to the column, and the eluate was collected in 0.6-ml fractions at a flow rate of 0.3 ml/min [13].

Electrophoresis and determination of N-terminal amino acid sequence: Apolipoproteins in lipoprotein fractions were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmli [17]. Five to 20% gradient gels (Bio-Rad Laboratories, Hercules, CA, U.S.A.) were used and stained with Comassie brilliant blue. A broad-range molecular marker (Bio-Rad Laboratories) was used for estimation of the molecular masses of proteins. The HDL fraction was separated by SDS-PAGE, and was transferred to a polyvinylidene difluoride membrane with a solution containing 25 mM Tris, 192 mM glycine, and 20% (v/v) methanol at 20 V for 1 hr. After being rinsed with water, the membrane was stained by Coomassie brilliant blue. The 42-kDa and 43-
kDa protein bands were cut from the membrane, and sequenced on a protein sequencer (Model 491, Applied Biosystems Japan Ltd., Tokyo, Japan) by standard procedures.

**Purification of bovine apoA-IV and preparation of anti-bovine apoA-IV antiserum:** Separation of apoA-IV was performed by preparative SDS-PAGE. After HDL was separated by SDS-PAGE, gels were stained with Coomassie brilliant blue, and the band corresponding to apoA-IV (42-kDa) was cut out. Elution of apoA-IV from gel pieces was carried out with an electric system. Purified bovine apoA-IV was emulsified in an equal volume of Freund's complete adjuvant, and injected into rabbits. After two weeks, the protein emulsified in Freund's incomplete adjuvant was administered as a booster. One week after the booster injection, blood was obtained from abdominal veins. The specificity of the antiserum obtained was estimated by immunoblot analysis. Five to 20% gradient gels (Bio-Rad Laboratories) were used for SDS-PAGE of bovine plasma and apoA-IV, and gels were stained with Coomassie brilliant blue or were immunoblotted with anti-bovine apoA-IV antiserum. Bovine apoA-IV was detected by chemiluminescence (ECL kit, Amersham Biosciences).

**ELISA:** The ELISA was essentially performed as described for apoE, with modifications [23]. Samples were diluted with PBS or 1 M urea solution. A polyclonal rabbit anti-bovine apoA-IV antibody was used for coating, and a polyclonal rabbit anti-bovine apoA-IV antibody conjugated to biotin was used as the secondary antibody. After incubation of the plates with alkaline phosphatase-conjugated streptavidin (Boehringer Mannheim GmbH, Mannheim, Germany), detection was done with the ELISA substrate system (alkaline phosphatase substrate kit, Bio-Rad Laboratories). A standard curve was prepared for each assay by appropriate dilution of the standard plasma, for which the apoA-IV concentration had already been determined by immunoblot and densitometric analysis.

**Analysis of data:** Data were analyzed by one-way ANOVA.

**RESULTS**

**Characterization of bovine apoA-IV by SDS-PAGE:** Apolipoproteins in lipoprotein fractions were separated by SDS-PAGE and detected by Coomassie brilliant blue staining (Fig. 1). Major bands were apoA-I in the HDL fraction and apoB100 in the LDL fraction. A minor band, whose molecular weight was estimated to be 42-kDa, was found in the VLDL, LDL and HDL fractions. Another band, whose molecular weight was estimated to be 43-kDa, was found in the HDL fractions. The N-terminal amino acid sequence determined for the 42-kDa protein is shown in Table 1. The 42-kDa protein had high homology to apoA-IV of mammals (human 70%, baboon 60%, rat 60% and mouse 65%). The 43-kDa protein had high homology to paraoxonase/arylesterase of mammals (unpublished data).

**Purification of bovine apoA-IV and specificity of anti-bovine apoA-IV antiserum:** Bovine apoA-IV was isolated from plasma by preparative SDS-PAGE. SDS-PAGE and Coomassie brilliant blue staining confirmed the purity of the apoA-IV, and only one band was stained (Fig. 2, lane 2). The specificity of the anti-bovine apoA-IV antiserum was confirmed by SDS-PAGE and immunoblotting (Fig. 2, lanes 1 and 3).

**ELISA for bovine apoA-IV:** The two standard curves made from a series of standard plasma diluted with PBS or urea are shown in Fig. 3. Standard plasma diluted with urea had higher absorbance than those diluted with PBS. The intraassay coefficient of variance for dilution with urea was 5.6% (n=8), and in the case of PBS it was 18.3% (n=8). That is to say, diluting samples with urea produced a better result than diluting them with PBS. In the following assay, we used urea for sample dilution. The interassay coefficient of variance for dilution with urea was 11.8% (n=9).

**Distribution of apoA-IV among lipoprotein subclasses in bovine plasma:** The distribution of apoA-IV in lipoprotein fractions separated by ultracentrifugation was evaluated in bovine plasma (Fig. 4). ApoA-IV was mostly recovered in the non-lipoprotein fractions (d>1.21 g/ml, 90%). Among
VLDL, LDL and HDL, the percentage of apoA-IV was unaltered. The distribution of apoA-IV in lipoprotein fractions separated by gel filtration chromatography is shown in Fig. 5. The elution positions of VLDL, LDL and HDL were determined based on the elution patterns of cholesterol, triglyceride, and the most abundant protein constituents in lipoprotein fractions separated by ultracentrifugation, respectively. The elution position of albumin represented that of the major proteins in non-lipoprotein fractions. As shown in Fig. 5, apoA-IV was predominantly found in HDL and non-lipoprotein fractions and was also detected in VLDL fractions at a low level. No detectable apoA-IV was observed in LDL fractions.

Table 1. N-terminal amino acid fragment of bovine 42-kDa protein

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Hyphen indicates the same amino acid as the apoA-IV fragment of another species.

Fig. 2. SDS-PAGE and immunoblot analysis of bovine apoA-IV. Plasma (20 µl of 20-fold dilution, lanes 1 and 3) and purified bovine apoA-IV (injection volume 20 µl, lane 2) were applied to gradient gel (5 to 20%). Proteins were stained by Coomassie brilliant blue (lanes 1 and 2) or were blotted and probed with anti-bovine apoA-IV antiserum (lane 3). Bars at left indicate molecular masses of marker proteins.
DISCUSSION

It has been reported that apoA-IV is found in several classes of bovine plasma lipoproteins [3, 10], but its N-terminal amino acid fragment had not been determined. In the present study, we determined the N-terminal amino acid fragment of 42-kDa and 43-kDa proteins in the HDL fraction. Because the 42-kDa protein had high homology to apoA-IV of mammals, it was identified as bovine apoA-IV. The 43-kDa protein had high homology to paraoxonase/arylesterase of mammals (unpublished data). Bovine paraoxonase/arylesterase was purified [7], and known to be associated with HDL [15]. This protein protects against the accumulation of lipid peroxides in LDL [19].

An ELISA was developed to determine the apoA-IV concentration in bovine plasma. In our system, urea was required for sample dilution to obtain sensitive and reliable values. It was considered that urea was involved in the dissociation of apoA-IV from lipoproteins, and facilitated the reaction between apoA-IV and the antibody.

It was reported that more than 90% of apoA-IV was unasassociated with the major lipoprotein fractions [24]. This report was coincident with the result that bovine apoA-IV was recovered in the non-lipoprotein fractions separated by ultracentrifugation. It is thought that there was artificial loss from lipoprotein fractions because VLDL or HDL separated by gel filtration chromatography contained apoA-IV. The underlying cause of the proposed ultracentrifugal loss of lipoprotein apolipoproteins is probably attributable to the combined effects of high ionic strength and high centrifugal force. When lipoprotein fractions were separated by gel filtration chromatography, apoA-IV was eluted in HDL and non-lipoprotein fractions [18, 20]. These reports were also consistent with the results of this study.

The plasma concentrations of apoA-IV in humans have been reported to be 13.0 ± 2.6 [18], 13.1 ± 1.8 [25], 14.15 ± 3.66 [24] and 37.4 ± 4.0 [4] mg/dl. In the rabbit, the apoA-

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Values are presented as the mean ± SD. a,b,c) within columns, values with different letters are significantly different at P<0.05 (one-way ANOVA).
IV concentration was found to be $5.32 \pm 0.76 \text{ mg/dl}$ [20]. The concentration of bovine apoA-IV in serum was similar to that of the rabbit and lower than in humans, but we can expect that these values essentially depend on the nutritional state.

ApoA-IV synthesis by the rat small intestine increases in response to acute and chronic dietary triglyceride [1]. Fat feeding significantly increases the apoA-IV level in plasma from normal healthy male subjects at 4 hr [4] and small intestine synthesis of apoA-IV is increased in newborn piglets by dietary lipid absorption [5]. The plasma concentration of apoA-IV increases when humans consume diets rich in unsaturated fatty acids [16]. It is provable that the increase in plasma apoA-IV during the lactating stage can be attributed to the increase in dietary fat intake, because, in general, the food intake of lactating cows increases for milk production, compared with that of non-lactating cows. Similarly, the decrease in plasma apoA-IV in fasting was probably caused by lower food intake.

In the present study, we demonstrated that bovine apoA-IV was associated with HDL, and there was a possibility that the increase in apoA-IV during the lactating stage was accompanied by an increase in HDL. In bovine plasma, most cholesterol is associated with HDL [3]. The apoA-IV concentrations in individual cows did not correlate well with cholesterol. In fasting, the concentration of apoA-IV decreased but cholesterol was not changed. These results suggested that the concentration of apoA-IV was not affected by the changes in plasma HDL. Further study is necessary to elucidate the factors in the change in apoA-IV in bovine plasma.

**REFERENCES**


